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Occupational safety and health aspects of corporate social responsibility reporting in Japan from 2004 to 2012

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Abstract

Background: A number of companies publish corporate social responsibility (CSR) reporting in booklets and other publicly available formats. The purpose of this paper is to clarify the nine-year (2004–2012) trend of occupational safety and health (OSH) activities as described in CSR reporting (by industry sector and company size).

Methods: We investigated CSR reporting on the website in all Japanese companies listed on the first section of the Tokyo Stock Exchange. The data were extracted from CSR reporting of each company every year from 2004 to 2012. We counted the pages dedicated to information on OSH activities by industry sector and company size and calculated the rate of OSH divided by total CSR-related activities.

Results: The number of companies publishing CSR reports increased in all industry sectors, although the rate of inclusion of OSH activity within CSR reports increased only among sectors such as construction, manufacturing, transportation, and commerce. Among all company size, CSR reporting increased constantly throughout all observed years. The proportion of companies that had described OSH in CSR reporting increased from 2004 to 2012, and 76.5% companies had described OSH activities in 2012. The average number of pages of CSR-related report was 34.2 in 2004, increasing to 43.1 in 2012. The proportion of described pages of OSH activities in total CSR reporting increased gradually, and 2.7% in 2012. The focus of CSR reporting gradually shifted from 'environment' to 'social activity including OSH'.

Conclusions: Majority of companies are putting more emphasis on OSH in CSR reporting in Japan.

Keywords: Reporting, Corporate social responsibility, Occupational safety and health, Yearly trend, Category of industry, Size of company

Background

Corporate social responsibility (CSR) is defined by the European Commission as a concept whereby companies integrate social and environmental concerns into their business operations and interaction with their stakeholders on a voluntary basis [1]. In developed countries, the concept of CSR has developed tremendously during the past decade, leading the International Standardized Organization (ISO) to publish ISO 26000 in 2010 [2]. ISO 26000:2010 has seven core subjects, one being labor practices. Labor practices include health and safety at work, which is related to the promotion and maintenance of the highest degree of physical, mental and social wellbeing of workers and the prevention of harm to health caused by working conditions. CSR is connected to occupational safety and health (OSH) in research practices [3, 4]. OSH is an important aspect of CSR, as OSH is one of the indicators used to measure companies' overall progress in CSR [5].

A number of companies publish CSR-related reports in booklets and other publicly available formats such as portable document format (PDF) files, which can be obtained from company website. This accountability mechanism requires the ongoing implementation of CSR activities [6]. Various guidelines for reporting CSR activities have been published, including the influential guidelines of the Global Reporting Initiative

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(GRI), first launched in 2000 [7]. GRI version 4 (G4) is the latest version of the GRI's Sustainability Reporting Guidelines [8]. Many companies use this document as a reference for developing CSR-related reports. GRI Guidelines recommend to describe OSH activities such as G4-LA5: Percentage of total workforce represented in formal joint management–worker health and safety committees that help monitor and advise on occupational health and safety programs, G4-LA6: Type of injury and rates of injury, occupational diseases, lost days, and absenteeism, and total number of work-related fatalities, by region and by gender, G4-LA7: Workers with high incidence or high risk of diseases related to their occupation, G4-LA8: Health and safety topics covered in formal agreements with trade unions, and G2-LA14: Evidence of substantial compliance with the ILO Guidelines for Occupational Health Management Systems.

According to the most recent review including 53 studies about CSR reporting [9], almost all studies analyze only tens of corporate reports. From this small number, it may be difficult to generalize and grasp entire trends and transitions in CSR reporting. This limited analysis likewise applies to CSR reporting focusing on OSH [4]. Few studies analyze yearly trends, which limits the ability to track industry dynamism.

The purpose of this study is to investigate and analyze CSR-related reports according to category of industry and size of company (number of employees) from 2004 to 2012 in all Japanese companies listed in the first section of the Tokyo Stock Exchange (TSE First Section) to understand the trends and development of OSH activities in CSR-related reports.

Methods

Data sources

The subjects of this study were the companies listed on the TSE First Section on October 15th each year from 2004 to 2012. The company list was obtained from the autumn catalogue of the Japanese company quarterly journal "Kaisha Shikiho" [10]. We listed the company names, categories of industry, and sizes of companies from the Kaisha Shikiho each year of the study period, gathering company data from each company's formal announcement.

Investigation

Investigations were carried out every year from 2004 to 2012 by several of our researchers during February and March of the following year. First, we investigated whether or not each company had published a CSR-related report using a PDF file and brochure over the internet. We considered CSR-related publications to be reports if they were eight or more pages long, including front and back covers. Second, each report was classified by its title; titles included environmental report, environmental and social report, CSR report, sustainability report, and others (including responsible care report). Third, we classified all pages of each report as referring to environmental activity, social activity, or other classifications. We found OSH most often in sections on social activity. We investigated OSH items like policy on OSH, health and safety committees, type and rates of injury, formal agreements on OSH with trade unions, occupational safety and health management system, countermeasure for mental health, and so on.

Quality control of the investigation

We conducted the investigation year by year and developed evaluation standards, producing a manual of protocols for investigation of CSR-related reports. Before starting the analysis, we investigated the same reports independently as a trial and shared the results of our analyses. If the results were not perfectly matched, we discussed the discrepancy, identified the cause and made modifications. This process was conducted each year of our investigation.

Statistical analysis

We calculated two proportions for companies each year from 2004 to 2012 by category of industry and by size of company (number of employees). One is the proportion of companies of the TSE First Section that published CSR-related reports (the published rate), and the other is the proportion of companies publishing CSR-related reports that described OSH activities (the described rate). Yearly changes from 2004 to 2012 were analyzed using the Cochran-Armitage test for trends in proportions. Calculation of the proportions was performed using IBM SPSS Statistics, Version 22.0 (IBM Corp., Chicago, IL, USA) and statistical analysis was performed using STATA/SE 13.1 software for windows (STATA Corp., College Station, Texas, USA). *P*-values of less than 0.05 were considered statistically significant.

Ethics

We used only publicly available information in the study and did not use personally identifiable information.

Results

Descriptions of the Japanese companies listed in the TSE First Section by category of industry and size of company (number of employees) are summarized in Table 1. The number of companies in the TSE First Section in Japan increased from 1583 in 2004 to 1736 in 2008 and decreased to 1717 in 2012. The number of companies, led by 'mining', 'transportation, information and communication', 'commerce', 'real estate' and 'services', increased by more than 10% from 2004 to 2012.

Table 1 Description of all Japanese companies listed on the first section of the Tokyo Stock Exchange by category of industry and size of company in 2004–2012

	2004		2005		2006		2007		2008		2009		2010		2011		2012		Increase rate during nine years
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	
Total	1583		1661		1705		1723		1736		1734		1707		1702		1717		
Category of industry																			
Fisheries, agriculture and forestry	6	0.4	6	0.4	6	0.4	6	0.3	5	0.3	5	0.3	5	0.3	5	0.3	5	0.3	83.3%
Mining	6	0.4	7	0.4	6	0.4	6	0.3	6	0.3	6	0.3	7	0.4	7	0.4	7	0.4	116.7%*
Construction	104	6.6	105	6.3	105	6.2	104	6.0	105	6.0	103	5.9	100	5.9	98	5.8	97	5.6	93.3%
Manufacturing	821	51.9	842	50.7	853	50.0	860	49.9	856	49.3	861	49.7	845	49.5	840	49.4	844	49.2	102.8%
Electricity and gas	16	1.0	17	1.0	17	1.0	17	1.0	17	1.0	17	1.0	17	1.0	17	1.0	17	1.0	106.3%
Transportation, information and communication	138	8.7	145	8.7	155	9.1	158	9.2	166	9.6	169	9.7	165	9.7	166	9.8	169	9.8	122.5%*
Commerce	248	15.7	272	16.4	282	16.5	285	16.5	279	16.1	285	16.4	291	17.0	298	17.5	300	17.5	121.0%*
Finance and insurance	140	8.8	145	8.7	151	8.9	146	8.5	152	8.8	142	8.2	135	7.9	128	7.5	129	7.5	92.1%
Real estate	37	2.3	45	2.7	49	2.9	52	3.0	54	3.1	49	2.8	46	2.7	47	2.8	48	2.8	129.7%*
Services	67	4.2	77	4.6	81	4.8	89	5.2	96	5.5	97	5.6	96	5.6	96	5.6	101	5.9	150.7%*
Size of company																			
-49	41	2.6	51	3.1	48	2.8	52	3.0	51	2.9	59	3.4	66	3.9	72	4.2	70	4.1	170.7%*
50–299	190	12.0	219	13.2	240	14.1	243	14.1	257	14.8	266	15.3	263	15.4	265	15.6	274	16.0	144.2%*
300–999	562	35.5	602	36.2	624	36.6	631	36.6	606	34.9	599	34.5	570	33.4	575	33.8	594	34.6	105.7%
1000–2999	518	32.7	522	31.4	528	31.0	526	30.5	524	30.2	523	30.2	523	30.6	520	30.6	501	29.2	96.7%
3000–4999	124	7.8	123	7.4	112	6.6	122	7.1	129	7.4	135	7.8	136	8.0	131	7.7	133	7.7	107.3%
5000–9999	92	5.8	86	5.2	91	5.3	89	5.2	84	4.8	84	4.8	77	4.5	71	4.2	69	4.0	75.0%
10,000-	52	3.3	54	3.3	56	3.3	55	3.2	60	3.5	61	3.5	64	3.7	56	3.3	59	3.4	113.5%*
uncertain	4	0.3	4	0.2	6	0.4	5	0.3	25	1.4	7	0.4	8	0.5	12	0.7	17	1.0	

N Number of the companies by category of industry and by size of company by number of employees

*Those which increased more than 10% from 2004 to 2012

Analysis by category of industry

The number and proportion of the companies publishing CSR-related reports and the companies that described OSH activities in CSR-related reports by category of industry from 2004 to 2012 are shown in Table 2. The number of companies that published CSR-related reports in 2004 was 413 (26.1%); this figure increased to 663 (38.6%) in 2012. The number of companies in all categories of industry except 'electricity and gas' increased from 2004 to 2012. The number of companies that described OSH activities was 211 (51.1%) in 2004, increasing to 507 (76.5%) in 2012. The companies classified as 'construction', 'manufacturing', 'transportation, information and communication', and 'commerce' showed an upward trend from 2004 to 2012, but the others did not show such a tendency.

Analysis by company size

The number and proportion of the companies publishing CSR-related reports and the companies describing OSH activities in CSR-related reports by company size from 2004 to 2012 are shown in Table 3. CSR reporting increased among companies with between 50 and 9999 employees throughout the observation period. This upward trend was especially strong in the companies with between 300 and 9999 employees. The number of companies that described OSH activities increased among companies of all sizes throughout the observation period. This upward trend was especially strong among companies with between 50 and 2999 employees.

The titles and lengths of CSR-related reports

The titles of CSR-related reports (environmental report, environmental and social report, CSR report, sustainability report and others) are shown in Fig. 1. Overall, 289 companies (70.0%) in 2004 used 'environmental report' as their report title, whereas 87 companies (13.1%) in 2012 used the same wording. In contrast, 24 companies (5.8%) in 2004 used 'CSR report' as the report title, whereas 333 companies (50.2%) in 2012 used the same wording.

The number of pages by content type is shown in Table 4. The average number of pages was 34.2 in 2004, increasing to 43.1 in 2012. The total number of pages was divided into three parts: environment activity, social activity (including OSH), and others (not environment activity, not social activity). The proportion of pages dedicated to environment activity decreased, while those that contained social activity (including OSH) and others increased.

Discussion

The purpose of this study was to investigate and analyze CSR-related reports by category of industry and size of company (number of employees) from 2004 to 2012 in all Japanese companies listed on the TSE First Section, to understand the trends and developments of CSR-related reports. We focused on OSH activities because they have to date received scant attention, although OSH is an important aspect of CSR. Key findings of this study were that the focus of CSR reporting in Japan gradually shifted from environment to social activity including OSH, and that the number of companies describing OSH in CSR reporting increased from 2004 to 2012.

The publication proportion of CSR-related reports increased in all categories except in 'electricity and gas', suggesting a growing awareness of CSR across all sectors. Of the 'electricity and gas' companies, 93.8% already published reports in 2004, and this trend was maintained through 2012 because of the sector's importance in civilian life. In tertiary industries such as services, the awareness of CSR was not as high as that in primary and secondary industries. In terms of the company size by number of employees, the larger the size, the higher the publication proportion of CSR-related reports was every year from 2004 to 2012, showing that larger companies exhibited a higher interest in CSR. Independent of company size, the publication proportion of CSR-related reports increased from 2004 to 2012.

Changes in the social environment from 2004 to 2012 led to this upward trend. One factor was economic circumstances, but the publication rate of CSR-related reports may have little influence on a company's economic circumstances. Economic fluctuations, as represented by Lehman's collapse in September 2008, had significant impact on business and financial conditions across the majority of industrial sectors. After Lehman's fall, there was no decrease in the number of companies publishing reports. This tendency may be attributed to two reasons. First, the companies that had already published CSR-related reports did not withdraw from publishing even if business conditions had deteriorated. Second, business and financial conditions may not have a direct impact on CSR reporting. Such speculation needs to be confirmed by further research.

The majority of report titles have recently changed from 'Environmental report' to 'CSR report' (Fig. 1), as observed in a past study [11]. The proportion of 'Environmental reports' among all CSR-related reports fell from 70.0% in 2004 to 13.1% in 2012, whereas 'CSR reports' increased from 5.8% in 2004 to 50.2% in 2012. In Japan, Environmental Reporting Guidelines were published in 1997 [12]. The change of the report title indicates that companies may have come to give higher value to social aspects of CSR.

OSH activities are labor practices and are described as social activity in CSR-related reports. The proportion of

Table 2 The companies which published corporate social responsibility (CSR) related reports divided by total number of companies listed on the first section of the Tokyo Stock Exchange by category of industry in 2004–2012

	2004			2005			2006			2007			2008		
	n(CSR)	n(CSR)/N	(%)	n	n(CSR)/N	(%)	n	n(CSR)/N	(%)	n	n(CSR)/N	(%)	n	n(CSR)/N	(%)
Total	413	26.1		494	29.7		569	33.4		555	32.2		572	32.9	
Category of industry															
Fisheries, agriculture and forestry	0	0.0		2	33.3		1	16.7		2	33.3		3	60.0	
Mining	0	0.0		1	14.3		1	16.7		2	33.3		3	50.0	
Construction	24	23.1		36	34.3		42	40.0		39	37.5		46	43.8	
Manufacturing	303	36.9		356	42.3		403	47.2		386	44.9		388	45.3	
Electricity and gas	15	93.8		16	94.1		15	88.2		16	94.1		16	94.1	
Transportation, information and communication	19	13.8		23	15.9		31	20.0		29	18.4		36	21.7	
Commerce	38	15.3		37	13.6		46	16.3		41	14.4		46	16.5	
Finance and insurance	11	7.9		15	10.3		22	14.6		30	20.5		24	15.8	
Real estate	2	5.4		5	11.1		4	8.2		6	11.5		4	7.4	
Services	1	1.5		3	3.9		4	4.9		4	4.5		6	6.3	
	n(OSH)	n(OSH)/n(CSR)	(%)	n(OSH)	n(OSH)/n(CSR)	(%)	n(OSH)	n(OSH)/n(CSR)	(%)	n(OSH)	n(OSH)/n(CSR)	(%)	n(OSH)	n(OSH)/n(CSR)	(%)
Total	211	51.1		286	57.9		395	69.4		379	68.3		423	74.0	
Category of industry															
Fisheries, agriculture and forestry				1	50.0		0	0.0		1	50.0		2	66.7	
Mining				1	100.0		1	100.0		2	100.0		2	66.7	
Construction	8	33.3		13	36.1		23	54.8		22	56.4		28	60.9	
Manufacturing	176	58.1		215	60.4		296	73.4		297	76.9		304	78.4	
Electricity and gas	8	53.3		11	68.8		12	80.0		10	62.5		9	56.3	
Transportation, information and communication	7	36.8		13	56.5		19	61.3		15	51.7		30	83.3	
Commerce	7	18.4		19	51.4		28	60.9		24	58.5		25	54.3	
Finance and insurance	4	36.4		9	60.0		11	50.0		4	13.3		17	70.8	
Real estate	1	50.0		2	40.0		3	75.0		2	33.3		3	75.0	
Services	0	0.0		2	66.7		2	50.0		2	50.0		3	50.0	

n.s. Not significant*nCSR* number of companies which published CSR-related reports*N* total number of companies listed in TSE1*n(CSR)* number of the companies which published CSR-related reports*n(OSH)* number of the companies that described occupational safety and health activities in CSR-related reports

Table 2 The companies which published corporate social responsibility (CSR) related reports divided by total number of companies listed on the first section of the Tokyo Stock Exchange by category of industry in 2004–2012 *(Continued)*

	2009			2010			2011			2012			
	n	n(CSR)/N	(%)	n	n(CSR)/N	(%)	n	n(CSR)/N	(%)	n	n(CSR)/N	(%)	*P* for trend
Total	609	35.1		625	36.6		619	36.4		663	38.6		$p < .001$
Category of industry													
Fisheries, agriculture and forestry	3	60.0		3	60.0		3	60.0		3	60.0		$p < .001$
Mining	3	50.0		3	42.9		3	42.9		3	42.9		$p < .05$
Construction	54	52.4		56	56.0		53	54.1		55	56.7		$p < .001$
Manufacturing	412	47.9		414	49.0		397	47.3		427	50.6		$p < .001$
Electricity and gas	17	100.0		15	88.2		14	82.4		14	82.4		n.s.
Transportation, information and communication	40	23.7		34	20.6		37	22.3		43	25.4		$p < .01$
Commerce	42	14.7		52	17.9		59	19.8		66	22.0		$p < .01$
Finance and insurance	25	17.6		30	22.2		33	25.8		29	22.5		$p < .001$
Real estate	6	12.2		8	17.4		9	19.1		9	18.8		$p < .05$
Services	7	7.2		10	10.4		11	11.5		14	13.9		$p < .001$
	n(OSH)	n(OSH)/n(CSR)	(%)	n(OSH)	n(OSH)/n(CSR)	(%)	n(OSH)	n(OSH)/n(CSR)	(%)	n(OSH)	n(OSH)/n(CSR)	(%)	*P* for trend
Total	448	73.6		482	77.1		458	74.0		507	76.5		$p < .001$
Category of industry													
Fisheries, agriculture and forestry	2	66.7		2	66.7		1	33.3		2	66.7		n.s.
Mining	3	100.0		2	66.7		3	100.0		3	100.0		n.s.
Construction	37	68.5		44	78.6		42	79.2		47	85.5		$p < .001$
Manufacturing	313	76.0		322	77.8		306	77.1		337	78.9		$p < .001$
Electricity and gas	13	76.5		12	80.0		10	71.4		12	85.7		n.s.
Transportation, information and communication	36	90.0		31	91.2		32	86.5		37	86.0		$p < .001$
Commerce	23	54.8		38	73.1		39	66.1		42	63.6		$p < .001$
Finance and insurance	15	60.0		22	73.3		15	45.5		14	48.3		n.s.
Real estate	3	50.0		4	50.0		6	66.7		7	77.8		n.s.
Services	3	42.9		5	50.0		4	36.4		6	42.9		n.s.

Table 3 The companies which published corporate social responsibility (CSR) related reports divided by total number of companies listed on the first section of the Tokyo Stock Exchange by size of company in 2004–2012

	2004			2005			2006			2007			2008		
	n(CSR)	n(CSR)/N	(%)	n	n(CSR)/N	(%)	n	n(CSR)/N	(%)	n	n(CSR)/N	(%)	n	n(CSR)/N	(%)
Size of company by number of employees															
-49	3	7.3		9	17.6		8	16.7		4	7.7		3	5.9	
50–299	20	10.5		37	16.9		25	10.4		31	12.8		36	14.0	
300–999	75	13.3		148	24.6		135	21.6		131	20.8		134	22.1	
1000–2999	145	28.0		164	31.4		213	40.3		199	37.8		197	37.6	
3000–4999	67	54.0		59	48.0		71	63.4		78	63.9		84	65.1	
5000–9999	59	64.1		37	43.0		66	72.5		64	71.9		61	72.6	
10,000-	43	82.7		38	70.4		50	89.3		46	83.6		52	86.7	
uncertain	1	25.0		2	50.0		1	16.7		2	40.0		5	20.0	
	n(OSH)	n(OSH)/n(CSR)	(%)	n(OSH)	n(OSH)/n(CSR)	(%)	n(OSH)	n(OSH)/n(CSR)	(%)	n(OSH)	n(OSH)/n(CSR)	(%)	n(OSH)	n(OSH)/n(CSR)	(%)
Size of company by number of employees															
-49	2	66.7		2	22.2		4	50.0		4	100.0		2	66.7	
50–299	7	35.0		22	59.5		13	52.0		20	64.5		25	69.4	
300–999	26	34.7		76	51.4		78	57.8		77	58.8		86	64.2	
1000–2999	69	47.6		92	56.1		145	68.1		129	64.8		147	74.6	
3000–4999	37	55.2		39	66.1		55	77.5		60	76.9		64	76.2	
5000–9999	37	62.7		27	73.0		51	77.3		49	76.6		53	86.9	
10,000-	33	76.7		26	68.4		48	96.0		39	84.8		41	78.8	
uncertain	0	0.0		2	100.0		1	100.0		1	50.0		5	100.0	

N total number of companies listed in TSE1*n(CSR)* number of the companies which published CSR-related reports*n(OSH)* number of the companies that described occupational safety and health activities in CSR-related reports

Table 3 The companies which published corporate social responsibility (CSR) related reports divided by total number of companies listed on the first section of the Tokyo Stock Exchange by size of company in 2004–2012 *(Continued)*

	2009			2010			2011			2012			
	n	n(CSR)/N	(%)	n	n(CSR)/N	(%)	n	n(CSR)/N	(%)	n	n(CSR)/N	(%)	*P* for trend
Size of company by number of employees													
-49	6	10.2		8	12.1		12	16.7		15	21.4		0.134
50–299	32	12.0		37	14.1		40	15.1		51	18.6		$p < .05$
300–999	145	24.2		142	24.9		133	23.1		152	25.6		$p < .001$
1000–2999	214	40.9		228	43.6		229	44.0		241	48.1		$p < .001$
3000–4999	89	65.9		90	66.2		90	68.7		91	68.4		$p < .001$
5000–9999	67	79.8		58	75.3		57	80.3		50	72.5		$p < .001$
10,000-	55	90.2		58	90.6		47	83.9		52	88.1		0.051
uncertain	1	14.3		4	50.0		11	91.7		11	64.7		
	n(OSH)	n(OSH)/n(CSR)	(%)	n(OSH)	n(OSH)/n(CSR)	(%)	n(OSH)	n(OSH)/n(CSR)	(%)	n(OSH)	n(OSH)/n(CSR)	(%)	*P* for trend
Size of company by number of employees													
-49	5	83.3		5	62.5		8	66.7		12	80.0		$p < .05$
50–299	24	75.0		29	78.4		29	72.5		40	78.4		$p < .001$
300–999	97	66.9		101	71.1		89	66.9		101	66.4		$p < .001$
1000–2999	157	73.4		172	75.4		169	73.8		182	75.5		$p < .001$
3000–4999	66	74.2		70	77.8		64	71.1		74	81.3		$p < .01$
5000–9999	54	80.6		51	87.9		46	80.7		42	84.0		$p < .01$
10,000-	45	81.8		51	87.9		43	91.5		47	90.4		$p < .05$
uncertain	0	0.0		3	75.0		10	90.9		9	81.8		-

Fig. 1 Detail on the titles of CSR-related reports from 2004 to 2012

companies describing OSH activities among those publishing CSR-related reports (the described rate) increased yearly from 2004 to 2012, and the rate was 76.5% in 2012. By industry category, the described rate was more than 80% in 2012 in 'mining', 'construction', 'electricity and gas', and 'transportation, information and communication'. The change of the described rate from 2004 to 2012 was enormous, especially in 'construction' (33.3% in 2004 to 85.5% in 2012) and in 'transportation, information and communication' (36.8% in 2004 to 86.0% in 2012). The accident severity rate in 'construction' and the rate of work time lost to injuries in 'transportation, information and communication' were higher in 2012 than in 2004 [13]. However, the described rate was less than 50% in 2012 in 'finance and insurance', and 'services'. This suggests that OSH activities were not considered part of CSR in these categories of industry. The 12th Occupational Safety & Health Program of Ministry of Health, Labour and Welfare in Japan observed that occupational accidents increased by 16.7% in tertiary industries from 2002 to 2011; there were 43,053 casualties (worker's death, illness or injuries due to a job-related reason with absence of 4 days or more) reported in 2002 and 50,243 in 2011 [14]. These data suggest that OSH activities need special attention in such sectors like 'finance and insurance' and 'services'.

Considering the size of company by number of employees, the described rate of OSH in CSR-related reports increased from 2004 to 2012, to more than 65% in 2012 among companies of all sizes. The frequency and severity of work time lost to injuries and accidents was higher in smaller-sized companies [13], whereas the number of accidents and injuries was higher in larger companies.

In regard to OSH process, the Global Reporting Initiative (GRI) guidelines recommend to describe Occupational Safety and Health Management Systems (OSHMS) (G2-LA14) [15]. The Ministry of Health, Labour and Welfare in Japan also promotes OSH activities based on OSHMS. 47 companies (22.3%) described OSHMS in CSR reporting in 2004, and 157 companies (31.0%) did in 2012.

Internationally, the GRI developed reporting guidelines in 2000 [7], and updated the indicators as G2 in 2002 [15], G3 in 2006 [16], and G4 in 2013 [8]; all GRI guidelines recommend including OSH activities. In Japan, however, no guidelines mention describing OSH sections in CSR reporting. Overall, 76.5% of companies in Japan in 2012 described OSH activities in their CSR reporting, and 75.3% of the

Table 4 Mean page count of corporate social responsibility (CSR) related reports and the percentage of pages of each report content (environment, social activity, and other) in all companies listed on the first section of the Tokyo Stock Exchange in 2004–2012

Year	2004	2005	2006	2007	2008	2009	2010	2011	2012	*P* for trend
Total pages (mean)	34.19	34.08	37.48	39.27	39.72	38.65	40.09	40.66	43.05	
Proportion (%) of described pages in total										
Environment activity	66.8	59.1	53.4	40.2	35.9	35.7	34.9	31.7	30.5	$p < .001$
Social activity	14.0	14.0	20.2	16.7	20.2	16.9	19.6	16.8	23.5	$p < .001$
Occupational safety and health[a]	1.6	2.1	3.2	2.9	3.1	3.4	3.9	3.3	2.7	$p < .001$
Other (not environment, not social activity)	19.2	26.9	26.4	43.1	43.9	47.5	45.6	51.5	46.0	$p < .001$

[a]Occupational safety and health is a part of social activity

OSH descriptions included mental health activities for employees. Although the burden of mental disorders increased between 1990 and 2010 and mental health is one of the major issues around the world [17], the most recent guidelines do not clearly describe mental health activities.

Strengths and limitations

This study has several strengths and limitations. This is one of the first studies to clarify the trend of OSH activities in CSR reporting from 2004 to 2012. To our knowledge, there are no studies that analyze the OSH aspect of CSR-related reports to reveal a 9-year trend. Second, our study checked all CSR-related reports and PDF files downloaded from the websites of Japanese companies listed on the TSE First Section from 2004 to 2012. Some companies disclosed CSR information only on their website and not through CSR-related reports or PDF files. In 2012, 633 companies had published reports of at least six pages, and 859 companies had published CSR information in paper format, as PDF files or as text on their website. Internet-based reporting became more popular because it is a cost effective means of disseminating corporate information [9].

There are at least three limitations to our study. First, although the concept of CSR includes accountability of CSR activities for different stakeholders, the reports do not describe all CSR activities. Second, we analyzed reporting only by companies listed on the TSE First Section, the leading companies in Japan. To be able to generalize our results, further analysis including small- and medium-sized companies would need to be undertaken. Third, the companies in this study include a few that were not listed continuously on the First Section of the TSE every year from 2004 to 2012 (5.4% of the companies in the First Section of the TSE from 2004 to 2012).

Conclusions

This study discloses that about 40% of the companies listed on the TSE First Section have published CSR-related reports and that the percentage of companies publishing CSR reports increased from 2004 to 2012 regardless of industry sector. CSR reporting increased yearly among the companies with between 50 and 9999 employees.

This study demonstrates that about 75% of the companies publishing CSR-related reports describe OSH activities, and the described rate increased yearly from 2004 to 2012. Employees are important stakeholders for companies and are fundamental human capital in resource management. For other stakeholders like stockholders, the information about OSH activities for employees is useful in judging the company's situation. While our results highlight OSH in CSR reporting, the next step is to analyze the content of reports in more detail.

Abbreviations

CSR: Corporate social responsibility; GRI: Global reporting initiative; OSH: Occupational safety and health; TSE First Section: the First section of the Tokyo Stock Exchange

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Availability of data and materials

Data supporting these findings are held by the Occupational Health Training Center of University of Occupational and Environmental Health, JAPAN, and requests for information should be directed to the corresponding author.

Authors' contributions

TN, KM, TM, FK and MN planned the study. TN, AN, and KM drafted the manuscript. TN, TM, FK and MN checked CSR reporting. TN and AN performed the statistical analysis. All the authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All data used for the study is openly available.

Publisher's Note

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References

1. Zwetsloot G, Starren A. European Agency for Safety and Health at work.: corporate social responsibility and safety and health at work. Luxembourg: Office for Official Publications of the European Communities. 2004. [https://osha.europa.eu/en/tools-and-publications/publications/reports/210].
2. International Organisation for Standardization. Guidance on social responsibility (ISO 26000:2010). 2010. [http://www.iso.org/iso/catalogue_detail?csnumber=42546].

3. Zanko M, Dawson P. Occupational health and safety Management in Organizations. A Review Int J Manag Rev. 2012;14:328–44.
4. Koskela M. Occupational health and safety in corporate social responsibility reports. Safety Sci. 2014;68:294–308.
5. Segal J-P, Sobczak A, Triomphe C-E. Corporate social responsibility and working conditions. Ireland: European Fundation for the Improvement of Living and Working Conditions; 2003.
6. Gray R. Taking a long view on what we now know about social and environmental accountability and reporting. Electronic Journal of Radical Organisational Theory. 2005;9:1–31.
7. Global Reporting Initiative: Sustainability reporting guidelines on economic, environmental, and social performance. 2000 [https://www.globalreporting.org/Pages/default.aspx].
8. Global Reporting Initiative: G4 sustainability reporting guidelines. 2013 [https://www.globalreporting.org/information/g4/Pages/default.aspx].
9. Morhardt JE. Corporate social responsibility and sustainability reporting on the internet. Bus Strateg Environ. 2009;19:436–52.
10. Tōyō Keizai Shinpōsha. Japan company handbook. Tokyo: Toyo Keizai Shinposha; 2004-2012.
11. Susanne B, Carolina G: From environmental accountability to corporate social responsibility? Reflections on the CSR boom in Japan from the perspective of business management and civil society groups. 2008 [http://www.dijtokyo.org/doc/JS20_Brucksch-Gruenschloss.pdf].
12. Ministry of the Environment, Japan: Environmental Reporting Guidelines. 1997. [http://www.env.go.jp/policy/j-hiroba/04-4.html].
13. Ministry of Health, Labour and Welfare, Japan: Survey on Industrial Accidents. 2014. [http://www.mhlw.go.jp/bunya/roudoukijun/anzeneisei11/rousai-hassei/].
14. Ministry of Health, Labour and Welfare, Japan: Survey on Industrial Accidents. [http://www.mhlw.go.jp/english/database/db-l/industrial_accidents.html].
15. Global Reporting Initiative: Sustainability Reporting Guidelines. 2002. [https://www.globalreporting.org/Pages/default.aspx]. (2002).
16. Global Reporting Initiative: G3 Sustainability Reporting Guidelines. 2006. [https://www.globalreporting.org/Pages/default.aspx].
17. Whiteford HA, Degenhardt L, Rehm J, Baxter AJ, Ferrari AJ, Erskine HE, Charlson FJ, Norman RE, Flaxman AD, Johns N, et al. Global burden of disease attributable to mental and substance use disorders: findings from the global burden of disease study 2010. Lancet. 2013;382:1575–158.

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Editorial

Work to live, to die, or to be happy?

Happiness is the meaning and the purpose of life, the whole aim and end of human existence.

- Aristotle

If the government cannot create happiness for its people, then there is no purpose for government to exist.

- Legal code of Bhutan (1729)

Gross National Happiness is more important than Gross Domestic Product.

- His Majesty Jigme Singye Wangchuck, the Fourth King of Bhutan

Although the concept of "happiness" has existed for centuries as quoted above, it is not until recently that "happiness at work" has gained attention in occupational health. Nowadays, convincing evidence supports that happier people live longer[1], healthier[2], be more productive[3], and are relatively more successful in their careers[4]. We can thus view employees' happiness as one of the major sources of positive outcomes in the workplace.

Corresponding to these facts, World Happiness Report was first published in April 2012 in support of the meeting by the United Nations on happiness and well-being, chaired by the Prime Minister of Bhutan[5]. Based on this report, Japan has ranked the 53rd happiest country in 2016 among 157 countries, down from 46th in 2015, 43rd in 2013, and 40th in 2012. What makes Japan sitting in this ranking? We all acknowledge that Japan has prospered through rapid economic growth during the period of 20 years between 1950 and 1970. And even now, we still hold the world's third largest economy. Indeed, there must be good reasons to explain this paradox.

Why Japanese Workers Seems to be Unhappy?

As an occupational epidemiologist myself, I would like to discuss several reasons for being not so happy in Japanese workers based on time use and also propose a hint to overcome such situation. First, Japanese people are notorious for being one of the poorest sleepers in the world. We are ranked the second shortest sleep country among 29 countries surveyed[6]. French, who slept the most, spend on an average of 8 hours and 50 minutes a day while Japanese spend 7 hours and 50 minutes. Japanese people wake up early as 7:00 AM in the morning but go to bed at 11:30 PM[7]. Based on the NHK national time use survey initiated in 1970, sleeping time for Japanese people keeps decreasing by 75 seconds every year. The survey on state of employees' health by the Ministry of Health, Labor and Welfare in 2013 uncovered that 46.5% of workers are sleeping less than 6 hours on a workday. The prospective UK million women study revealed that sleeping less than 7 hours (as compared to 7 hours) was the second strongest factor reducing happiness next to physical disabilities[8]. Poor sleep can not only be a source of irritability and stress, but also unhappiness as well.

Second, Japanese are working too long. OECD data suggests that average annual hours worked among Japanese was ranked the 21st (1,729 hours in 2014) among 38 countries but the statistic include not only those working under full-time condition but also part-time and part-year condition which is increasing rapidly in the Japanese society. Counting non-full-time condition contribute to reduce average annual hours worked. Scientific evidence suggest that long working hours is a major contributor for reducing resting time and sleep[9].

Third, because of both poor sleep and long working hours, work-life balance is largely unbalanced. Besides this, combination of long working hours with poor sleep may develop poor mental health[10] and even increase the chance of committing suicides (Karo-jisatsu) and death from overwork (Karoshi)[11]. What we should focus on is to balance between work, sleep/resting, and leisure time, and creating meaningful and challenging work. We also need to reduce working condition such as unusual work time, holiday work, unpaid work as well as eliminating wasting time on pointless paperwork and irrelevant procedures.

It is high time for Japanese society to think about how we work and what we work for. A paper evaluating the difference of work styles and meaning of work between Europeans and Americans phrased that "Europeans work to live and Americans live to work[12]." There is no right or wrong answer to this question of which attitude is bet-

ter or more appropriate, but rephrasing this word would be possible by adding the Japanese style: "Europeans work to live, Americans live to work, and Japanese work to die." I am certainly sure that "work to die" is a wrong answer. At the same time, I would like to call for research to design happy workplaces.

References

1) Diener E, Chan MY (2011) Happy people live longer: Subjective well-being contributes to health and longevity. Appl Psychol **3**, 1–43. [CrossRef]
2) Pressman SD, Cohen S (2005) Does positive affect influence health? Psychol Bull **131**, 925–71. [Medline] [CrossRef]
3) Oswald A, Proto E, Sgroi D (2015) Happiness and productivity. J Labor Econ **33**, 789–822. [CrossRef]
4) Lyubomirsky S, King L, Diener E (2005) The benefits of frequent positive affect: does happiness lead to success? Psychol Bull **131**, 803–55. [Medline] [CrossRef]
5) World Happines Report (2016) Edited by Helliwell J, Layard R, Sachs J.
6) OECD (2009) Measuring leisure time in OECD countries.
7) Walch OJ, Cochran A, Forger DB (2016) A global quantification of "normal" sleep schedules using smartphone data. Sci Adv **2**, e1501705. [Medline] [CrossRef]
8) Liu B, Floud S, Pirie K, Green J, Peto R, Beral V, for the Million Women Study Collaborators (2015) Does happiness itself directly affect mortality? The prospective UK Million Women Study. Lancet **387**, 874–81. [Medline] [CrossRef]
9) Virtanen M, Ferrie JE, Gimeno D, Vahtera J, Elovainio M, Singh-Manoux A, Marmot MG, Kivimaki M (2009) Long working hours and sleep disturbances: the Whitehall II prospective cohort study. Sleep **32**, 737–45. [Medline]
10) Nakata A (2011) Work hours, sleep sufficiency, and prevalence of depression among full-time employees: a community-based cross-sectional study. J Clin Psychiatry **72**, 605–14. [Medline] [CrossRef]
11) Takeuchi A, Sakano N, Miyatake N (2014) Combined effects of working hours, income, and leisure time on suicide in all 47 prefectures of Japan. Ind Health **52**, 137–40. [Medline] [CrossRef]
12) Okulicz-Kozaryn A (2011) Europeans work to live and Americans live to work (Who is happy to work more: Americans or Europeans?). J Happiness Stud **12**, 225–43. [CrossRef]

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Psychosocial correlates of cortisol levels in fingernails among middle-aged workers

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SHORT COMMUNICATION

Psychosocial correlates of cortisol levels in fingernails among middle-aged workers

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ABSTRACT

It was recently suggested that cortisol levels in fingernails reflect cumulative hormone exposure over a relatively long period. This exploratory study cross-sectionally investigated the relationships between fingernail cortisol level and psychosocial stress in a sample of middle-aged workers (94 men and 29 women). The participants were asked to grow their fingernails for ~2 weeks and then provide fingernail samples from every digit by using nail clippers. Further, they completed questionnaires for assessment of exposure to psychosocial stress in the past (stressful life events in the workplace in the previous year; e.g. change to a different line of work) and in the present (job stress and perceived stress). Results of a regression analysis adjusting for the effects of demographic variables showed that experience of stressful life events, but not job stress and perceived stress, was associated with elevated fingernail cortisol level. These findings indicate the potential of fingernail samples to retrospectively reflect individual differences in cortisol levels related to past psychosocial stress.

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Cortisol; fingernail; job stress; stressful life events; hair cortisol; perceived stress

Introduction

Psychosocial stress triggers a number of physiological changes such as activation of the sympathetic nervous system and hypothalamic–pituitary–adrenal (HPA) axis. One consequence of this activation is the release of cortisol, an adrenal cortex hormone secreted in response to acute stress (Dickerson & Kemeny, 2004). Traditionally, cortisol has been measured in blood and saliva samples. The analysis of saliva and blood hormone level provides an index for a short period. Several recent studies have shown that endogenous hormones such as cortisol can reliably be measured in scalp hair (Russell, Koren, Rieder, & Van Uum, 2012). Scalp hair grows at an average rate of 1.0 cm/month; therefore, 1.0 cm of scalp hair may reflect hormone levels secreted in 1 month. While analysis of saliva and blood hormone levels provides an index for a short period, hair sample analysis provides a retrospective index of cumulative hormone exposure over a longer period, which could have advantages in the investigation of cortisol levels and chronic stress.

It was recently suggested that cortisol levels in fingernails also reflect cumulative hormone exposure over a relatively long period (de Berker, André, & Baran, 2007; Warnock et al., 2010). Neutrally charged endogenous hormones passively diffuse from capillaries into the nail matrix, and are incorporated into keratin during nail formation (de Berker et al., 2007). In this process, free cortisol may be incorporated into keratin through the same mechanism by which cortisol is incorporated into hair (Russell et al., 2012). Fingernails grow at an average rate of 1.0 mm/10 days (Gupta et al., 2005); therefore, 1.0 mm of fingernail may retrospectively reflect hormone levels over 10 days. In addition, previous work that traced fluoride in nails found that 3–4 months were required for nails to fully extend from the nail matrix (Buzalaf, Pessan, & Alves, 2006). Therefore, fingernail samples may reflect cortisol levels in the 3–4 months prior to clipping. In a recent study (Izawa et al., 2015), it was reported that cortisol levels in fingernails were associated with cortisol levels in the past that were assessed by hair and saliva samples. However, no previous studies clearly demonstrated the relationship between fingernail cortisol and psychosocial stress as well demographic variables, and minimal information is available on the fundamental aspects of fingernail cortisol.

This exploratory study cross-sectionally investigated the relationships between fingernail cortisol level and psychosocial stress in the past (stressful life events in the workplace in the previous year) and in the present (job stress and perceived stress) in a sample of middle-aged workers. This population was chosen because psychosocial stress in the workplace is associated with stress-related disease and could cause numerous biological changes (e.g. Eller et al., 2009). We expected that fingernail cortisol level would be positively associated with psychosocial stress, especially for stressful life events in the previous year, because fingernail samples reflect cumulative hormonal exposure in the past. Furthermore,

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we additionally investigated the effects of demographic variables (age, gender, body mass index, smoking status, and manicure use) on fingernail cortisol level.

Methods

Participants

White-collar workers were recruited from hospitals and research institutes in Kanagawa Prefecture in Japan. The sample consisted of hospital personnel and research institute staff who were mainly engaged in desk work. The sample initially comprised 140 workers; however, 17 were excluded for various reasons: six for recent steroid injection or medication use, two for antidepressant use, one for history of an adrenal gland tumor, one for recently giving birth to a child, three for missing questionnaire data, and four for containing statistical outliers regarding fingernail cortisol (see results section for additional details). Therefore, the final sample consisted of 123 workers (94 men; 29 women). Of the 123 participants, none reported adrenal gland disease such as Cushing's syndrome or Addison's disease, and none were taking steroid medications or antidepressants. Written informed consent was obtained from participants, and the ethical committees of the National Institute of Occupational Safety and Health approved the study.

Questionnaires

Stressful life events in the workplace were assessed using the following six items derived from the Social Readjustment Rating Scale (Holmes & Rahe, 1967): "collapse of the company", "disemployment", "job change", "change in job responsibility", "change to different line of work", and "merger and reorganization". Participants were asked whether they had experienced any of these six life events during the previous year using a yes/no format.

Job stress was assessed on a six-item measure derived from the Brief Job Stress Questionnaire (Shimomitsu, Yokoyama, Ono, Maruta, & Tanigawa, 1998), which was based on the job stress model of Karasek and Theorell (1990). Three items pertained to job demands (e.g. "have an extremely large amount of work to do") and three items concerned perceived job control (e.g. "can choose how and in what order to do work"). Each item was rated on a four-point Likert scale ranging from "disagree" to "agree", and the Cronbach's alpha coefficient of job demands and control items was .77 and .75, respectively. The job strain index was calculated by dividing job demands by job control. A job strain of six items indicated a balance between demands and control, with higher scores reflecting high demand coupled with low control.

Perceived stress was assessed using the Japanese version of the Perceived Stress Scale (Iwahashi, Tanaka, Fukudo, & Hongo, 2002; PSS). The PSS is a 14-item questionnaire with five response options that assess the perceived degree of stressfulness of situations over the past month (e.g. "how often have you felt that things were going your way?"). The internal reliability (alpha coefficients) range from .82 to .89.

Procedure

For collection of fingernail samples, participants were asked to grow their fingernails for ~2 weeks and provide samples from every digit by using nail clippers with a catcher to minimize tissue loss. Participants with manicured hands were asked to remove any nail polish before clipping. Fingernail samples were transferred into a Ziploc bag and frozen at −30 °C prior to the assay.

Participants also completed a questionnaire that assessed smoking status, height, weight, and frequency of nail polish use at the time of sample collection. Collection of fingernail samples and the questionnaire survey were conducted once in January 2012 or in February 2012.

Nail hormone extraction and enzyme immunoassay

Our nail hormone extraction method was identical to that used in a previous study (Izawa et al., 2015). Each sample was transferred into a 15-mL Falcon tube. Then, 5 mL of isopropanol was added, and the tube was vortexed twice for 60 s. This washing procedure was repeated two times. Samples were air-dried overnight. Dried samples were transferred to a 2.0-mL polypropylene micro-tube with a zirconia ball (diameter, 5 mm) and ground for 40 min using a mixer mill (Retsch MM300, Haan, Germany) set at 30 Hz. Fifteen milligrams of fingernail powder was transferred to another 2.0-mL micro-tube, followed by the addition of 1.5 mL of pure methanol. The micro-tube was slowly rotated for 24 h at room temperature to allow for steroid extraction. Following this, the micro-tube was centrifuged at 10,000 rpm for 2 min. One milliliter of the clear supernatant was transferred into a 50-mL Falcon tube, and subjected to evaporation for ~20 min at 60 °C until completely dry.

Cortisol level was determined by an enzyme immunoassay method using the EIA Kit (Salimetrics LLC, State College, PA). The evaporated samples were re-suspended in 100 μl of the assay diluent included in the EIA Kit, and the levels of cortisol in the diluent were analyzed according to the manufacturer's instructions. The inter-assay and intra-assay variations were <6.41 and 3.65%, respectively. The findings are presented as pg cortisol/mg fingernail (pg/mg). The lowest detectable level of cortisol is 0.56 pg/mg. We previously confirmed that the dilution curve of cortisol levels measured in serially diluted fingernail extracts significantly paralleled the cortisol standard curve from the assay kit (Izawa et al., 2015).

Statistical analyses

Cortisol levels in fingernails were logarithmically transformed (base 10) because their distribution in this sample was skewed. Further, the Smirnov–Grubbs tests for transformed values were performed to find statistical outliers. Correlational analyzes, independent *t*-tests, and a multiple linear regression analysis were subsequently conducted to evaluate the effect of stressful life events in the workplace, job strain, and perceived stress on cortisol level in fingernails. Age, gender (male/female), body mass index (BMI), and smoking status (yes/no) were included in the regression analysis.

Table 1. Demographics of the participants.

Demographic variable	*N* or mean ± SD
Male/female	94/29
Age (years), mean ± SD	43.4 ± 10.2
BMI (kg/m^2), mean ± SD	23.2 ± 3.5
Smoking status (no/yes)	91/32
Stressful life events in the workplace (0/1+)	75/48
Job strain (demand/control), mean ± SD	1.1 ± 0.5
Perceived stress, mean ± SD	22.5 ± 6.4
Cortisol level in fingernails (pg/mg), mean ± SD	4.6 ± 2.4

Table 2. Results of the multiple regression analysis for cortisol level in fingernails.

Independent variables	b (SE)		β	*p*
Gender (male/female)	−0.050	(0.044)	−.104	.263
Age	0.001	(0.002)	.051	.581
BMI	−0.002	(0.005)	−.039	.664
Smoking status (no/yes)	0.114	(0.041)	.248	.006
Stressful life events in the workplace (0/1+)	0.087	(0.036)	.209	.019
Job strain	0.056	(0.036)	.152	.125
Perceived stress	−0.003	(0.003)	−.082	.406

The distribution of the number of experienced stressful life events was skewed; we coded this variable as "0" (did not experience any stressful events) and "1" (experienced one or more stressful events).

Furthermore, the effect of nail polish could be evaluated in 19 women, and nail hormone levels were compared between women who used nail polish ($N = 11$) and those who did not ($N = 8$) using an independent *t*-test. All statistical calculations were performed using PASW Statistics 18 for Windows (SPSS Inc., Tokyo, Japan).

Results

The Smirnov–Grubbs test identified four statistical outliers ranging from 46.0 to 142.4 pg/mg, which were excluded from analyzes. The means and standard deviation (SD) of fingernail cortisol, as well as demographic and psychosocial variables, are presented in Table 1. Participants who experienced one or more stressful life events (i.e. the 1+ group) experienced an average of 1.4 (±0.5) stressful life events. Fingernail cortisol levels between women who used nail polish (mean ± SD: 4.3 ± 4.1 pg/mg) and those who did not (3.4 ± 1.0 pg/mg) were not significantly different.

The means and SD of fingernail cortisol in the participants without and with stressful life events in the workplace were 4.2 ± 2.1 and 5.2 ± 2.7 pg/mg, respectively. Cortisol levels significantly differed between the groups [$t(121) = 2.62$, $p = .010$]. Job strain and perceived stress did not correlate significantly with fingernail cortisol levels. The results of a multiple linear regression analysis are also shown in Table 2. Smoking status and experience of stressful life events in the workplace were significantly associated with higher fingernail cortisol levels.

We found a moderate correlation between job stress and perceived stress ($r = .45$, $p < .01$), implying a possibility of collinearity in the regression analysis. However, excluding job stress or perceived stress from the regression analysis did not alter the results.

Discussion

This exploratory study investigated the relationship between fingernail cortisol level and psychosocial stress (stressful life events, job stress, and perceived stress) in a sample of middle-aged workers. We found that stressful life events in the workplace, but not job strain and perceived stress, were associated with higher cortisol levels in fingernails after adjusting for the effects of demographic factors. Fingernail samples reflect cumulative hormonal exposure in the past. Therefore, it could be interpreted that fingernail cortisol is associated with psychosocial stress in the past (stressful life events in the previous year), rather than in the present (job stress and perceived stress). Life events in the workplace such as changing to a different line of work are generally recognized as stressful (Holmes & Rahe, 1967). A recent retrospective study (Grassi-Oliveira et al., 2012) found a positive association between hair cortisol levels and number of negative life events, consistent with the findings of this study. To the best of our knowledge, this is the first study to demonstrate a relationship between fingernail cortisol level and psychosocial stress.

In this study, we also investigated the effects of demographic variables on fingernail cortisol, and found that smokers had higher fingernail cortisol levels. This result was consistent with previous findings on salivary cortisol, in which smokers exhibited higher cortisol levels (e.g. Steptoe & Ussher, 2006). However, we did not find significant associations between fingernail cortisol and other demographic variables (age, gender, BMI, and manicure use). BMI has been frequently reported to be associated with hair cortisol (Stalder et al., 2012). However, the lower mean BMI and comparatively limited BMI range of this study could have affected its results.

Fingernail samples could have some advantages, compared with use of saliva and hair samples. Salivary cortisol is known to have large diurnal rhythms and reflect acute increases in hormones, which would confound an investigation of the relationship between cortisol and chronic stress. In contrast, fingernail cortisol level would not be affected by such transient increases and diurnal rhythms. Furthermore, many strands are needed for the measurement of hair cortisol, and hair self-sampling may be difficult for some participants. In contrast, fingernail samples can be self-collected, and only small amounts are required.

This study has certain limitations, which warrant careful interpretation of its findings. First, in this study, we only investigated fingernail cortisol, but not salivary or hair cortisol. More information on relationships between fingernail and salivary or hair cortisol is a key component to advancing the study of fingernail cortisol. Second, we did not account for individual differences in nail growth rate, which could affect observed steroid concentrations. Further, in this restricted study design, participants clipped their fingernails at home (i.e. not in accurately controlled conditions). Third, we did not assess the effects of stressful life events outside the workplace or those of positive life events. While we did ask

participants whether they experienced stressful life events outside the workplace (e.g. death of spouse, divorce, marital separation), the experience rate of most events was quite low, which made it difficult to correlate these events with hormone levels. Fourth, for the assessment of job stress, we did not clearly define the time period of job stress that participants should consider. However, reported job stress may largely reflect participants' experience of stress in the present, but not in the past, because job stress is relatively unstable and variable across time (Kawada & Otsuka, 2014). Fifth, the sample size in this study was small, particularly that of the nail polish use analysis. Therefore, associations of psychosocial stress with fingernail cortisol could not be investigated separately for male and female participants. Furthermore, this study was conducted in healthy middle-aged workers, so not all age groups were represented in our sample.

In conclusion, we demonstrated that experience of stressful life events in the workplace in the previous year was associated with elevated fingernail cortisol level in a sample of middle-aged workers. Thus, we provided evidence that fingernail samples may retrospectively reflect individual differences in cortisol levels related to past psychosocial stress.

Disclosure statement

The authors report no conflicts of interest.

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References

de Berker, D.A., André, J., & Baran, R. (2007). Nail biology and nail science. *International Journal of Cosmetic Science*, *29*, 241–275. doi: 10.1111/j.1467-2494.2007.00372.x

Buzalaf, M.A., Pessan, J.P., & Alves, K.M. (2006). Influence of growth rate and length on fluoride detection in human nails. *Caries Research*, *40*, 231–238. doi: 10.1159/000092231

Dickerson, S.S., & Kemeny, M.E. (2004). Acute stressors and cortisol responses: a theoretical integration and synthesis of laboratory research. *Psychological Bulletin*, *130*, 355–391. doi: 10.1037/0033-2909.130.3.355

Eller, N.H., Netterstrøm, B., Gyntelberg, F., Kristensen, T.S., Nielsen, F., Steptoe, A., & Theorell, T. (2009). Work-related psychosocial factors and the development of ischemic heart disease: *a systematic review*. *Cardiology Review*, *17*, 83–97. doi: 10.1097/CRD.0b013e318198c8e9

Grassi-Oliveira, R., Pezzi, J.C., Daruy-Filho, L., Viola, T.W., Francke, I.D., Leite, C.E., & Brietzke, E. (2012). Hair cortisol and stressful life events retrospective assessment in crack cocaine users. *The American Journal of Drug and Alcohol Abuse*, *38*, 535–538. doi: 10.3109/00952990.2012.694538

Gupta, G.R., Dhruw, V.K., Athawal, B.K., Siddiqui, P., Yousuf Agrawal, H.K., & Chandra, H. (2005). Human nail growth pattern and medicolegal aspect. *Journal of Indian Academy of Forensic Medicine*, *27*, 87–91. Retrieved from: http://medind.nic.in/jal/t05/i2/jalt05i2p87.pdf

Holmes, T.H., & Rahe, R.H. (1967). The Social Readjustment Rating Scale. *Journal of Psychosomatic Research*, *11*, 213–218. doi: 10.1016/0022-3999(67)90010-4

Iwahashi, S., Tanaka, Y., Fukudo, S., & Hongo, M. (2002). The development of the Japanese version of the Perceived Stress Scale. *Shinshinigaku*, *42*, 459–466. [in Japanese].

Izawa, S., Miki, K., Tsuchiya, M., Mitani, T., Midorikawa, T., Fuchu, T., … Togo, F. (2015). Cortisol level measurements in fingernails as a retrospective index of hormone production. *Psychoneuroendocrinology*, *54*, 24–30. doi: 10.1016/j.psyneuen.2015.01.015

Karasek, R.A., & Theorell, T. (1990). *Healthy work*. New York: Basic Books.

Kawada, T., & Otsuka, T. (2014). Change in job stress and job satisfaction over a two-year interval using the Brief Job Stress Questionnaire. *Work*, *49*, 107–111. doi: 10.3233/WOR-131658

Russell, E., Koren, G., Rieder, M., & Van Uum, S. (2012). Hair cortisol as a biological marker of chronic stress: current status, future directions and unanswered questions. *Psychoneuroendocrinology*, *37*, 589–601. doi: 10.1016/j.psyneuen.2011.09.009

Shimomitsu, T., Yokoyama, K., Ono, Y., Maruta, T., & Tanigawa, T. (1998). Development of a novel brief of job stress questionnaire. In S. Kato (Ed), *Report of the research grant for the prevention of work-related diseases from the Ministry of Labour (in Japanese)* (pp. 107–112). Tokyo: Japanese Ministry of Labour.

Stalder, T., Steudte, S., Alexander, N., Miller, R., Gao, W., Dettenborn, L., & Kirschbaum, C. (2012). Cortisol in hair, body mass index and stress-related measures. *Biological Psychology*, *90*, 218–223. doi: 10.1016/j.biopsycho.2012.03.010

Steptoe, A., & Ussher, M. (2006). Smoking, cortisol and nicotine. *International Journal of Psychophysiology: Official Journal of the International Organization of Psychophysiology*, *59*, 228–235. doi: 10.1016/j.ijpsycho.2005.10.011

Warnock, F., McElwee, K., Seo, R.J., McIsaac, S., Seim, D., Ramirez-Aponte, T., … Young, A.H. (2010). Measuring cortisol and DHEA in fingernails: a pilot study. *Neuropsychiatric Disease and Treatment*, *6*, 1–7. Retrieved from: https://www.ncbi.nlm.nih.gov/pubmed/20169040

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ORIGINAL ARTICLE

Ikarisoside A inhibits acetylcholine-induced catecholamine secretion and synthesis by suppressing nicotinic acetylcholine receptor-ion channels in cultured bovine adrenal medullary cells

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Abstract Ikarisoside A is a natural flavonol glycoside derived from plants of the genus *Epimedium*, which have been used in Traditional Chinese Medicine as tonics, antirheumatics, and aphrodisiacs. Here, we report the effects of ikarisoside A and three other flavonol glycosides on catecholamine secretion and synthesis in cultured bovine adrenal medullary cells. We found that ikarisoside A (1–100 μM), but not icariin, epimedin C, or epimedoside A, concentration-dependently inhibited the secretion of catecholamines induced by acetylcholine, a physiological secretagogue and agonist of nicotinic acetylcholine receptors. Ikarisoside A had little effect on catecholamine secretion induced by veratridine and 56 mM K^+. Ikarisoside A (1–100 μM) also inhibited $^{22}Na^+$ influx and $^{45}Ca^{2+}$ influx induced by acetylcholine in a concentration-dependent manner similar to that of catecholamine secretion. In *Xenopus* oocytes expressing α3β4 nicotinic acetylcholine receptors, ikarisoside A (0.1–100 μM) directly inhibited the current evoked by acetylcholine. It also suppressed ^{14}C-catecholamine synthesis and tyrosine hydroxylase activity induced by acetylcholine at 1–100 μM and 10–100 μM, respectively. The present findings suggest that ikarisoside A inhibits acetylcholine-induced catecholamine secretion and synthesis by suppression of nicotinic acetylcholine receptor-ion channels in bovine adrenal medullary cells.

Keywords Adrenal medulla · Catecholamine secretion · *Epimedium* · Flavonoids · Ikarisoside A · Nicotinic acetylcholine receptor

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Introduction

Flavonoids, a group of secondary metabolites with variable phenolic structure, which exist widely in plants (Nijveldt et al. 2001; Ren and Zuo 2012), may exert potential benefits associated with reduced risks of age- and life style-related diseases such as cardiovascular diseases, diabetes, and some cancers (Lu et al. 2013; Yanagihara et al. 2014). Ikarisoside A is one of the flavonol derivatives derived from plants of the genus *Epimedium*, which have been used in Traditional Chinese Medicine as tonics, antirheumatics, and aphrodisiacs (Dou et al. 2006). Previous studies reported that the total flavonoid fraction of *Epimedium* extract suppresses urinary calcium excretion and improves bone properties in ovariectomized mice (Chen et al. 2011), and that other extracts with structures similar to that of ikarisoside A, such as icariin, can stimulate osteogenic activities (Zhou et al. 2013) and have

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anti-inflammatory effects (Lai et al. 2013). Furthermore, ikarisoside A also has pharmacological effects such as antioxidant and anti-inflammatory effects (Choi et al. 2008) as well as anti-osteoporosis effects (Choi et al. 2010).

In the human body, the most abundant catecholamines are adrenaline, noradrenaline, and dopamine, all of which are produced from phenylalanine and/or tyrosine. Catecholamines are produced mainly in the chromaffin cells of the adrenal medulla, the postganglionic fibers of the sympathetic nervous system, and the central nervous system. Catecholamines play very important roles in heart rate, blood pressure, blood glucose levels, and the general reactions of the sympathetic nervous system.

Adrenal medullary cells derived from embryonic neural crests are functionally homologous to sympathetic postganglionic neurons. In bovine adrenal medullary cells, catecholamine secretion is associated with the activation of three types of ionic channels: nicotinic acetylcholine receptor (nAChR)-ion channels, voltage-dependent Na^+ channels, and voltage-dependent Ca^{2+} channels (Wada et al. 1985b). ACh induces Na^+ influx via nAChR-ion channels, then, it induces Ca^{2+} influx and subsequent catecholamine secretion (Wada et al. 1985b). On the other hand, stimulation of catecholamine synthesis induced by ACh is associated with the activation of tyrosine hydroxylase in cultured bovine adrenal medullary cells (Yanagihara et al. 1987; Tsutsui et al. 1994). The conversion of tyrosine to L-3,4-dihydroxyphenylalanine (DOPA) is the rate-limiting step of catecholamine biosynthesis (Nagatsu et al. 1964). Adrenal medullary cells have provided a good model for the detailed analysis of a drug's actions on catecholamine secretion and synthesis (Kajiwara et al. 2002; Toyohira et al. 2005; Shinohara et al. 2007).

In our previous study, we isolated 20 flavonol glycosides from *Epimedium* species, including ikarisoside A, icariin, epimedoside A, and epimedin C (Mizuno et al. 1988). Ikarisoside A showed neurite outgrowth activity in cultured PC12h cells (Kuroda et al. 2000). There is, however, little evidence regarding ikarisoside A's effects on sympathetic nervous system activity. In the present study, we investigated the effects of four flavonol glycosides on bovine adrenal medullary cell functions and found that ikarisoside A, but not the other three flavonol glycosides, inhibited ACh-induced catecholamine secretion and synthesis by suppression of nAChR-ion channels in the cells.

Materials and methods

Materials

Oxygenated Krebs-Ringer phosphate (KRP) buffer was used throughout unless stated otherwise. Its composition is as follows (in mM): 154 NaCl, 5.6 KCl, 1.1 $MgSO_4$, 2.2 $CaCl_2$, 0.85 NaH_2PO_4, 2.15 Na_2HPO_4, and 10 glucose, adjusted to pH 7.4. Drugs and reagents were obtained from the following sources: Eagle's minimum essential medium (Eagle's MEM) (Nissui Pharmaceutical, Tokyo, Japan); collagenase (Nitta Zerachin, Osaka, Japan); calf serum (Cell Culture Technologies, Gravesano, Switzerland). ACh and veratridine were from Sigma (St. Louis, MO, USA). L-[U-^{14}C]tyrosine was from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA); $^{45}CaCl_2$, $^{22}NaCl$, and L-[1-^{14}C]tyrosine from Perkin-Elmer Life Sciences (Boston, MA, USA).

Isolation of flavonol glycosides from the leaves of *Epimedium* species

The leaves of *Epimedium diphyllum* were collected at Miyazaki Prefecture, Japan. Ikarisoside A and other flavonol glycosides were purified by high performance liquid chromatography, as reported previously (Mizuno et al. 1988). Ikarisoside A and other flavonol glycosides were dissolved in 100 % dimethyl sulfoxide (DMSO) and then diluted in a reaction medium before use at a final DMSO concentration not exceeding 0.5 %, unless otherwise specified. DMSO (0.5 %) did not influence the basal and ACh-induced catecholamine secretion in the present study (data not shown).

Primary culture of bovine adrenal medullary cells

Bovine adrenal medullary cells were isolated by collagenase digestion of adrenal medullary slices according to the method as reported previously (Yanagihara et al. 1979, 1996). Cells were suspended in Eagle's MEM containing 10 % calf serum, 3 μM cytosine arabinoside, and several antibiotics, and maintained in monolayer culture at a density of 4×10^6 cells/dish (35 mm dish; Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) or 10^6 cells/well (24-well plate; Corning Life Sciences, Lowell, MA, USA) at 37 °C under a humidified atmosphere of 5 % CO_2 and 95 % air. The cells were used for experiments between 2 and 5 days of culture.

Catecholamine secretion from cultured bovine adrenal medullary cells

The secretion of catecholamines was measured as described previously (Yanagihara et al. 1979). Cells (10^6/well) were washed three times with oxygenated KRP buffer, then firstly preincubated with or without ikarisoside A (0.3–100 μM) or other flavonol glycosides (10 μM) at 37 °C for 10 min, and incubated with or without ikarisoside A (0.3–100 μM) or other flavonol glycosides (10 μM) in the presence or absence of various secretagogues (300 μM ACh, 100 μM veratridine or 56 mM K^+) at 37 °C for another 10 min. After the reaction, the incubation medium was transferred immediately to a test tube containing perchloric acid (final concentration, 0.4 M) for the

full stop of the reaction. Catecholamines (noradrenaline and adrenaline) secreted into the medium were adsorbed onto aluminum hydroxide and estimated by the ethylenediamine condensation method (Weil-Malherbe and Bone 1952) using a fluorescence spectrophotometer (F-2500; Hitachi, Tokyo, Japan) with excitation and emission wavelengths of 420 and 540 nm, respectively.

$^{22}Na^{+}$ and $^{45}Ca^{2+}$ influx

The influx of $^{22}Na^{+}$ and $^{45}Ca^{2+}$ was measured as reported previously (Wada et al. 1985a, b). After preincubation with or without ikarisoside A (0.3–100 μM) at 37 °C for 10 min, cells (4×10^6/dish) were incubated with 1.5 μCi of $^{22}NaCl$ or 1.5 μCi of $^{45}CaCl_2$ at 37 °C for 5 min with or without 300 μM ACh and ikarisoside A (0.3–100 μM) in KRP buffer. After incubation, the cells were washed three times with ice-cold KRP buffer, solubilized in 10 % Triton X-100, and counted for radioactivity of $^{22}Na^{+}$ and $^{45}Ca^{2+}$ by a gamma counter (ARC-2005, Aloka, Tokyo, Japan) and a liquid scintillation counter (TRI-CARB 2900TR, PACKARD INSTRUMENT CO., Meriden, CT, USA), respectively.

^{14}C-Catecholamine synthesis from [^{14}C]tyrosine in the cells

After preincubation for 10 min, cells (4×10^6/dish) were incubated with 20 μM L-[U-^{14}C]tyrosine (1.0 μCi) KRP buffer in the presence or absence of various concentrations of ikarisoside A (0.3–100 μM) and 300 μM ACh at 37 °C for 20 min. After removing the incubation medium by aspiration, cells were harvested in 0.4 M perchloric acid and centrifuged at 1600×*g* for 10 min. ^{14}C-Catecholamines were separated further by ion exchange chromatography on Duolite C-25 columns (H^{+}-type, 0.4 × 7.0 cm) (Yanagihara et al. 1987) and counted for the radioactivity by a liquid scintillation counter (TRI-CARB 2900TR, PACKARD INSTRUMENT CO., Meriden, CT, USA). ^{14}C-Catecholamine synthesis was expressed as the sum of the ^{14}C-catecholamines (adrenaline, noradrenaline, and dopamine).

Tyrosine hydroxylase activity in situ

After preincubation with or without ikarisoside A (0.3–100 μM) for 10 min, cells (10^6/well) were exposed to 200 μl of KRP buffer with or without ikarisoside A (0.3–100 μM) and 300 μM ACh, supplemented with 18 μM L-[1-^{14}C]tyrosine (0.2 μCi) for 10 min at 37 °C. Upon addition of the labeled tyrosine, each well was sealed immediately with an acrylic tube capped with a rubber stopper and fitted with a small plastic cup containing 200 μl of NCS-II tissue solubilizer (GE Healthcare UK Ltd. Little Chalfont, Buckinghamshire, UK) to absorb the $^{14}CO_2$ released by the cells and counted for the radioactivity (Bobrovskaya et al. 1998).

Expression of α3β4 nAChRs in *Xenopus* oocytes and electrophysiological recordings

The complementary DNAs (cDNAs) encoding the α3 and β4 subunits of rat neuronal nAChR, subcloned into pcDNAI/Neo (Invitrogen, Carlsbad, CA) vector, were kindly provided from Dr. James W. Patrick (Division of Neuroscience, Baylor College of Medicine, TX, USA). After linearization of cDNA with *Not*I, complementary RNAs (cRNAs) were transcribed using T7 RNA polymerase from the mMESSAGE mMACHINE kit (Ambion, Austin, TX, USA). Adult female *Xenopus laevis* frogs were obtained from Kyudo Co., Ltd. (Saga, Japan). *Xenopus* oocytes and cRNA microinjection were prepared as described previously (Ueno et al. 2004; Horishita and Harris 2008). cRNAs of α3 and β4 subunits were co-injected at a same ratio (10–20 ng/50 nL) into *Xenopus* oocytes, and electrophysiological recordings were performed 2–6 days after injection. Oocytes were placed in a 100 μl recording chamber and perfused at 2 ml/min with extracellular Ringer solution (110 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1.8 mM $BaCl_2$, pH 7.5) containing 1.0 μM atropine sulfate. Ca^{2+} in the solution was replaced with Ba^{2+} to minimize the effects of secondarily activated Ca^{2+}-dependent Cl^{-} channels. Recording electrodes (1–3 MΩ) were filled with 3 M KCl, and the whole-cell voltage clamp was achieved through these two electrodes using a Warner Instruments model OC-725C (Warner, Hamden, CT, USA) at −70 mV. We measured the peak of the transient inward current in response to ACh that was applied for 30 s and examined the effects of ikarisoside A on a concentration of ACh that produced 50 % of the maximal effect (EC_{50}) of ACh. The EC_{50} was determined for each oocyte by 1 mM ACh that produces a maximal current. Ikarisoside A stocks were prepared in 100 % DMSO and diluted in bath solution to a final DMSO concentration not exceeding 0.1 %. Ikarisoside A was preapplied for 2 min to allow an equilibration with its site of interaction before ACh was added and its effect on the cation currents was determined. In all cases, between two currents, there was 10 min interval under washing with normal Ringer solution.

Statistical analysis

All experiments were performed in duplicate or triplicate, and each experiment was repeated at least three times. All values are given as means ± SEM. The significance of differences between means was evaluated using one-way analysis of variance (ANOVA). When a significant *F* value was found by ANOVA, Dunnett's or Scheffe's test for multiple comparisons was used to identify differences among the groups. Values were considered statistically different when *P* was less than

0.05. Statistical analyses were performed using PRISM for Windows version 5.0J software (Abacus Concept, Berkeley, CA, USA).

Results

The structures of four flavonol glycosides isolated from *Epimedium*

The four flavonol glycosides ikarisoside A, icariin, epimedin C, and epimedoside A were isolated from the leaves of *E. diphyllum* as reported previously (Mizuno et al. 1988). The structures of these four flavonol glycosides are shown in Fig. 1.

Effects of the flavonol glycosides on catecholamine secretion induced by various secretagogues in adrenal medullary cells

None of the four flavonol glycosides (ikarisoside A, icariin, epimedin C, and epimedoside A) at 10 μM significantly affected the basal secretion of catecholamines (Fig. 2a). ACh (300 μM), an agonist of nAChRs, caused catecholamine secretion corresponding to 18.90 ± 0.38 % of the total catecholamines in the cells. When the cells were treated with the same four flavonol glycosides at 10 μM for 10 min, ikarisoside A strongly reduced catecholamine secretion induced by ACh, to 6.83 ± 0.51 % of the total, whereas the other three had little effect (Fig. 2a). Veratridine (100 μM), an activator of voltage-dependent Na^+ channels, and 56 mM K^+, which depolarizes cell membranes and then activates voltage-dependent Ca^{2+} channels, also caused catecholamine secretion corresponding to 26.52 ± 0.88 % (Fig. 2b) and 20.51 ± 0.70 % (Fig. 2c) of the total catecholamines, respectively. Treatment of cells with these flavonol glycosides at 10 μM did not affect catecholamine secretion induced by veratridine (Fig. 2b) and 56 mM K^+ (Fig. 2c).

Concentration-inhibition curves for the effects of ikarisoside A on catecholamine secretion, $^{45}Ca^{2+}$ influx, and $^{22}Na^+$ influx induced by ACh

We examined the effects of ikarisoside A on catecholamine secretion, $^{45}Ca^{2+}$ influx, and $^{22}Na^+$ influx induced by ACh. Treatment of cells with ikarisoside A at 1, 3, 10, 30, and 100 μM significantly inhibited ACh-induced secretion of catecholamines (18.22 ± 0.16 % of the total catecholamines in the cells) to 15.36 ± 0.38 %, 12.27 ± 0.40 %, 7.68 ± 0.30 %, 5.33 ± 0.47 %, and 4.95 ± 0.25 % of the total catecholamines in the cells, respectively (Fig. 3a). Ikarisoside A also inhibited ACh-induced $^{45}Ca^{2+}$ influx and $^{22}Na^+$ influx in a concentration-dependent manner (Fig. 3b, c). The half-maximal inhibitory concentration (IC_{50}) of ikarisoside A in catecholamine secretion, $^{45}Ca^{2+}$ influx, and $^{22}Na^+$ influx are 4.00, 9.90, and 2.96 μM, respectively.

Inhibitory mode of ikarisoside A on ACh-induced catecholamine secretion in adrenal medullary cells

To investigate the mechanism by which ikarisoside A inhibits ACh-induced catecholamine secretion, we examined whether or not the inhibitory effect of ikarisoside A on catecholamine secretion is overcome when the ACh concentration is

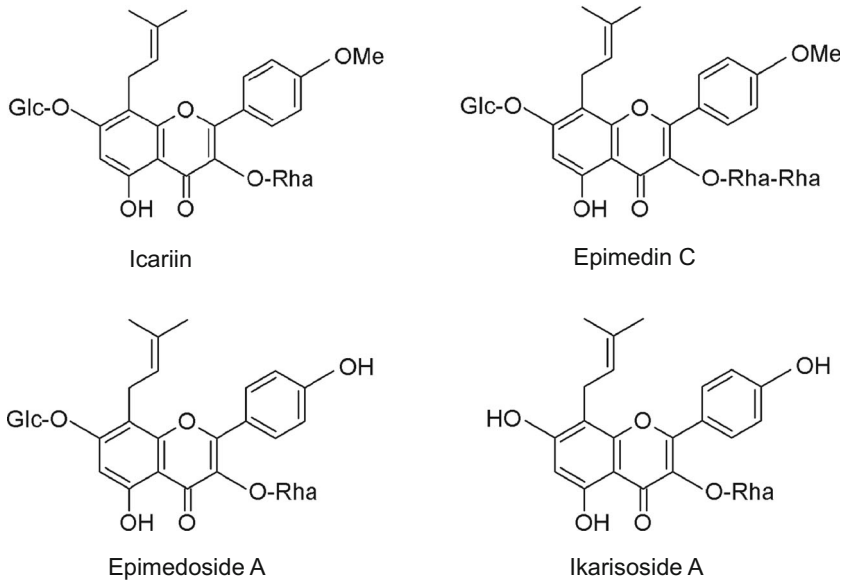

Fig. 1 Chemical structures of icariin, epimedin C, epimedoside A, ikarisoside A. The abbreviations Glc and Rha in the structures are glucose and rhamnose, respectively

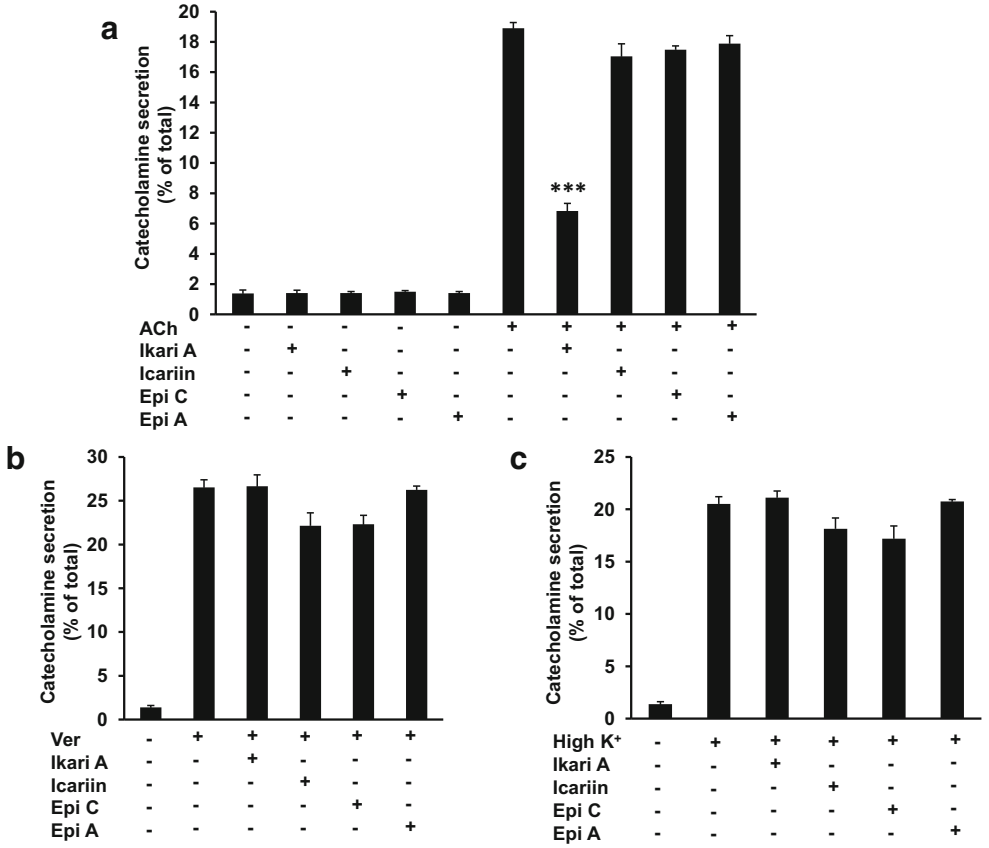

Fig. 2 Effects of ikarisoside A, icariin, epimedin C, or epimedoside A on catecholamine secretion induced by various secretagogues in cultured bovine adrenal medullary cells. After preincubation with cells with or without ikarisoside A (Ikari A) (10 μM), icariin (10 μM), epimedin C (Epi C) (10 μM), and epimedoside A (Epi A) (10 μM) for 10 min, the cells (10^6/well) were incubated with or without these four flavonol glycosides (10 μM), ACh (300 μM) (**a**), veratridine (100 μM) (**b**), or 56 mM K^+ (**c**) for another 10 min at 37 °C. Catecholamines secreted into the medium were expressed as a percentage of the total catecholamines in the cells. Data are means ± SEM from three separate experiments carried out in triplicate. ***$P < 0.001$, compared with ACh alone (analyzed by one-way ANOVA with Dunnett's multiple comparison post hoc test)

increased. Even when the ACh concentrations in the incubation medium increased from 3 to 300 μM, they did not overcome the inhibitory effect of ikarisoside A (Fig. 4a). Double-reciprocal plot analysis revealed that ikarisoside A exerts a noncompetitive type of inhibition on ACh-induced secretion of catecholamines (Fig. 4b).

Effects of ikarisoside A on ACh-induced inward current in *Xenopus* oocytes expressing α3β4 nAChRs

We examined the direct effects of ikarisoside A on ACh responses in *Xenopus* oocytes expressing rat α3β4 nAChRs. As shown in Fig. 5a, ikarisoside A reversibly inhibited ACh (0.2 mM)-induced currents. Ikarisoside A inhibited ACh-induced currents concentration dependently. It suppressed those currents to 80 ± 3 %, 69 ± 6 %, 43 ± 6 %, 32 ± 8 %, and 22 ± 5 % of the control at 0.1, 0.3, 1, 3, and 10 μM, respectively, and the inhibitory effects were significant from 0.10 μM onward; the IC_{50} was 0.48 μM (Fig. 5b).

Effect of aglycon of ikarisoside A on ACh-induced secretion of catecholamines

Ikarisoside A is a flavonol glycoside having one rhamnose at the 3 position in the chemical structure. 3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-8-(3-methylbut-2-enyl)-4*H*-chromen-4-one is the aglycon of ikarisoside A. We examined the effect of this aglycon on ACh-induced secretion of catecholamines. As shown in Fig. 6, the aglycon of ikarisoside A did not affect basal or ACh-induced secretion of catecholamines.

Effect of ikarisoside A on ^{14}C-catecholamine synthesis from [^{14}C]tyrosine and tyrosine hydroxylase activity

As shown in Fig. 7a, ACh (300 μM) increased the synthesis of ^{14}C-catecholamines from [^{14}C]tyrosine about 3-fold in bovine adrenal medullary cells. The concurrent treatment of cells with ikarisoside A inhibited the stimulatory effect of 300 μM ACh on ^{14}C-catecholamine synthesis in a concentration (1–

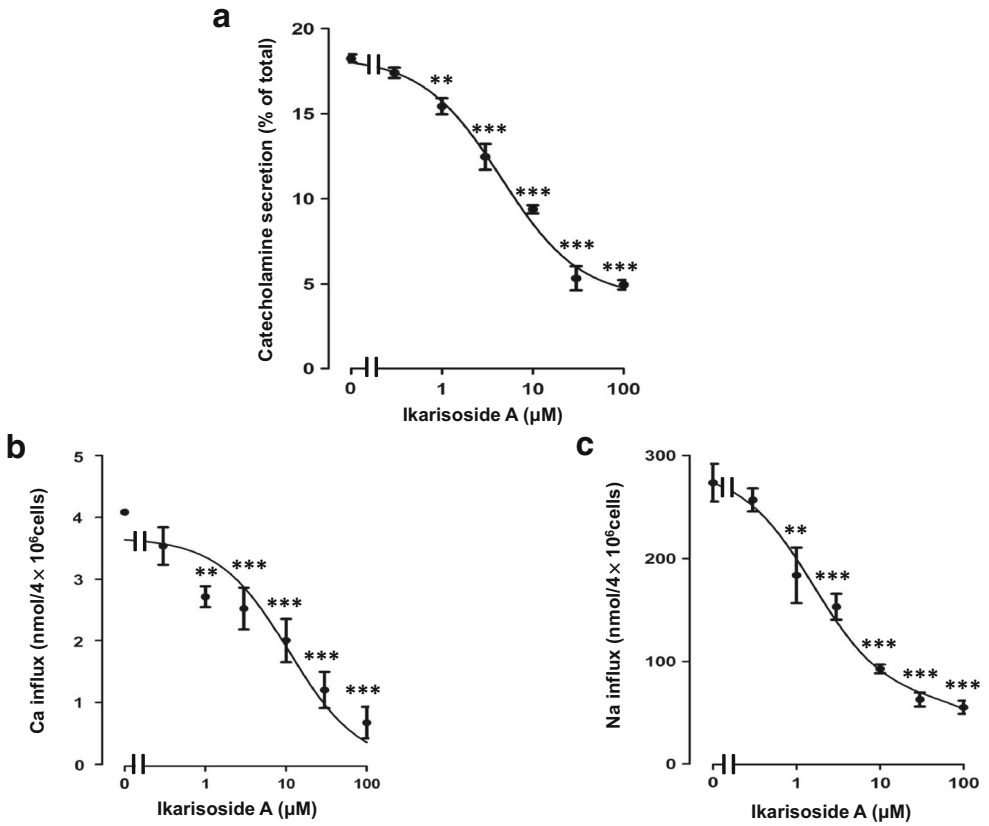

Fig. 3 Effects of ikarisoside A on catecholamine secretion (**a**), $^{45}Ca^{2+}$ influx (**b**), and $^{22}Na^{+}$ influx (**c**) induced by ACh. (**a**) After preincubation for 10 min with or without ikarisoside A (0.3–100 μM), cells were stimulated with ACh (300 μM) in the presence or absence of ikarisoside A (0.3–100 μM) for another 10 min at 37 °C. Catecholamines secreted into the medium were expressed as a percentage of the total catecholamines in the cells. **b**, **c** After preincubation for 10 min, cells were stimulated with ACh (300 μM) and 1.5 μCi of $^{45}CaCl_2$ (**b**) or $^{22}NaCl$ (**c**) in the presence or absence of ikarisoside A (0.3–100 μM) for another 5 min at 37 °C. $^{45}Ca^{2+}$ influx and $^{22}Na^{+}$ influx were measured and were expressed as nmol/4 × 10^6 cells. Data are means ± SEM from three separate experiments carried out in triplicate. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with ACh alone (analyzed by one-way ANOVA with Dunnett's multiple comparison post hoc test)

100 μM)-dependent manner (Fig. 7a), yielding an IC_{50} value of 2.85 μM. Ikarisoside A (1–100 μM) had little effect on the basal synthesis of ^{14}C-catecholamines.

We next examined the effect of ikarisoside A on tyrosine hydroxylase activity in the cells. After preincubation with or without ikarisoside A (0.1–100 μM) for 10 min, cells were incubated with 300 μM ACh in the absence or presence of ikarisoside A (0.1–100 μM) for another 10 min at 37 °C. Ikarisoside A (10–100 μM) inhibited the tyrosine hydroxylase activity induced by ACh and tended to inhibit the basal enzyme activity (Fig. 7b). The IC_{50} value of ikarisoside A for its inhibitory effect on the ACh-induced tyrosine hydroxylase activity was 9.13 μM (derived from the curve representing the difference between stimulated and basal tyrosine hydroxylase; not shown).

Discussion

In present study, we investigated the effects of four flavonol glycosides derived from the leaves of the genus *Epimedium*. We demonstrated that ikarisoside A, but not the other three, inhibited the secretion and synthesis of catecholamines induced by ACh in cultured bovine adrenal medullary cells. To our knowledge, this is the first direct evidence of an inhibitory effect of ikarisoside A on catecholamine secretion and synthesis in cultured bovine adrenal medullary cells.

Inhibitory effect of ikarisoside A on catecholamine secretion induced by ACh

The present study demonstrated that ikarisoside A significantly inhibits catecholamine secretion induced by ACh, but not by veratridine or 56 mM K^+ in adrenal medullary cells. We previously reported that ACh activates nAChR-ion channels, and induces Na^+ influx, subsequent Ca^{2+} influx, and finally catecholamine secretion. On the other hand, veratridine activates voltage-dependent Na^+ channels and 56 mM K^+ depolarizes cell membranes to activate voltage-dependent Ca^{2+} channels (Wada et al. 1984, 1985b). In the present study, ikarisoside A did not inhibit the stimulatory effects of veratridine and 56 mM K^+ on catecholamine secretion. Therefore,

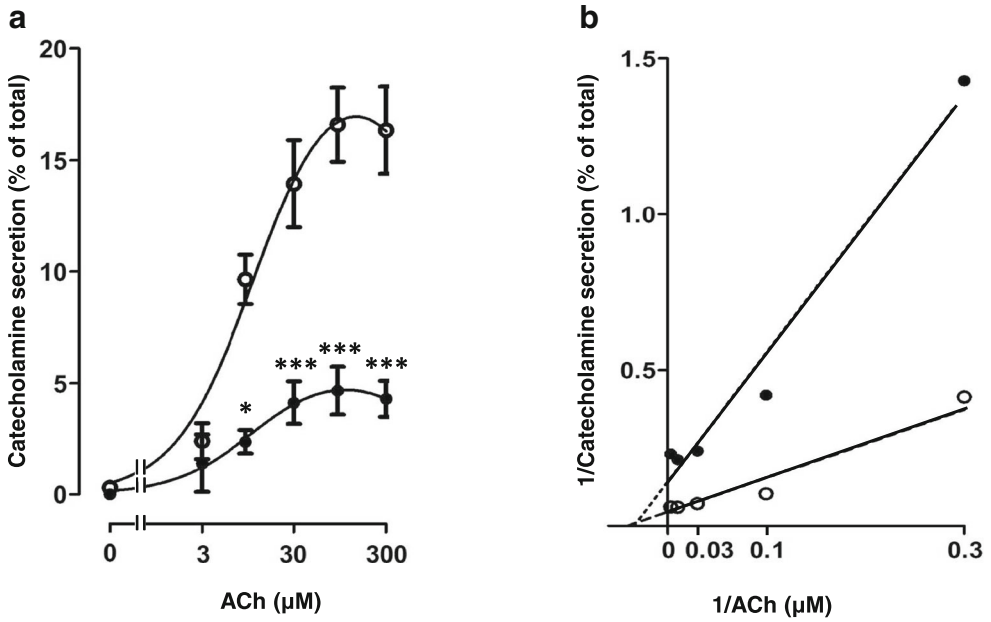

Fig. 4 Inhibitory mode of ikarisoside A on catecholamine secretion induced by ACh. **a** After preincubation for 10 min, cells were stimulated with (*black circle*) or without (*white circle*) ikarisoside A (10 μM) in the presence or absence of ACh (1–300 μM) for another 10 min at 37 °C. Catecholamines secreted into the medium were expressed as a percentage of the total catecholamines in the cells. Data are means ± SEM from three separate experiments carried out in triplicate. The data of ACh plus ikarisoside A are shown by subtracting basal secretion obtained in the presence of ikarisoside A. $*P < 0.05$ and $***P < 0.001$, compared with ACh alone (analyzed by one-way ANOVA with Dunnett's multiple comparison post hoc test). **b** Double-reciprocal plot analysis of the data in (**a**)

ikarisoside A seems to inhibit nAChR-ion channels but not voltage-dependent Na^+ channels or voltage-dependent Ca^{2+} channels. Ikarisoside A inhibited Ca^{2+} influx and Na^+ influx induced by ACh in a concentration-dependent manner similar

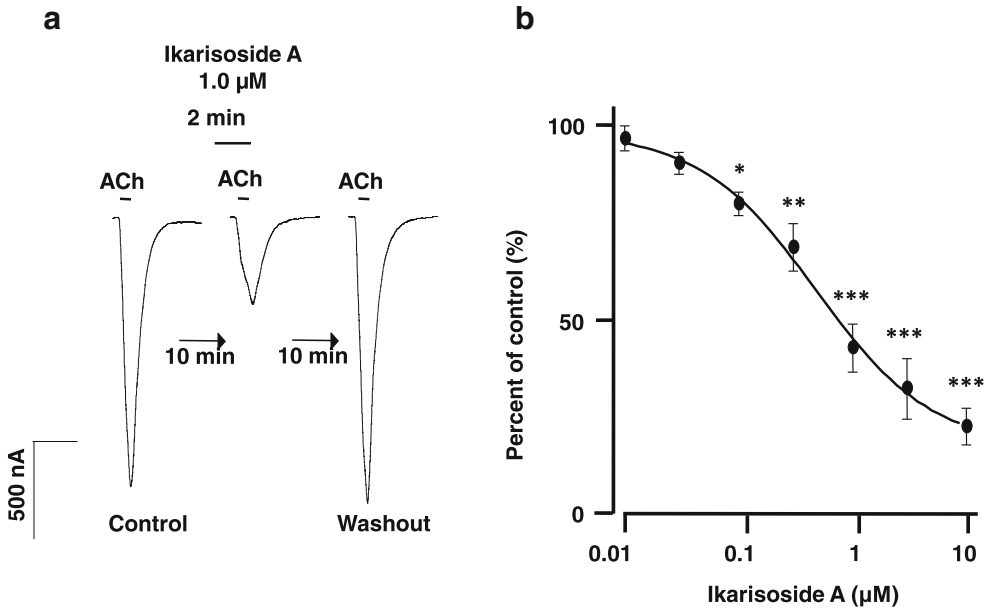

Fig. 5 Effects of ikarisoside A on peak ACh-induced inward currents in *Xenopus* oocytes expressing rat α3β4 nAChRs. **a** Representative traces from a single *Xenopus* oocyte are shown. The currents of ikarisoside A-treated oocytes were recorded 10 min after recording of the control currents, and the washout currents were obtained 10 min after ikarisoside A treatment. Ikarisoside A (1 μM) suppressed the currents induced by the EC_{50} (0.2 mM) of ACh, and the inhibitory effects were reversible. **b** Concentration-response curve for the inhibitory effects of ikarisoside A on ACh-induced currents. The peak current amplitude in the presence of ikarisoside A was normalized to that of the control and the effects are expressed as percentages of the control. Data are presented as means ± SEM from four separate experiments carried out in triplicate. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, compared to the control (based on one-way ANOVA with Dunnett's multiple comparison post hoc test). Nonlinear regression analysis was performed and the mean value of IC_{50} for ikarisoside A is 0.48 μM

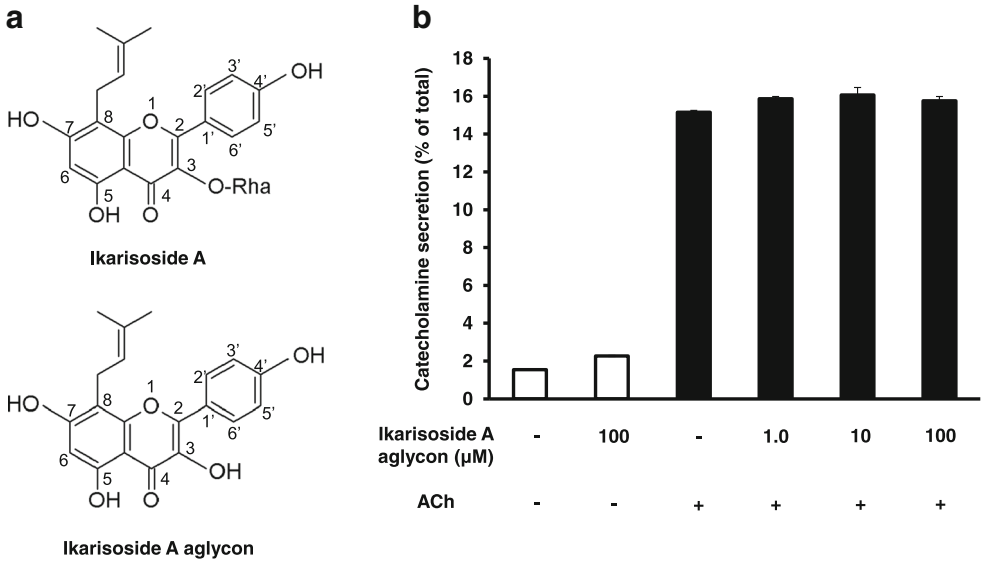

Fig. 6 Structure of ikarisoside A and its aglycon (**a**) and effect of aglycon of ikarisoside A on ACh-induced catecholamine secretion (**b**). **a** Structure of ikarisoside A and its aglycon (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-8-(3-methylbut-2-enyl)-4*H*-chromen-4-one). **b** After preincubation with cells with or without aglycon of ikarisoside A (1–100 μM) for 10 min, the cells (10^6/well) were incubated with or without aglycon of ikarisoside A (1–100 μM) and ACh (300 μM) for another 10 min at 37 °C. Catecholamines secreted into the medium were expressed as a percentage of the total catecholamines in the cells. Data are means ± SEM from three separate experiments carried out in triplicate

to that of catecholamine secretion. In the exocytotic secretion of catecholamines, Ca^{2+} plays an indispensable role as the coupler in the stimulus-secretion coupling (Douglas and Rubin 1961, 1963). From these findings, it is likely that ikarisoside A inhibits ACh-induced catecholamine secretion by suppressing nAChR-ion channels. We investigated the inhibitory mode of ikarisoside A on nAChR-ion channels. Even when the concentration of ACh was increased, the inhibitory

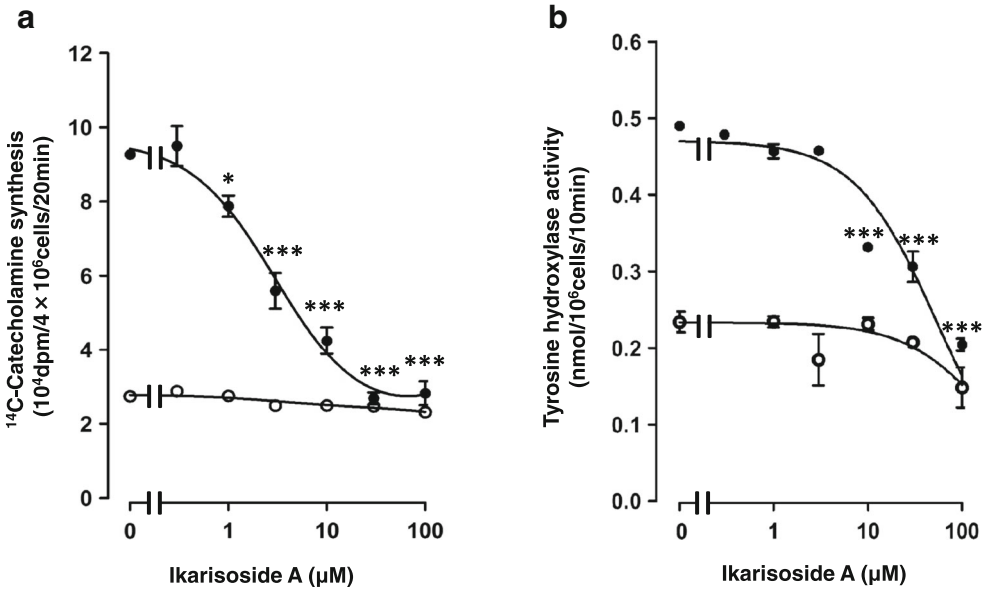

Fig. 7 Effects of ikarisoside A on ^{14}C-catecholamine synthesis from [^{14}C]tyrosine (**a**) and tyrosine hydroxylase activity (**b**) in the cells. **a** After preincubation for 10 min with or without ikarisoside A (0.1–100 μM), cells (4 × 10^6/dish) were incubated with L-[U-^{14}C] tyrosine (20 μM, 1 μCi) in the presence or absence of ikarisoside A (0.1–100 μM) and with (*black circle*) or without (*white circle*) 300 μM ACh at 37 °C for 20 min. The ^{14}C-catecholamines formed were measured. **b** After preincubation with or without ikarisoside A (0.1–100 μM) for 10 min, cells (10^6/well) were incubated with L-[1-^{14}C] tyrosine (18 μM, 0.2 μCi) in the presence or absence of ikarisoside A (0.1–100 μM) and with (*black circle*) or without (*white circle*) 300 μM ACh at 37 °C for 10 min, and tyrosine hydroxylase activity was measured. Data are means ± SEM from three separate experiments carried out in triplicate. *$P < 0.05$ and ***$P < 0.001$, compared with ACh alone (analyzed by one-way ANOVA with Dunnett's multiple comparison post hoc test)

effect of ikarisoside A on ACh-induced secretion of catecholamines was not overcome, suggesting a noncompetitive inhibition and that ikarisoside A acts at a site different from that for ACh binding. A previous review (Lena and Changeux 1993) reported that the site at which noncompetitive blockers act lies at the interface between the nicotinic receptor protein and the membrane lipids.

In the *Xenopus* oocytes expressed with α3β4 nAChRs, ikarisoside A directly inhibited ACh-induced current. The IC_{50} values of ikarisoside A for $^{22}Na^{+}$ influx in adrenal medullary cells and for Na^{+} current in the oocytes were 2.96 and 0.48 μM, respectively. The IC_{50} in the bovine adrenal medullary cells is 6.2-fold bigger than that of the drug in the oocyte system. Although the reason for the discrepancy of the IC_{50} between the two systems is not yet clear, the discrepancy may be explained in the following way. (1) A maximally effective concentration of ACh was used for the $^{22}Na^{+}$ influx experiments in bovine adrenal medullary cells but the half-maximal concentration was used for the Na^{+} current in the oocyte system. (2) In the oocyte expression system, there may be some changes in the test compound potency compared to that of the method using mammalian cells, i.e., a decrease (Lambert et al. 2001; Akk et al. 2008) or an increase (Pintado et al. 2000) in the sensitivity of test compounds. (3) Bovine adrenal medullary cells express multiple nAChR subtypes such as α3β4 (Criado et al. 1992; Garcia-Guzman et al. 1995), α3β4α5 (Campos-Caro et al. 1997), and α7 (Lopez et al. 1998). We should study above possibilities and examine the effect of ikarisoside A on the function of nAChRs in other mammalian cells.

Structure-activity relationship of ikarisoside A for inhibition of nAChR-ion channels

In the present study, we used four flavonol glycosides derived from the *Epimedium* species. Ikarisoside A, but not the other three flavonols, inhibited the functioning of nAChR-ion channels. Judging from the differences in their structures, ikarisoside A has a hydroxyl group at the 7 position in the structure whereas other three have a glucose moiety at this position, suggesting that a glucose moiety at the 7 position may induce stereo-specific interference when flavonol glycosides interact with nAChRs. Furthermore, the inhibition of ACh-induced secretion by ikarisoside A disappeared by the removal of the rhamnose moiety at the 3 position from ikarisoside A. These findings suggest that the rhamnose moiety at the 3 position of ikarisoside A is essential to inhibit the function of nAChR-ion channels.

Inhibitory effect of ikarisoside A on catecholamine synthesis

Ikarisoside A inhibited not only catecholamine secretion but also reduced catecholamine synthesis in ACh-stimulated cells. In the regulation of catecholamine synthesis, Ca^{2+} plays an important role as the coupler in the stimulus-synthesis coupling (Yanagihara et al. 1987) as well as in the stimulus-secretion coupling (Douglas and Rubin 1961, 1963). In the present study, we observed that ikarisoside A suppressed the $^{22}Na^{+}$ influx and the subsequent $^{45}Ca^{2+}$ influx by inhibiting nAChR-ion channels. Therefore, it is likely that ikarisoside A inhibits catecholamine synthesis and tyrosine hydroxylase activity induced by ACh via the suppression of Ca^{2+} influx in cultured bovine adrenal medulla cells. In harmony with this view, the IC_{50} values of ikarisoside A for inhibition of $^{22}Na^{+}$ and $^{45}Ca^{2+}$ influx and for inhibition of catecholamine synthesis and tyrosine hydroxylase are very similar.

Pharmacological significance of the inhibitory effects of ikarisoside A on adrenal medullary functions

The human serum concentration of ikarisoside A has not been reported yet. Several previous in vitro studies reported that ikarisoside A at 5.0–20 μM inhibits osteoclastogenic differentiation and nitric oxide synthase in murine monocyte/macrophage cell line RAW264.7 cells (Choi et al. 2008, 2010) and induces neurite outgrowth activity in PC12h cells at 10 μM (Kuroda et al. 2000). In the present study, we observed a significant inhibition of ikarisoside A at 0.1 and 1.0 μM in ACh-induced current in *Xenopus* oocytes and ACh-induced synthesis and secretion of catecholamines, respectively.

It is well known that adrenaline and noradrenaline have an important role in the regulation of normal function in the central and peripheral sympathetic nervous systems. Under strong and prolonged stress, an increased catecholamine release may occur, which possibly induces cardiovascular diseases such as hypertension, atherosclerosis, coronary heart disease, and heart failure (Yanagihara et al. 2014). Chronic heart failure is reported to be associated with the activation of the sympathetic nervous system as manifested by increased circulating catecholamines (Westfall and Westfall 2011). Furthermore, Hara et al. (2011) reported that the stress hormone adrenaline stimulates β_2-adrenoceptors, which activates the Gs protein/cyclic AMP-dependent protein kinase and the β-arrestin-mediated signaling pathway, reduces the p53 level, and induces DNA damage.

Our previous studies reported that daidzein, a soy isoflavone, (Liu et al. 2007) and nobiletin, a citrus polymethoxy flavone, (Zhang et al. 2010) suppress the secretion and synthesis of catecholamines induced by ACh in cultured bovine adrenal medullary cells. In addition to these flavonoids, ikarisoside A also may protect the hyperactive catecholamine system induced by strong stress or emotional excitation which evokes the secretion of ACh from the splanchnic nerves. Further in vivo experiments will provide more conclusive information on ikarisoside A and promote the development of a

therapeutic drug for stress-induced disorders associated with mental or cardiovascular diseases.

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Conflict of interest The authors declare that they have no competing interests.

References

Akk G, Li P, Bracamontes J, Reichert DE, Covey DF, Steinbach JH (2008) Mutations of the $GABA_A$ receptor α1 subunit M1 domain reveal unexpected complexity for modulation by neuroactive steroids. Mol Pharmacol 74:614–627

Bobrovskaya L, Cheah TB, Bunn SJ, Dunkley PR (1998) Tyrosine hydroxylase in bovine adrenal chromaffin cells: angiotensin II-stimulated activity and phosphorylation of Ser^{19}, Ser^{31}, and Ser^{40}. J Neurochem 70:2565–2573

Campos-Caro A, Smillie FI, Dominguez del Toro E, Rovira JC, Vicente-Agullo F, Chapuli J, Juiz JM, Sala S, Sala F, Ballesta JJ, Criado M (1997) Neuronal nicotinic acetylcholine receptors on bovine chromaffin cells: cloning, expression, and genomic organization of receptor subunits. J Neurochem 68:488–497

Chen WF, Mok SK, Wang XL, Lai KH, Lai WP, Luk HK, Leung PC, Yao XS, Wong MS (2011) Total flavonoid fraction of the herba epimedii extract suppresses urinary calcium excretion and improves bone properties in ovariectomised mice. Br J Nutr 105:180–189

Choi HJ, Eun JS, Park YR, Kim DK, Li R, Moon WS, Park JM, Kim HS, Cho NP, Cho SD, Soh Y (2008) Ikarisoside A inhibits inducible nitric oxide synthase in lipopolysaccharide-stimulated RAW 264.7 cells via p38 kinase and nuclear factor-κB signaling pathways. Eur J Pharmacol 601:171–178

Choi HJ, Park YR, Nepal M, Choi BY, Cho NP, Choi SH, Heo SR, Kim HS, Yang MS, Soh Y (2010) Inhibition of osteoclastogenic differentiation by Ikarisoside A in RAW 264.7 cells via JNK and NF-κB signaling pathways. Eur J Pharmacol 636:28–35

Criado M, Alamo L, Navarro A (1992) Primary structure of an agonist binding subunit of the nicotinic acetylcholine receptor from bovine adrenal chromaffin cells. Neurochem Res 17:281–287

Dou J, Liu Z, Liu S (2006) Structure identification of a prenylflavonol glycoside from epimedium koreanum by electrospray ionization tandem mass spectrometry. Anal Sci 22:449–452

Douglas WW, Rubin RP (1961) Mechanism of nicotinic action at the adrenal medulla: calcium as a link in stimulus-secretion coupling. Nature 192:1087–1089

Douglas WW, Rubin RP (1963) The mechanism of catecholamine release from the adrenal medulla and the role of calcium in stimulus-secretion coupling. J Physiol 167:288–310

Garcia-Guzman M, Sala F, Sala S, Campos-Caro A, Stuhmer W, Gutierrez LM, Criado M (1995) α-Bungarotoxin-sensitive nicotinic receptors on bovine chromaffin cells: molecular cloning, functional expression and alternative splicing of the α7 subunit. Eur J Neurosci 7:647–655

Hara MR, Kovacs JJ, Whalen EJ, Rajagopal S, Strachan RT, Grant W, Towers AJ, Williams B, Lam CM, Xiao K, Shenoy SK, Gregory SG, Ahn S, Duckett DR, Lefkowitz RJ (2011) A stress response pathway regulates DNA damage through β_2-adrenoreceptors and β-arrestin-1. Nature 477:349–353

Horishita T, Harris RA (2008) n-Alcohols inhibit voltage-gated Na^+ channels expressed in Xenopus oocytes. J Pharmacol Exp Ther 326:270–277

Kajiwara K, Yanagita T, Nakashima Y, Wada A, Izumi F, Yanagihara N (2002) Differential effects of short and prolonged exposure to carvedilol on voltage-dependent Na^+ channels in cultured bovine adrenal medullary cells. J Pharmacol Exp Ther 302:212–218

Kuroda M, Mimaki Y, Sashida Y, Umegaki E, Yamazaki M, Chiba K, Mohri T, Kitahara M, Yasuda A, Naoi N, Xu ZW, Li MR (2000) Flavonol glycosides from Epimedium sagittatum and their neurite outgrowth activity on PC12h cells. Planta Med 66:575–577

Lai X, Ye Y, Sun C, Huang X, Tang X, Zeng X, Yin P, Zeng Y (2013) Icaritin exhibits anti-inflammatory effects in the mouse peritoneal macrophages and peritonitis model. Int Immunopharmacol 16:41–49

Lambert JJ, Belelli D, Harney SC, Peters JA, Frenguelli BG (2001) Modulation of native and recombinant $GABA_A$ receptors by endogenous and synthetic neuroactive steroids. Brain Res Brain Res Rev 37:68–80

Lena C, Changeux JP (1993) Allosteric modulations of the nicotinic acetylcholine receptor. Trends Neurosci 16:181–186

Liu M, Yanagihara N, Toyohira Y, Tsutsui M, Ueno S, Shinohara Y (2007) Dual effects of daidzein, a soy isoflavone, on catecholamine synthesis and secretion in cultured bovine adrenal medullary cells. Endocrinology 148:5348–5354

Lopez MG, Montiel C, Herrero CJ, Garcia-Palomero E, Mayorgas I, Hernandez-Guijo JM, Villarroya M, Olivares R, Gandia L, McIntosh JM, Olivera BM, Garcia AG (1998) Unmasking the functions of the chromaffin cell α7 nicotinic receptor by using short pulses of acetylcholine and selective blockers. Proc Natl Acad Sci U S A 95:14184–14189

Lu MF, Xiao ZT, Zhang HY (2013) Where do health benefits of flavonoids come from? Insights from flavonoid targets and their evolutionary history. Biochem Biophys Res Commun 434:701–704

Mizuno M, Iinuma M, Tanaka T, Sakakibara N, Hanioka S (1988) Flavonol glycosides in Epimedium species. Chem Pharm Bull 36(9):3487–3490

Nagatsu T, Levitt M, Udenfriend S (1964) Tyrosine hydroxylase. The initial step in norepinephrine biosynthesis. J Biol Chem 239:2910–2917

Nijveldt RJ, van Nood E, van Hoorn DE, Boelens PG, van Norren K, van Leeuwen PA (2001) Flavonoids: a review of probable mechanisms of action and potential applications. Am J Clin Nutr 74:418–425

Pintado AJ, Herrero CJ, Garcia AG, Montiel C (2000) The novel Na^+/Ca^{2+} exchange inhibitor KB-R7943 also blocks native and expressed neuronal nicotinic receptors. Br J Pharmacol 130:1893–1902

Ren ZL, Zuo PP (2012) Neural regeneration: role of traditional Chinese medicine in neurological diseases treatment. J Pharmacol Sci 120:139–145

Shinohara Y, Toyohira Y, Ueno S, Liu M, Tsutsui M, Yanagihara N (2007) Effects of resveratrol, a grape polyphenol, on catecholamine secretion and synthesis in cultured bovine adrenal medullary cells. Biochem Pharmacol 74:1608–1618

Toyohira Y, Kubo T, Watanabe M, Uezono Y, Ueno S, Shinkai K, Tsutsui M, Izumi F, Yanagihara N (2005) Selective blockade of nicotinic acetylcholine receptors by pimobendan, a drug for the treatment of heart failure: reduction of catecholamine secretion and synthesis in adrenal medullary cells. Naunyn Schmiedeberg's Arch Pharmacol 371:107–113

Tsutsui M, Yanagihara N, Miyamoto E, Kuroiwa A, Izumi F (1994) Correlation of activation of Ca^{2+}/calmodulin-dependent protein kinase II with catecholamine secretion and tyrosine hydroxylase activation in cultured bovine adrenal medullary cells. Mol Pharmacol 46:1041–1047

Ueno S, Tsutsui M, Toyohira Y, Minami K, Yanagihara N (2004) Sites of positive allosteric modulation by neurosteroids on ionotropic γ-aminobutyric acid receptor subunits. FEBS Lett 566:213–217

Wada A, Izumi F, Yanagihara N, Kobayashi H (1985a) Modulation by ouabain and diphenylhydantoin of veratridine-induced ^{22}Na influx and its relation to ^{45}Ca influx and the secretion of catecholamines in cultured bovine adrenal medullary cells. Naunyn Schmiedeberg's Arch Pharmacol 328:273–278

Wada A, Takara H, Izumi F, Kobayashi H, Yanagihara N (1985b) Influx of ^{22}Na through acetylcholine receptor-associated Na channels: relationship between ^{22}Na influx, ^{45}Ca influx and secretion of catecholamines in cultured bovine adrenal medulla cells. Neuroscience 15:283–292

Wada A, Yashima N, Izumi F, Kobayashi H, Yanagihara N (1984) Involvement of Na influx in acetylcholine receptor mediated secretion of catecholamines from cultured bovine adrenal medulla cells. Neurosci Lett 47:75–80

Weil-Malherbe H, Bone AD (1952) The chemical estimation of adrenaline-like substances in blood. Biochem J 51:311–318

Westfall TC and Westfall DP (2011) Adrenergic agonists and antagonists, in Goodman & Gilman's the pharmacological basis of therapeutics, 12th edn (Brunton LL, Chabner BC, and Knollmann BC, eds), pp. 277-330. McGraw-Hill, NY

Yanagihara N, Isosaki M, Ohuchi T, Oka M (1979) Muscarinic receptor-mediated increase in cyclic GMP level in isolated bovine adrenal medullary cells. FEBS Lett 105:296–298

Yanagihara N, Oishi Y, Yamamoto H, Tsutsui M, Kondoh J, Sugiura T, Miyamoto E, Izumi F (1996) Phosphorylation of chromogranin A and catecholamine secretion stimulated by elevation of intracellular Ca^{2+} in cultured bovine adrenal medullary cells. J Biol Chem 271: 17463–17468

Yanagihara N, Wada A, Izumi F (1987) Effects of α_2-adrenergic agonists on carbachol-stimulated catecholamine synthesis in cultured bovine adrenal medullary cells. Biochem Pharmacol 36:3823–3828

Yanagihara N, Zhang H, Toyohira Y, Takahashi K, Ueno S, Tsutsui M (2014) New insights into the pharmacological potential of plant flavonoids in the catecholamine system. J Pharmacol Sci 124:123–128

Zhang H, Toyohira Y, Ueno S, Shinohara Y, Itoh H, Furuno Y, Yamakuni T, Tsutsui M, Takahashi K, Yanagihara N (2010) Dual effects of nobiletin, a citrus polymethoxy flavone, on catecholamine secretion in cultured bovine adrenal medullary cells. J Neurochem 114:1030–1038

Zhou J, Chen KM, Ge BF, Ma XN, Guo XY, Cheng K, Gao YH, Yan LJ, Shi WG (2013) Comparison between icariin and genistein in osteogenic activity of marrow stromal cells. Zhongguo Zhong Yao Za Zhi 38:1783–1788

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ARTICLE

Long-term dietary nitrite and nitrate deficiency causes the metabolic syndrome, endothelial dysfunction and cardiovascular death in mice

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Abstract

Aims/hypothesis Nitric oxide (NO) is synthesised not only from L-arginine by NO synthases (NOSs), but also from its inert metabolites, nitrite and nitrate. Green leafy vegetables are abundant in nitrate, but whether or not a deficiency in dietary nitrite/nitrate spontaneously causes disease remains to be clarified. In this study, we tested our hypothesis that long-term dietary nitrite/nitrate deficiency would induce the metabolic syndrome in mice.

Methods To this end, we prepared a low-nitrite/nitrate diet (LND) consisting of an amino acid-based low-nitrite/nitrate chow, in which the contents of L-arginine, fat, carbohydrates, protein and energy were identical with a regular chow, and potable ultrapure water. Nitrite and nitrate were undetectable in both the chow and the water.

Results Three months of the LND did not affect food or water intake in wild-type C57BL/6J mice compared with a regular diet (RD). However, in comparison with the RD, 3 months of the LND significantly elicited visceral adiposity, dyslipidaemia and glucose intolerance. Eighteen months of the LND significantly provoked increased body weight, hypertension, insulin resistance and impaired endothelium-dependent relaxations to acetylcholine, while 22 months of the LND significantly led to death mainly due to cardiovascular disease, including acute

Mika Kina-Tanada and Mayuko Sakanashi contributed equally to this work.

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myocardial infarction. These abnormalities were reversed by simultaneous treatment with sodium nitrate, and were significantly associated with endothelial NOS downregulation, adiponectin insufficiency and dysbiosis of the gut microbiota.
Conclusions/interpretation These results provide the first evidence that long-term dietary nitrite/nitrate deficiency gives rise to the metabolic syndrome, endothelial dysfunction and cardiovascular death in mice, indicating a novel pathogenetic role of the exogenous NO production system in the metabolic syndrome and its vascular complications.

Keywords Acute myocardial infarction · Cardiovascular death · Diet · Endothelial dysfunction · Metabolic syndrome · Mice · Nitrate · Nitric oxide · Nitrite

Abbreviations

AMPK	Adenosine monophosphate-activated protein kinase
CT	Computed tomography
eNOS	Endothelial nitric oxide synthase
EWAT	Epididymal white adipose tissue
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
iNOS	Inducible nitric oxide synthase
LND	Low-nitrite/nitrate diet
nNOS	Neuronal nitric oxide synthase
NOS	Nitric oxide synthase
PPAR-γ	Peroxisome proliferator-activated receptor-γ
RD	Regular diet
WAT	White adipose tissue
WT	Wild-type

Introduction

The metabolic syndrome is defined as a constellation of interrelated cardiovascular risk factors of metabolic origin, including visceral obesity, dyslipidaemia, hypertension, glucose intolerance and insulin resistance [1]. The metabolic syndrome is highly prevalent in industrial countries worldwide, and it has been reported that, in accordance with the most recent harmonised definition of the metabolic syndrome, 23% of the adult population (≥20 years of age) in the USA suffered from the metabolic syndrome in 2009–2010 [2, 3]. Greater global industrialisation is associated with rising rates of obesity, which are expected to dramatically increase the prevalence of the metabolic syndrome worldwide, especially as the population ages [1]. The metabolic syndrome is associated with increased risks of myocardial infarction, stroke, cardiovascular disease mortality and all-cause mortality [4]. It also confers higher risks of peripheral vascular disease, type 2 diabetes, renal disease, hepatic disease and cancer [5–9]. Several factors, including excessive food energy intake, lack of physical activity, genetic susceptibility and ageing, have been thought to be involved in the pathogenesis of the metabolic syndrome. However, the precise mechanisms in its development remain to be fully elucidated [1].

Nitric oxide (NO) exerts multiple biological actions, and is one of the most crucial signalling molecules in mammalian physiology and pathology [10–15]. It is endogenously synthesised from a precursor L-arginine by a family of NO synthases (neuronal [nNOS], inducible [iNOS] and endothelial [eNOS]) with stoichiometric production of L-citrulline. NO has a very short half-life of several seconds and is rapidly oxidised to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-). Although nitrite and nitrate were in the past regarded as mere inert metabolites of NO, recent studies have revealed that nitrate is reduced to nitrite and then to NO, so they serve as NO donors [16–18]. Green leafy vegetables, such as spinach and lettuce, and beetroot, are abundant in nitrate, and vegetables are the dominant source of dietary nitrate in humans, contributing to 60–80% of dietary nitrate intake [19, 20]. Potable tap water also contains nitrate and a small quantity of nitrite, and 15–20% of dietary nitrate intake is derived from tap water [19, 20]. It has been reported that cardiac and hepatic ischaemia–reperfusion injury in mice [21–23], cardiac allograft rejection in rats [23] and platelet aggregation in mice [24] are exacerbated by a low-nitrite/nitrate diet (LND) (a commercially available low-nitrite/nitrate chow plus potable ultrapure water, or the low-nitrite/nitrate chow alone) compared with a regular diet (RD). These results suggest that dietary nitrite/nitrate deficiency modulates disease conditions. Whether or not this deficiency spontaneously causes disease, however, remains to be clarified. In this study, we tested our hypothesis that long-term dietary nitrite/nitrate deficiency would give rise to the metabolic syndrome in mice.

Methods

Mice This study was approved by the Animal Care and Use Committee, University of the Ryukyus, Japan, and was carried out according to the Institutional Policy on the Care and Use of the Laboratory Animals. The experiments were performed in 6-week-old male wild-type (WT) C57BL/6J mice (Kyudo, Tosu, Japan). All the mice were maintained in temperature- and humidity-controlled rooms illuminated from 08:00 h to 20:00 h. Food and water intake was measured by placing the animals in metabolic cages for 24 h (see electronic supplementary material [ESM] Methods for further details).

Diet We prepared a purified amino acid-based low-nitrite/nitrate chow in which the contents of L-arginine, fat, carbohydrates, protein and energy were identical to a regular chow (Purina 5001; LabDiet, St Louis, MO, USA), and potable ultrapure Milli-Q water in which nitrite and nitrate levels were

undetectable (Merck Millipore, Darmstadt, Germany) (Tables 1 and 2). We randomly assigned the mice to diet groups, and either the low-nitrite/nitrate chow plus ultrapure water (LND) or the regular chow plus tap water (RD) was fed ad libitum to the mice from 6 weeks of age for 1.5–22 months (see ESM Methods).

Nitrite and nitrate levels The nitrite and nitrate contents of the chows were analysed using the diazotisation method and the cadmium reduction-diazotisation method, respectively (Japan Food Research Laboratories, Tokyo, Japan). The nitrite and nitrate levels in the plasma and drinking water were assessed using the HPLC-Griess system (ENO-20; Eicom, Kyoto, Japan) (see ESM Methods).

Blood pressure Systolic blood pressure was measured by the tail-cuff method under conscious conditions in a blinded manner (Model MK-2000; Muromachi Kikai, Tokyo, Japan) (see ESM Methods).

Glucose tolerance test Glucose 1 g/kg body weight was intraperitoneally injected into the mice under general anaesthesia with sodium pentobarbital (50 mg/kg, i.p.; Sigma-Aldrich, St Louis, MO, USA) after 18 h of fasting. Whole blood samples were collected from the tail, and blood glucose levels were evaluated using a portable blood glucose analyser (Glucocard MyDia; Arkray, Kyoto, Japan) (see ESM Methods).

Insulin tolerance test and plasma insulin levels The mice received 0.3 U/kg body weight of insulin (soluble human insulin, Humulin R; Eli Lilly, Indianapolis, IN, USA) injected into the intraperitoneal cavity under general anaesthesia with sodium pentobarbital (50 mg/kg, i.p.). The fasting plasma insulin levels were assessed using a commercially available ELISA kit (AKRIN-031; Shibayagi, Gunma, Japan) (see ESM Methods).

Visceral fat weight After euthanasia, epididymal white adipose tissue (EWAT) was removed and weighed (see ESM Methods).

Table 1 Composition of regular and low-nitrite/nitrate chow

Constituent	Regular chow	Low-nitrite/nitrate chow
Protein (% kJ)	28.5	28.5
Fat (% kJ)	13.5	13.5
Carbohydrate (% kJ)	58.0	58.0
L-arginine (g/kg)	14.1	14.1
Nitrite (μmol/kg)	<6.5	<6.5
Nitrate (μmol/kg)	548.3	<4.8

Values are expressed as the mean values of two samples

Table 2 Nitrate and nitrite levels in tap water and ultrapure water

Constituent	Tap water	Ultrapure water
Nitrite (μmol/l)	3.30	<0.01
Nitrate (μmol/l)	6.07	<0.01

Values are expressed as the mean values of two samples

Adipocyte hypertrophy and inflammation Epididymal and peri-renal white adipose tissue (WAT) was stained with an H&E solution. The circumferential length of each adipocyte was measured using a light microscope equipped with a CCD camera and morphometric analysis software (DS-Ri1CCD camera and NIS-Elements D 3.2 software; Nikon, Tokyo, Japan). To evaluate inflammation in the adipose tissues, aggregates of inflammatory cells (inflammatory foci consisting of more than ten inflammatory cells) were counted in the maximal cut surface of the EWAT and peri-renal WAT sections on a light microscope at ×40 magnification (see ESM Methods).

Plasma lipid profile Plasma lipid profile was assessed using a Dri-Chem autoanalyser (FDC4000; Fuji Film, Tokyo, Japan). Plasma LDL-cholesterol levels were determined by HPLC (Skylight Biotech, Akita, Japan) [25] (see ESM Methods).

Western blot analysis Western blot analysis was performed as previously reported [26] to detect nNOS, iNOS and eNOS, phosphorylated eNOS at serine 1177 and at threonine 495 (BD Transduction Laboratories, Franklin Lakes, NJ, USA), adiponectin, peroxisome proliferator-activated receptor-γ (PPAR-γ), adenosine monophosphate-activated protein kinase (AMPK), sirtuin 1 (Cell Signaling Technology, Danvers, MA, USA), p-AMPK (Santa Cruz Biotechnology, Dallas, TX, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sigma-Aldrich) (see ESM Methods). The validation of antibodies was performed by the supplier.

Serum cytokine and chemokine levels Serum cytokine/chemokine levels were measured using a Bio-Plex system (23-Plex, M60-009RDPD; Bio-Rad, CA, USA) [27].

16S ribosomal RNA gene sequencing The faecal DNA samples were run through a next-generation sequencer, as previously reported [28]. The number of each bacterial strain contained in the faecal contents was estimated as the genome equivalent by quantitative real-time PCR of 16S ribosomal RNA genes, followed by pyrosequencing of the 16S amplicons [28] (see ESM Methods).

Organ chamber experiment Thoracic aortic rings were mounted in microtissue organ bath chambers (MTBO-1; Labo Support, Osaka, Japan) filled with Krebs-Henseleit

solution, and the isometric contractile force of the rings was measured (see ESM Methods).

Micro-computed tomography imaging Mice were anesthetised with 2% isoflurane (Wako Pure Chemical Industries, Osaka, Japan), and computed tomography (CT) images were acquired by three-dimensional micro-CT (R_mCT2; Rigaku Corporation, Tokyo, Japan) (see ESM Methods).

Statistical analyses Most of our results are expressed as mean ± SEM. Statistical analyses were performed using a Student's *t* test, or ANOVA followed by Bonferroni's post hoc test. The results of sequencing reads of gut bacteria are expressed as the median and interquartile range, and the statistical analysis was carried out using a Wilcoxon rank-sum test [29]. Kaplan–Meier survival curves were compared using the logrank test. A value of $p < 0.05$ was considered to be statistically significant (see ESM Methods).

Results

Three month LND markedly reduced plasma nitrite/nitrate levels The 3 month LND markedly reduced plasma nitrite/nitrate levels compared with the RD (Fig. 1a). To examine the underlying mechanism, we evaluated NOS levels in isolated aorta and visceral fat. eNOS protein levels in the aorta were comparable in the two diets (Fig. 1b), whereas, intriguingly, levels in EWAT were markedly lower with the LND than the RD (Fig. 1c), accounting for the markedly reduced plasma nitrite/nitrate levels induced by the LND. No nNOS or iNOS was found in EWAT with either diet (ESM Fig. 1a).

Three month LND resulted in metabolic syndrome-like conditions There were no significant differences in food intake (Fig. 1d), water intake (ESM Fig. 1b), or body weight (ESM Fig. 1c) between the 3 month LND and RD. However, the 3 month LND significantly increased EWAT weight (Fig. 1e), epididymal white adipocyte size (Fig. 1f) and plasma levels of total cholesterol (Fig. 2a), LDL-cholesterol (Fig. 2b) and small

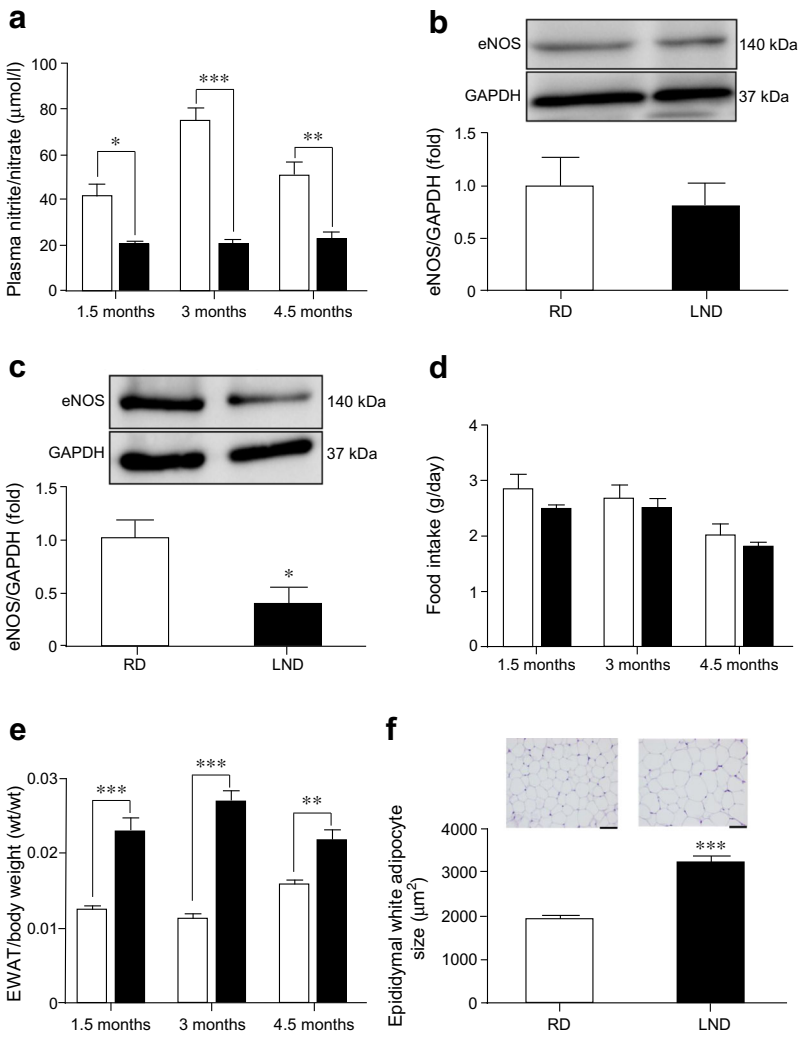

Fig. 1 Effects of 1.5–4.5 months of the LND on plasma nitrite/nitrate, eNOS levels, food intake and visceral fat in WT mice. (**a**) Plasma nitrite/nitrate levels (n = 8–12). (**b**, **c**) eNOS protein levels in the aorta (**b**; n = 6) and EWAT induced by 3 months of the LND (**c**; n = 10). (**d**, **e**) Food intake (n = 10–12) and relationship of EWAT to body weight (n = 10–12). (**f**) Epididymal white adipocyte size induced by 3 months of the LND (n = 10). Scale bars, 50 μm. White bars, RD; black bars, LND. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

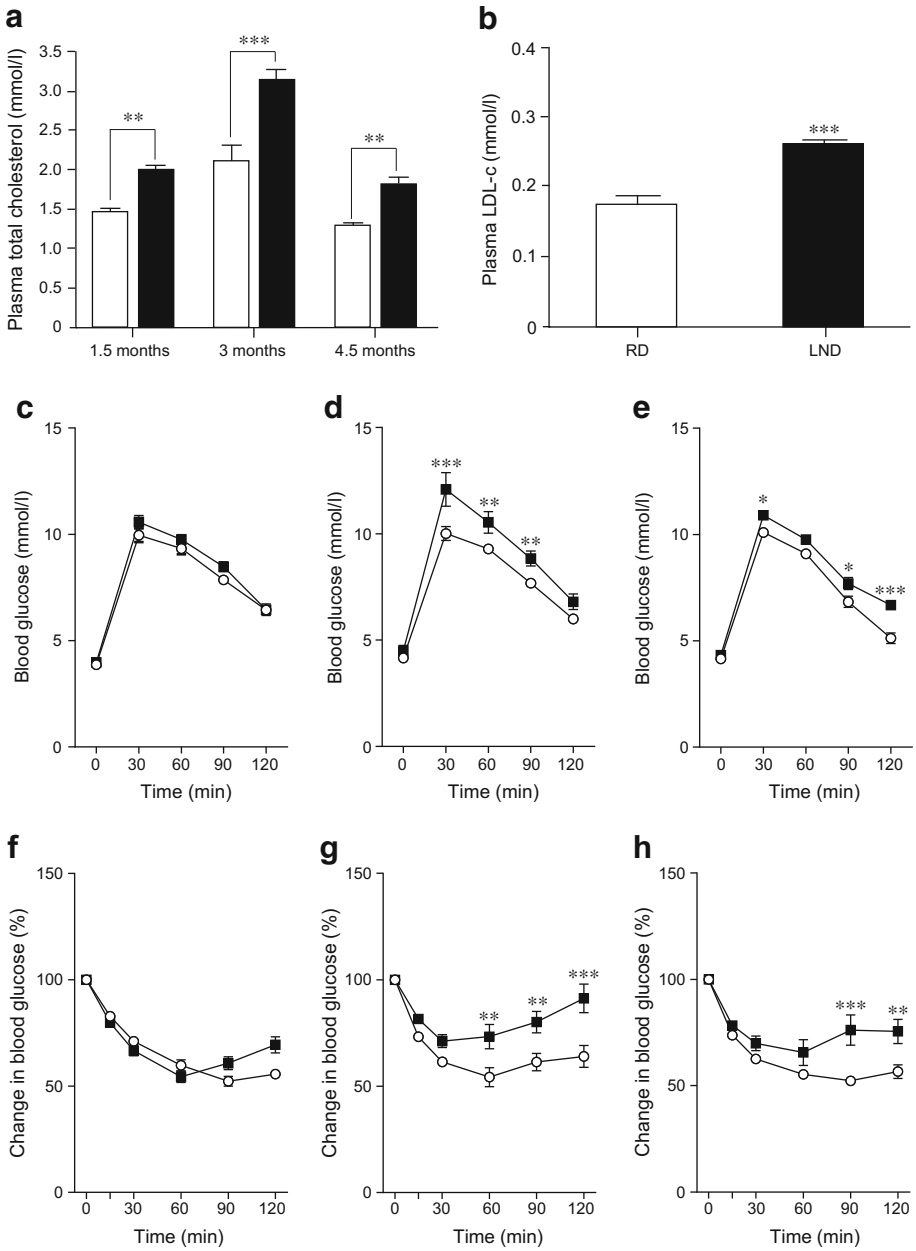

Fig. 2 Effects of 1.5–4.5 months of the LND on plasma lipid, blood glucose and insulin response. (**a**) Plasma total cholesterol levels (n = 10–12). White bars, RD; black bars, LND. (**b**) Plasma LDL-cholesterol (LDL-c) levels induced by 3 months of the LND (n = 12). (**c**–**e**) Blood glucose levels after i.p. injection of 1 g/kg glucose after 1.5 (**c**), 3 (**d**) and 4.5 (**e**) months of the LND (n = 11–12). (**f**–**h**) Percentage change in blood glucose levels after i.p. injection of 0.3 U/kg insulin after 1.5 (**f**), 3 (**g**) and 4.5 (**h**) months of the LND (n = 8–12). White bars and white circles, RD; black bars and black squares, LND. *p < 0.05, **p < 0.01, ***p < 0.001

dense LDL-cholesterol (ESM Fig. 1d), and non-significantly elevated plasma triacylglycerol levels compared with the RD (p = 0.54) (Table 3). Furthermore, the 3 month LND significantly augmented blood glucose levels after i.p. glucose injection and aggravated blood glucose-lowering responses to i.p. insulin injection (Fig. 2d, g). Arterial blood pressure levels were similar with both diets (ESM Fig. 2a).

We next studied the time course of the metabolic effects at 1.5, 3 and 4.5 months after the start of the LND. The effects on EWAT weight (Fig. 1e), plasma total cholesterol levels (Fig. 2a), blood glucose levels after glucose injection (Fig. 2c–e) and blood glucose-lowering responses to insulin (Fig. 2f–h, ESM Fig. 1e–g) were time-dependent and appeared to reach a plateau at 3 months. There were no significant differences in fasting plasma insulin levels between the mice fed the LND or the RD at any of the time points (ESM Fig. 2b).

Table 3 Plasma triacylglycerol levels at 1.5, 3 and 4.5 months after the start of RD or LND

Diet	Plasma triacylglycerol levels (mmol/l)		
	1.5 months	3 months	4.5 months
RD (n = 6–12)	1.23 ± 0.07	0.94 ± 0.05	1.06 ± 0.07
LND (n = 6–12)	1.25 ± 0.07	1.41 ± 0.19	1.21 ± 0.19

Statistical analysis was performed by two-way factorial ANOVA followed by Bonferroni's post hoc test for multiple comparisons, and no significant differences were seen

Adiponectin levels in EWAT were markedly lower for the 3 month LND compared with the RD (Fig. 3a). The EWAT levels of PPAR-γ, total AMPK and p-AMPK, but not sirtuin 1, were also markedly reduced after 1 week of the LND in comparison to the RD (Fig. 3b–e).

Simultaneous oral treatment with 2 mmol/l sodium nitrate for 3 months significantly reversed the reduced plasma nitrite/nitrate levels induced by the LND (Fig. 4a). It also non-significantly improved the LND-induced gain in EWAT weight ($p = 0.06$) (Fig. 4b), and significantly ameliorated the epididymal white adipocyte hypertrophy, hyper-small dense LDL-cholesterolaemia, impaired glucose tolerance, reduced blood glucose-lowering responses to insulin, eNOS downregulation and adiponectin insufficiency induced by the LND (Fig. 4c–h, ESM Fig. 2c).

Dysbiosis of gut microbiota was noted in the LND-fed mice Although there was no significant difference between the two diets in the number of total sequencing reads (ESM Fig. 3a), there were significantly fewer operational taxonomic units, which represent the kind of gut bacteria, with the LND than the RD, suggesting less diversity of gut microbiota (Fig. 5a). Significantly different gut bacteria in each rank are shown in Fig. 5b, c and ESM Fig. 3b–j. In the phylum rank, there were more Actinobacteria with the LND than the RD (ESM Fig. 3b). In the class rank, there were more Actinobacteria and fewer Betaproteobacteria with the LND (ESM Fig. 3c, d). In the order rank, Bifidobacteriales was more numerous with the LND, and Burkholderiales and Bacillales were less numerous (ESM Fig. 3e, f). In the family (ESM Fig. 3g, h), the genus (Fig. 5b, c) and the species (ESM Fig. 3i, j) ranks, more than three gut bacteria were more prevalent, and more than two gut bacteria were less prevalent with the LND. On the other hand, *Bacteroides fragilis* was to a certain degree present with the RD, but absent with the LND (median and interquartile range 312 [118–446] for the RD vs 0 for the LND; $n = 5$). There were no significant differences between the two diets in Firmicutes, Bacteroidetes or Firmicutes/Bacteroidetes ratio, which might change in disease states of the metabolic syndrome [30–33], or *Akkermansia muciniphila*, which might improve the metabolic syndrome [34, 35] (data not shown). *Bacteroides uniformis* CECT 7771, which might improve the metabolic syndrome [36], was not detected in either diet.

An 18 month LND resulted in more severe metabolic syndrome and endothelial dysfunction Although the metabolic effects of the LND appeared to reach a plateau at 3 months, we explored the effects of an extremely long period of LND (18 months) for confirmation. There were no significant differences in food or water intake between the 18 month LND and RD (ESM Fig. 4a, b), but, notably, 18 months of the LND resulted in more severe metabolic syndrome, eliciting a significant gain in body weight, which was not seen until 4.5 months after the start of the LND (Fig. 6a). Micro-CT

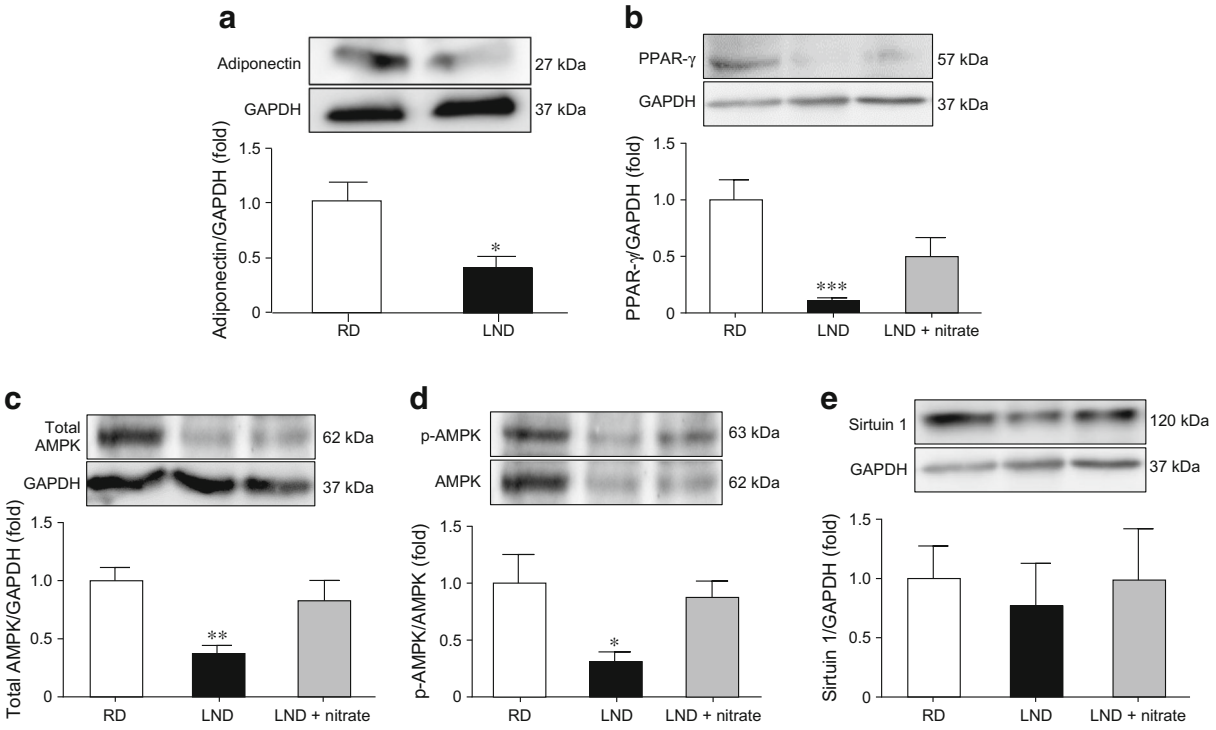

Fig. 3 Effects of 3 months of the LND and nitrate supplementation on adiponectin, and effects of 1 week of the LND and nitrate supplementation on PPAR-γ, total AMPK, p-AMPK and sirtuin 1 levels in EWAT. (**a**) Adiponectin levels ($n = 5$). (**b**–**e**) PPAR-γ, total AMPK, p-AMPK and sirtuin 1 levels ($n = 7$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

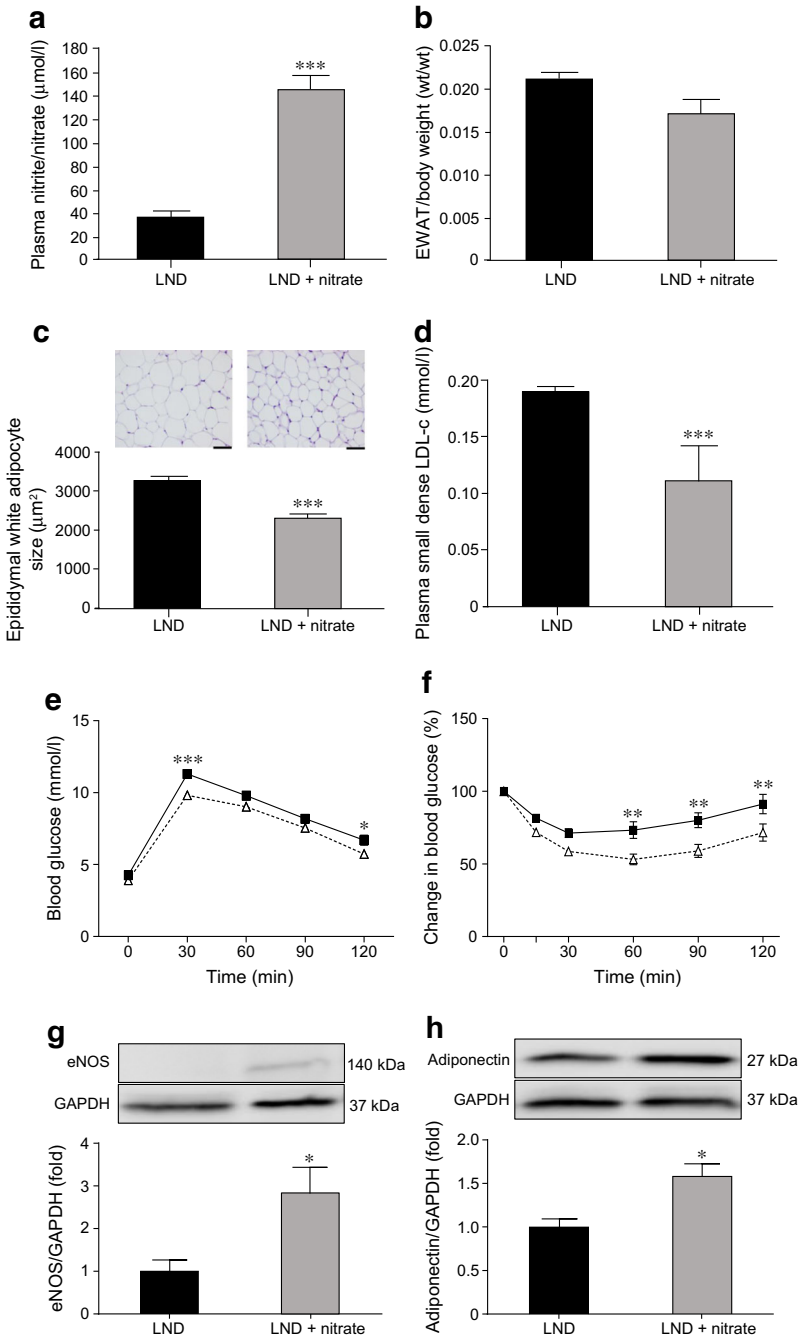

Fig. 4 Effects of 3 months of nitrate supplementation on metabolic syndrome-like conditions induced by the LND. (**a**) Plasma nitrite/nitrate levels (n = 6–12). (**b**) Relationship of EWAT to body weight (n = 7–8). (**c**) Epididymal white adipocyte size (n = 10). Scale bar, 50 μm. (**d**) Plasma small dense LDL-cholesterol (LDL-c) levels (n = 10–12). (**e**) Blood glucose levels after i.p. injection of 1 g/kg glucose (n = 8). (**f**) Percentage change in blood glucose levels after i.p. injection of 0.3 U/kg insulin (n = 9–10). Black squares, LND; white triangles, LND + nitrate. (**g**, **h**) eNOS (n = 5–6) and adiponectin (n = 3) levels in EWAT. *p < 0.05, **p < 0.01, ***p < 0.001

imaging indicated that body fat was markedly increased, by 3.2 times, in the 18 month LND-fed mice (Fig. 6b, c), and this was specifically due to an increase in visceral fat (Fig. 6d) and not subcutaneous fat (ESM Fig. 4c). The 18 month LND elevated levels of plasma small dense LDL-cholesterol and blood glucose following glucose injection, and significantly blunted blood glucose-lowering responses to insulin (Fig. 6e–g, ESM Fig. 4d). In addition, it significantly increased levels of fasting blood glucose, fasting plasma insulin and arterial blood pressure (Fig. 7a–c), findings that were not observed until 4.5 months after the start of the LND. eNOS protein levels in the EWAT were also significantly decreased with the 18 month LND (ESM Fig. 4e).

As the 18 month LND induced severe metabolic syndrome, we next examined vascular reactivity. In isolated aortas, contractions in response to phenylephrine, an adrenergic α_1-receptor agonist, and endothelium-independent relaxations to diethylamine NONOate, an NO donor, were comparable between the two diets (ESM Fig. 4f, g), whereas endothelium-dependent relaxation in response to acetylcholine, a physiological eNOS activator, was significantly impaired with the LND (Fig. 7d). eNOS protein levels were significantly diminished in

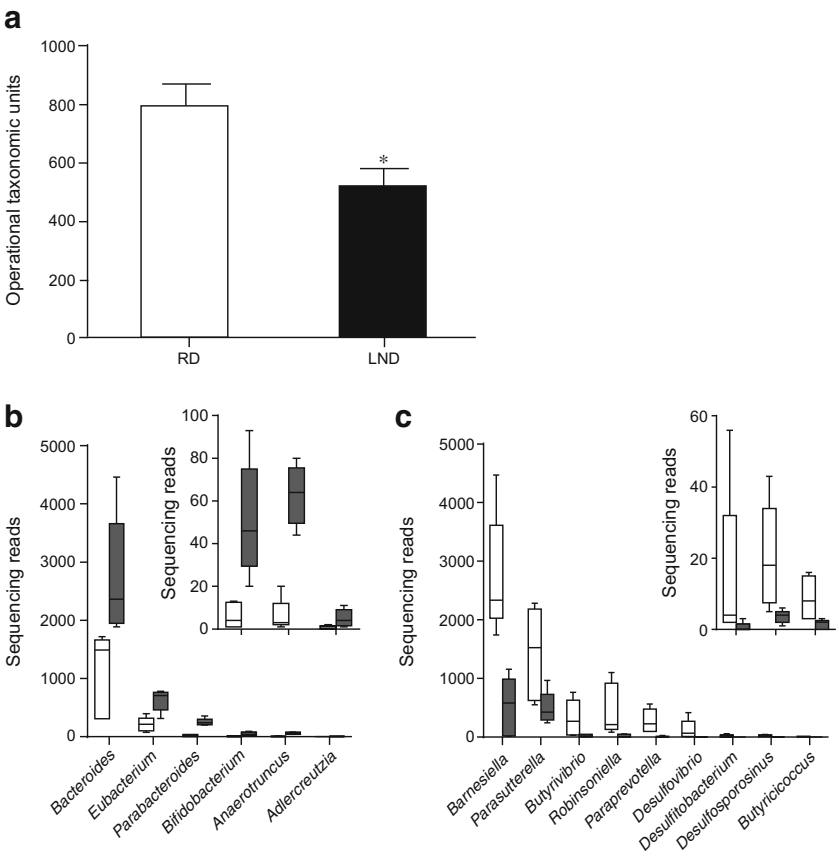

Fig. 5 Effects of 3 months of the LND on gut microbiota. (**a**) Operational taxonomic units ($n = 5$ each). (**b**, **c**) Significantly higher (**b**) or lower (**c**) numbers of gut bacteria in the genus rank ($p < 0.05$, $n = 5$ each). White boxes, RD; grey boxes, LND. Insets are the magnified views of sequencing reads of the rightmost three gut bacteria in parts **b** and **c**. *$p < 0.05$

the aortas (Fig. 7e), and there was a non-significant tendency with the LND towards lower levels of phosphorylation of eNOS at serine 1177 ($p = 0.12$), which is an index of eNOS activation, without affecting the phosphorylation levels of eNOS at threonine 495, which is an index of eNOS inactivation (ESM Fig. 4h, i).

Simultaneous treatment with sodium nitrate for 18 months inhibited these metabolic abnormalities, the endothelial dysfunction and the aortic and EWAT eNOS downregulation induced by the LND (Figs 6, 7a–e, ESM Fig. 4c–e).

There were no significant increases in the serum levels of 23 inflammatory markers measured using the Bio-Plex system between the 1.5 month LND and RD (Table 4). There were also no significant differences in the number of inflammatory foci in the EWAT and peri-renal WAT between the 3 month LND and RD (data not shown). However, the 18 month LND non-significantly increased the number of inflammatory foci in the EWAT ($p = 0.08$) and significantly augmented those in the peri-renal WAT compared with the RD (Fig. 7f, g).

A 22 month LND led to cardiovascular death We experienced sudden death in some of the LND-fed mice. During the 22 months of follow-up, none (0/24) of the RD-fed mice, but 31.8% (7/22) of the LND-fed mice, died. The survival rate was significantly worse in the LND-fed than in the RD-fed mice, and co-treatment with sodium nitrate improved the reduced survival (Fig. 8a). We performed a post mortem histopathological analysis to identify the cause of death in the seven dead LND-fed mice. We were not able to investigate the cause of death in one mouse because of strong post mortem putrefaction. We judged that one mouse had died of an acute anterior wall myocardial infarction (Fig. 8b), and that one mouse died of malignant lymphoma of the lung, liver (Fig. 8g), kidney and spleen. In the other four mice, we noted coronary perivascular fibrosis, pulmonary congestion and acute renal tubular necrosis (Fig. 8d–f), findings that are observed in cardiac sudden death. Myocardial fibrosis, which might have resulted from myocardial infarction, was seen in one mouse (Fig. 8c), malignant lymphoma of the liver and spleen was observed in one mouse, and no other pathological findings that could explain the cause of death were seen in any of the mice.

Discussion

In previous studies, the effects of an LND on cardiac and liver ischaemia–reperfusion injury [21–23], cardiac allograft rejection [23] and platelet aggregation [24] were investigated using a commercially available low-nitrite/nitrate chow, but the contents of L-arginine, fat, carbohydrates, protein and energy were

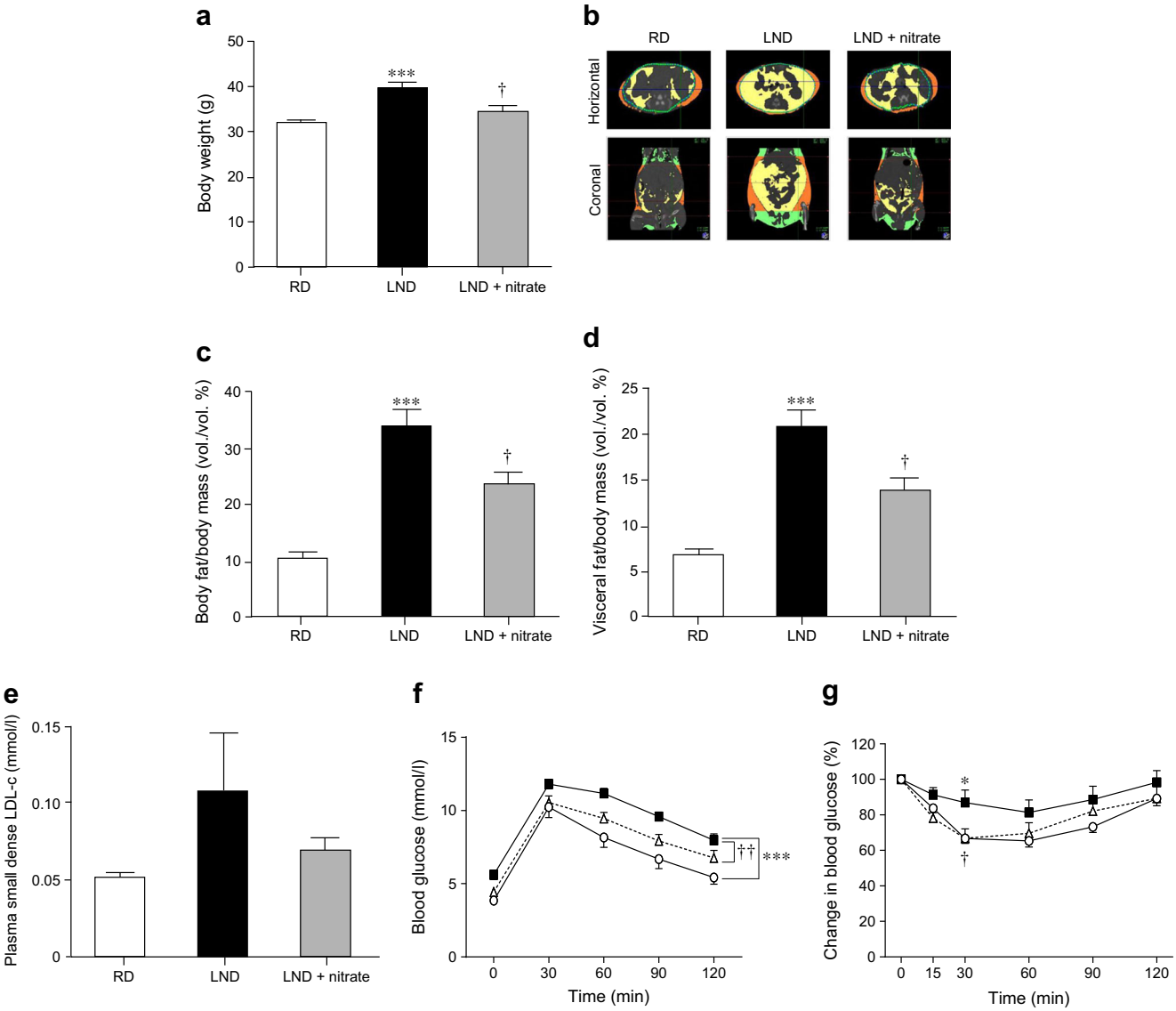

Fig. 6 Effects of 18 months of the LND and nitrate supplementation on body weight, body fat, plasma small dense LDL-cholesterol, blood glucose and insulin sensitivity. (**a**) Body weight (n = 12–16). (**b**) Micro-CT images. Yellow, visceral fat; orange, subcutaneous fat. (**c**) Relationship of body fat to body mass (n = 11–12). (**d**) Relationship of visceral fat to body mass (n = 11–12). (**e**) Levels of plasma small dense LDL-cholesterol (LDL-c; n = 7–9). (**f**) Blood glucose levels after i.p. injection of 1 g/kg glucose (n = 12–14). (**g**) Percentage change in blood glucose levels after i.p. injection of 0.3 U/kg insulin (n = 10). White circles, RD; black squares, LND; white triangles, LND + nitrate. $^{*}p < 0.05$, $^{***}p < 0.001$ for RD vs LND; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ for LND vs LND + nitrate

considerably different between the commercially available low-nitrite/nitrate chow and a regular chow. In this study, we employed a low-nitrite/nitrate chow in which the contents of those ingredients were identical with the regular chow.

We previously bred mice in which all three NOS isoforms were completely disrupted (triply n/i/eNOSs$^{-/-}$ mice) [37], and indicated that both their plasma nitrite/nitrate concentrations and urinary nitrite/nitrate excretion were extremely low, at less than 10% of the normal levels of WT mice [38]. These results suggested that in vivo NO synthesis is predominantly regulated by endogenous NOSs, and that the contribution of the exogenous NO production system to that regulation might be minor. However, contrary to these suggestions, another study showed that a 1 week LND, compared with an RD, markedly decreased plasma nitrite/nitrate levels in WT mice showing normal NOS activities [21]; however, the possible underlying mechanisms remain to be clarified.

In this study, we obtained a similar finding in that 1.5–4.5 months of the LND markedly reduced plasma nitrite/nitrate levels in WT mice. We then examined the causative mechanisms, and found that eNOS levels in the visceral fat, but not in the aorta, were markedly suppressed in the 3 month LND-fed mice, accounting for the markedly depressed plasma

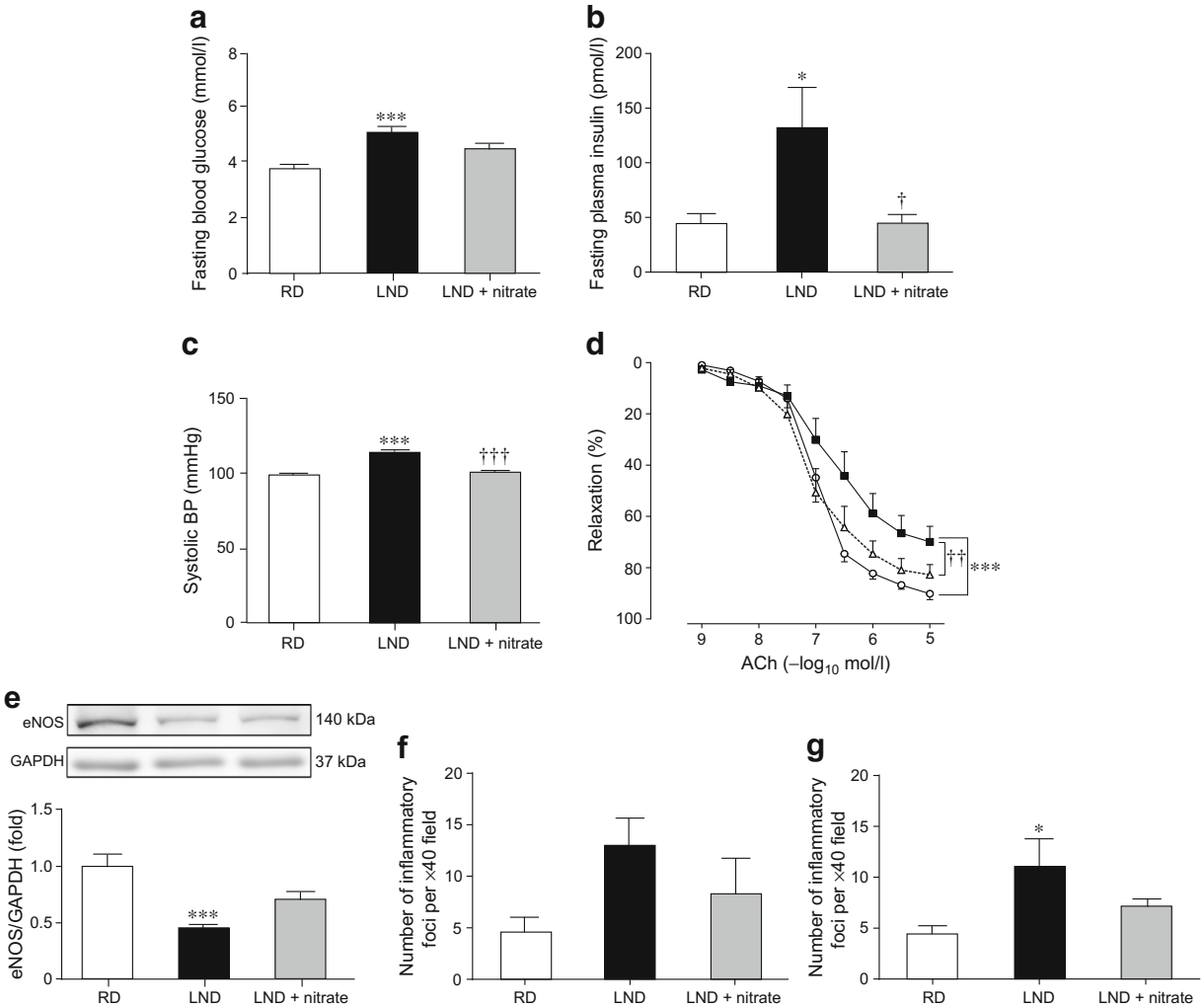

Fig. 7 Effects of 18 months of the LND and nitrate supplementation on fasting blood glucose, blood pressure, vascular reactivity, eNOS levels and inflammation. (**a**) Fasting blood glucose levels (n = 12–14). (**b**) Fasting plasma insulin levels (n = 6–7). (**c**) Systolic BP (n = 12–15). (**d**) Endothelium-dependent relaxation in response to acetylcholine (ACh) (n = 6). White circles, RD; black squares, LND; white triangles, LND + nitrate. (**e**) eNOS protein levels in the aorta (n = 6). (**f**, **g**) Number of inflammatory foci in EWAT (**f**) and peri-renal WAT (**g**) (n = 12–14). $^{*}p < 0.05$, $^{***}p < 0.001$ for RD vs LND; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ for LND vs LND + nitrate

nitrite/nitrate levels induced by the LND. We also found that adiponectin levels in the visceral fat were remarkably low in the 3 month LND-fed mice. Adiponectin has been reported to up-regulate eNOS [39], so the adiponectin insufficiency may have mediated the visceral adipose eNOS downregulation induced by the LND. It has also been reported that eNOS enhances adiponectin levels in adipocytes [40]. Thus, there may be a vicious cycle of adiponectin insufficiency and eNOS downregulation, and this vicious cycle may contribute to the markedly diminished plasma nitrite/nitrate levels induced by the LND.

The levels of PPAR-γ, total AMPK and p-AMPK were significantly lower in the EWAT in the 1 week LND-fed mice compared with the RD-fed mice. As it has been reported that PPAR-γ and AMPK increase levels of adiponectin levels in adipocytes [41, 42], it is conceivable that reduced PPAR-γ and AMPK in the visceral fat were involved in the adiponectin insufficiency induced by the LND.

The 3 month LND-fed mice exhibited visceral obesity with adipocyte hypertrophy, hyper-LDL-cholesterolaemia and hyper-small dense LDL-cholesterolaemia and glucose intolerance, and the 18 month LND-fed mice manifested body weight gain, hypertension, insulin resistance and endothelial dysfunction. These changes eventually resulted in death due to cardiovascular disease, including acute myocardial infarction. These abnormalities were reversed by concurrent nitrate supplementation, indicating that the observed effects were indeed caused by dietary nitrite/nitrate deficiency. It is thus evident that long-term dietary nitrite/nitrate deficiency can cause the metabolic syndrome, endothelial dysfunction and cardiovascular death in mice. The 3 and 4.5 month LND significantly reduced blood glucose-lowering responses to insulin, but did not significantly affect fasting plasma insulin levels. Thus, before 18 months, the effects of LND on glucose tolerance could be due to the result of those on pancreatic beta cell function.

Table 4 Serum levels of 23 inflammatory markers in mice fed the RD and LND for 1.5 months

Marker	Serum levels (pg/ml)		*p* value
	RD	LND	
IL-1α	31.3 ± 4.5	28.0 ± 3.0	0.55
IL-1β	643.9 ± 67.2	558.5 ± 72.1	0.40
IL-2	110.4 ± 18.2	93.4 ± 19.2	0.54
IL-3	27.5 ± 4.6	23.5 ± 4.2	0.53
IL-4	51.7 ± 8.3	46.6 ± 9.3	0.69
IL-5	89.5 ± 14.2	72.8 ± 13.1	0.40
IL-6	67.3 ± 8.6	55.7 ± 8.3	0.34
IL-9	580.2 ± 54.8	544.4 ± 33.7	0.60
IL-10	217.9 ± 56.5	134.3 ± 23.4	0.19
IL-12p40	459.1 ± 26.7	401.8 ± 31.3	0.18
IL-12p70	1084.5 ± 118.6	800.0 ± 139.3	0.14
IL-13	724.3 ± 104.6	714.4 ± 108.3	0.95
IL-17	342.7 ± 30.0	275.5 ± 30.2	0.13
TNF-α	847.6 ± 103.5	632.7 ± 107.4	0.17
IFN-γ	111.6 ± 14.5	85.7 ± 15.7	0.24
MCP-1	746.2 ± 72.7	590.0 ± 70.2	0.14
MIP-1α	74.4 ± 8.4	59.0 ± 10.0	0.26
MIP-1β	131.6 ± 19.0	105.1 ± 16.8	0.31
G-CSF	184.7 ± 22.4	128.6 ± 18.2	0.07
GM-CSF	456.9 ± 19.2	437.8 ± 25.7	0.56
KC	137.6 ± 16.9	118.7 ± 8.1	0.33
Eotaxin	1459.7 ± 75.0	1397.2 ± 104.0	0.63
RANTES	34.2 ± 2.2	23.4 ± 1.3	0.0004

n = 8–10 mice for each group

G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte/macrophage-colony stimulating factor; KC, keratinocyte-derived chemokine; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal cell expressed and secreted

A clustering of cardiovascular risk factors (i.e. the metabolic syndrome) could have contributed to the development of endothelial dysfunction and cardiovascular death in the LND-fed mice. On the other hand, hyper-LDL-cholesterolaemia and hyper-small dense LDL-cholesterolaemia, both of which are independent cardiovascular risk factors [43], were also noted in the LND-fed mice. The small dense LDL-cholesterol particle can easily penetrate the vascular wall because of its small particle size, and is related more strongly to the risk of cardiovascular disease. It is thus likely that those factors may also have been independently involved in the occurrence of endothelial dysfunction and cardiovascular death in the LND-fed mice.

The 18 month LND-fed mice displayed an impairment of endothelium-dependent relaxation in response to acetylcholine, a physiological eNOS activator, along with aortic eNOS downregulation, suggesting the presence of coronary vasospasm. Coronary vasospasm-elicited myocardial ischaemia can lead to fatal cardiac arrhythmia and/or cardiogenic shock. On the other hand, the dead LND-fed mice showed acute myocardial infarction, myocardial fibrosis that might have resulted from myocardial infarction and coronary perivascular fibrosis. They also exhibited pulmonary congestion and acute renal tubular necrosis, both of which are seen in sudden cardiac death. Taking these findings together, we thought that 83.3% (5/6) of the dead LND-fed mice showed changes consistent with cardiovascular death.

The data obtained at 3 and 4.5 months are the most important and significant, as the changes in glucose metabolism, lipid biology, body fat distribution and gut microbiome occurred independent of food intake and body weight. On the other hand, the morbidity and mortality experiments at 18 and 22 months, respectively, are complicated by greater weight gain in the LND group, making it difficult to separate the effects of obesity from those of the nitrite/nitrate deficiency.

We conducted the glucose and insulin tolerance tests under anaesthesia. Whereas it has been reported that high-fat diet-fed C57BL/6J mice showed glucose intolerance compared with their RD-fed counterparts whether they were tested under conscious or anesthetised conditions [44], it has also been indicated that the use of anaesthesia could influence glucose tolerance in C57BL/6J mice [45]. Therefore, the use of anaesthesia could be a limitation of this study.

An increased number of inflammatory foci, decreased eNOS levels and lower adiponectin levels were noted in the visceral fat of the LND-fed mice, and improvements in the LND-induced metabolic syndrome by nitrate supplementation were linked to ameliorations of these changes. As it has been reported that inflammation, eNOS downregulation and adiponectin insufficiency contribute to the occurrence and progression of the metabolic syndrome [46–48], it is possible that those factors were involved in the development of the metabolic syndrome induced by the LND.

The following lines of evidence suggest a causal role of dysbiosis of the gut microbiota in the development of the metabolic syndrome. First, it has been reported that the composition of gut microbiota differs largely between lean individuals and patients with the metabolic syndrome [33]. Second, it has been indicated that transplantation of gut microbiota from obese humans with metabolic abnormalities into germ-free mice results in the development of obesity and metabolic abnormalities in the mice [49]. Third, it has been shown that the transfer of gut microbiota from lean healthy human participants into individuals with the metabolic syndrome improves insulin resistance in the latter group [33]. In our study, there were significantly fewer operational taxonomic units with the LND than with the RD, suggesting less diversity of gut microbiota in the LND-fed mice. There also were significantly different constituents of gut microbiota in a variety of the hierarchy ranks with the LND and the RD. In

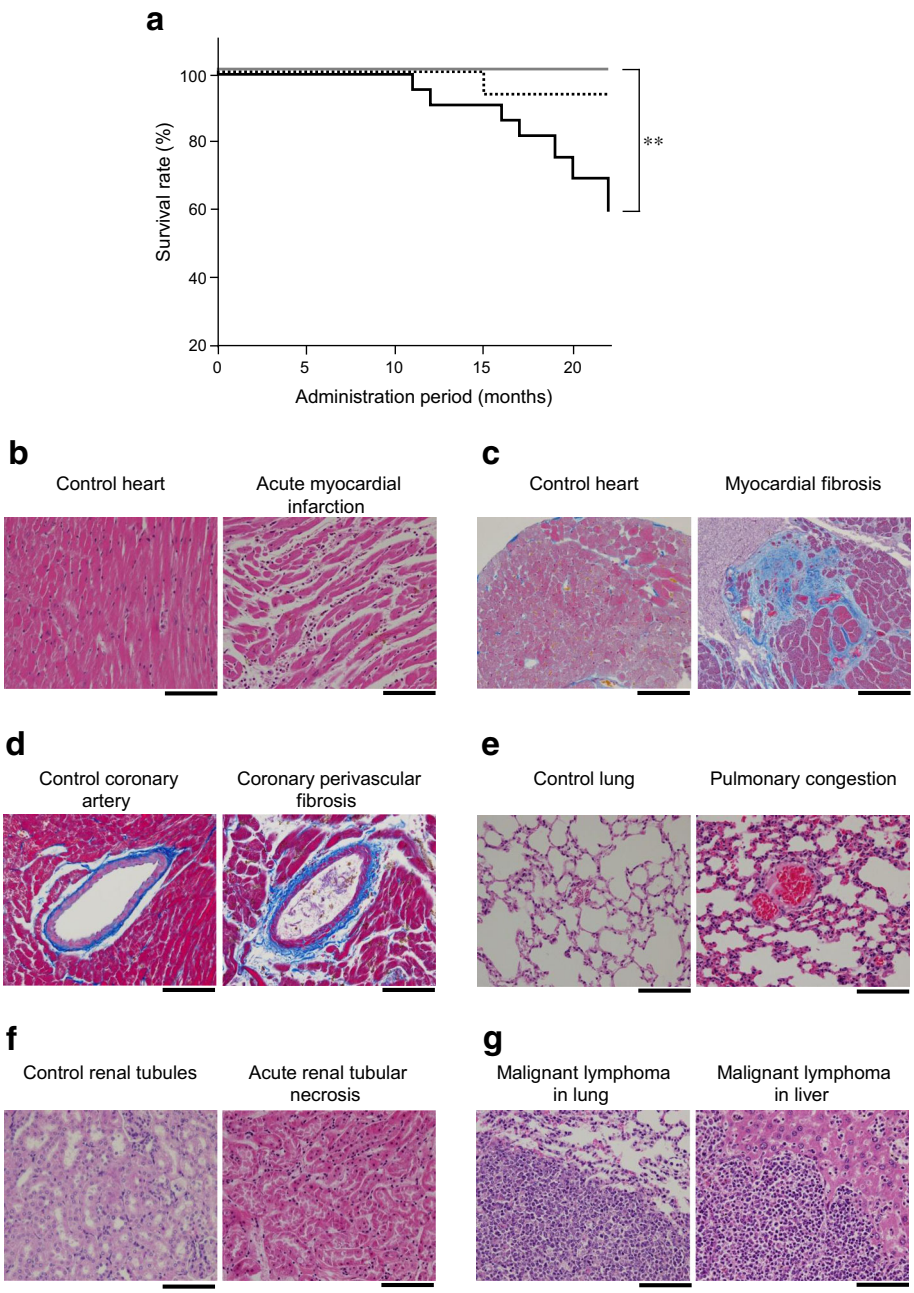

Fig. 8 Effects of 22 months of the LND and nitrate supplementation on survival rate and post mortem findings in LND-fed mice. (**a**) Survival rate (n = 15–24). Grey line, RD; black line, LND; dotted line, LND + nitrate. **$p < 0.01$. (**b**) Acute myocardial infarction. (**c**) Myocardial fibrosis. (**d**) Coronary perivascular fibrosis. (**e**) Pulmonary congestion. (**f**) Acute renal tubular necrosis. (**g**) Malignant lymphoma. Scale bars, 100 μm

agreement with these results, Vrieze et al. indicated that less diversity and fewer distinct constituents of gut microbiota were recognised in individuals with the metabolic syndrome, and that the improvement of insulin resistance after transfer of the gut microbiota was accompanied by an amelioration of the reduced diversity and distinct constituents of gut microbiota [33]. It is thus possible that dysbiotic gut microbiota were involved in the pathogenesis of the metabolic syndrome induced by the LND.

We completely matched the energy contents in the two diets, and food consumption was comparable between the LND and RD at 1.5, 3, 4.5 and 18 months after the start of the diet, suggesting a similar energy intake in the LND and RD. Nevertheless, the LND-fed mice developed the metabolic syndrome. Therefore, we may have succeeded for the first time in identifying specific dietary ingredients that cause the metabolic syndrome even in the absence of excessive intake of energy.

In summary, we were able to demonstrate that long-term dietary nitrite/nitrate deficiency gave rise to the metabolic syndrome, endothelial dysfunction and eventually cardiovascular death in mice, indicating a novel pathogenetic role of the exogenous NO production system in the metabolic syndrome and its vascular complications.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Contribution statement MK-T. and MS designed the study, acquired, analysed and interpreted data, and drafted the article. AT acquired, analysed and interpreted data, and drafted the article. TK, TM, KN, TU, JN, CK, MI, HK, YTa, Yto, S-iK, JO and KS acquired, analysed, and interpreted data. HSu and HM designed the study, analysed and interpreted data. HSh, NY, SM, YO, MM and AA analysed and interpreted data. MT designed the study, analysed and interpreted data, drafted the article. All authors critically revised the article for important intellectual content and gave final approval of the version to be published. MT is the guarantor of this work.

References

1. Eckel R (2015) The Metabolic Syndrome. In: Kasper DL, Fauci AS, Hauser SL, Longo DL, Jameson JL, Loscaldo J (eds) Harrison's Principles of Internal Medicine. Mc Graw Hill Education, New York, pp 2449–2454
2. Beltran-Sanchez H, Harhay MO, Harhay MM, McElligott S (2013) Prevalence and trends of metabolic syndrome in the adult U.S. population, 1999-2010. J Am Coll Cardiol 62:697–703
3. Alberti KG, Eckel RH, Grundy SM et al (2009) Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation 120:1640–1645
4. Mottillo S, Filion KB, Genest J et al (2010) The metabolic syndrome and cardiovascular risk a systematic review and meta-analysis. J Am Coll Cardiol 56:1113–1132
5. Chen J, Muntner P, Hamm LL et al (2004) The metabolic syndrome and chronic kidney disease in U.S. adults. Ann Intern Med 140: 167–174
6. Esposito K, Chiodini P, Colao A, Lenzi A, Giugliano D (2012) Metabolic syndrome and risk of cancer: a systematic review and meta-analysis. Diabetes Care 35:2402–2411
7. Garg PK, Biggs ML, Carnethon M et al (2014) Metabolic syndrome and risk of incident peripheral artery disease: the cardiovascular health study. Hypertension 63:413–419
8. Hamaguchi M, Kojima T, Takeda N et al (2005) The metabolic syndrome as a predictor of nonalcoholic fatty liver disease. Ann Intern Med 143:722–728
9. Hanley AJ, Karter AJ, Williams K et al (2005) Prediction of type 2 diabetes mellitus with alternative definitions of the metabolic syndrome: the Insulin Resistance Atherosclerosis Study. Circulation 112:3713–3721
10. Ignarro LJ (1990) Biosynthesis and metabolism of endothelium-derived nitric oxide. Annu Rev Pharmacol Toxicol 30:535–560
11. Moncada S, Higgs A (1993) The L-arginine-nitric oxide pathway. N Engl J Med 329:2002–2012
12. Murad F (2006) Shattuck Lecture. Nitric oxide and cyclic GMP in cell signaling and drug development. N Engl J Med 355:2003–2011
13. Tsutsui M, Shimokawa H, Otsuji Y, Ueta Y, Sasaguri Y, Yanagihara N (2009) Nitric oxide synthases and cardiovascular diseases: insights from genetically modified mice. Circ J 73:986–993
14. Tsutsui M, Shimokawa H, Otsuji Y, Yanagihara N (2010) Pathophysiological relevance of NO signaling in the cardiovascular system: novel insight from mice lacking all NO synthases. Pharmacol Ther 128:499–508
15. Tsutsui M, Tanimoto A, Tamura M et al (2015) Significance of nitric oxide synthases: Lessons from triple nitric oxide synthases null mice. J Pharmacol Sci 127:42–52
16. Kevil CG, Lefer DJ (2011) Review focus on inorganic nitrite and nitrate in cardiovascular health and disease. Cardiovasc Res 89: 489–491
17. Lundberg JO, Weitzberg E, Gladwin MT (2008) The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. Nat Rev Drug Discov 7:156–167
18. Omar SA, Webb AJ (2014) Nitrite reduction and cardiovascular protection. J Mol Cell Cardiol 73:57–69
19. Milkowski A, Garg HK, Coughlin JR, Bryan NS (2010) Nutritional epidemiology in the context of nitric oxide biology: a risk-benefit evaluation for dietary nitrite and nitrate. Nitric Oxide 22:110–119
20. Weitzberg E, Lundberg JO (2013) Novel aspects of dietary nitrate and human health. Annu Rev Nutr 33:129–159
21. Bryan NS, Calvert JW, Elrod JW, Gundewar S, Ji SY, Lefer DJ (2007) Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury. Proc Natl Acad Sci U S A 104:19144–19149
22. Bryan NS, Calvert JW, Gundewar S, Lefer DJ (2008) Dietary nitrite restores NO homeostasis and is cardioprotective in endothelial nitric oxide synthase-deficient mice. Free Radic Biol Med 45:468–474
23. Zhan J, Nakao A, Sugimoto R et al (2009) Orally administered nitrite attenuates cardiac allograft rejection in rats. Surgery 146: 155–165
24. Park JW, Piknova B, Huang PL, Noguchi CT, Schechter AN (2013) Effect of blood nitrite and nitrate levels on murine platelet function. PLoS One 8:e55699
25. Usui S, Hara Y, Hosaki S, Okazaki M (2002) A new on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides in lipoproteins by HPLC. J Lipid Res 43:805–814
26. Uchida T, Furuno Y, Tanimoto A et al (2014) Development of an experimentally useful model of acute myocardial infarction: 2/3 nephrectomized triple nitric oxide synthases-deficient mouse. J Mol Cell Cardiol 77:29–41
27. Satoh K, Satoh T, Kikuchi N et al (2014) Basigin mediates pulmonary hypertension by promoting inflammation and vascular smooth muscle cell proliferation. Circ Res 115:738–750
28. Kim SW, Suda W, Kim S et al (2013) Robustness of gut microbiota of healthy adults in response to probiotic intervention revealed by high-throughput pyrosequencing. DNA Res 20:241–253
29. Qin N, Yang F, Li A et al (2014) Alterations of the human gut microbiome in liver cirrhosis. Nature 513:59–64
30. Lecomte V, Kaakoush NO, Maloney CA et al (2015) Changes in gut microbiota in rats fed a high fat diet correlate with obesity-associated metabolic parameters. PLoS One 10:e0126931
31. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI (2005) Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A 102:11070–11075
32. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444:1027–1031

33. Vrieze A, Van Nood E, Holleman F et al (2012) Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. Gastroenterology 143:913–916.e917
34. Everard A, Belzer C, Geurts L et al (2013) Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. Proc Natl Acad Sci U S A 110:9066–9071
35. Shin NR, Lee JC, Lee HY et al (2014) An increase in the Akkermansia spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. Gut 63:727–735
36. Gauffin Cano P, Santacruz A, Moya A, Sanz Y (2012) Bacteroides uniformis CECT 7771 ameliorates metabolic and immunological dysfunction in mice with high-fat-diet induced obesity. PLoS One 7:e41079
37. Morishita T, Tsutsui M, Shimokawa H et al (2005) Nephrogenic diabetes insipidus in mice lacking all nitric oxide synthase isoforms. Proc Natl Acad Sci U S A 102:10616–10621
38. Nakata S, Tsutsui M, Shimokawa H et al (2008) Spontaneous myocardial infarction in mice lacking all nitric oxide synthase isoforms. Circulation 117:2211–2223
39. Hattori Y, Suzuki M, Hattori S, Kasai K (2003) Globular adiponectin upregulates nitric oxide production in vascular endothelial cells. Diabetologia 46:1543–1549
40. Koh EH, Kim M, Ranjan KC et al (2010) eNOS plays a major role in adiponectin synthesis in adipocytes. Am J Physiol Endocrinol Metab 298:E846–E853
41. Bouskila M, Pajvani UB, Scherer PE (2005) Adiponectin: a relevant player in PPARgamma-agonist-mediated improvements in hepatic insulin sensitivity? Int J Obes 29(Suppl 1):S17–S23
42. Daval M, Foufelle F, Ferre P (2006) Functions of AMP-activated protein kinase in adipose tissue. J Physiol 574:55–62
43. Sniderman AD, Furberg CD, Keech A et al (2003) Apolipoproteins versus lipids as indices of coronary risk and as targets for statin treatment. Lancet 361:777–780
44. Andrikopoulos S, Blair AR, Deluca N, Fam BC, Proietto J (2008) Evaluating the glucose tolerance test in mice. Am J Physiol Endocrinol Metab 295:E1323–E1332
45. Windelov JA, Pedersen J, Holst JJ (2016) Use of anesthesia dramatically alters the oral glucose tolerance and insulin secretion in C57Bl/6 mice. Physiol Rep 4:e12824
46. Duplain H, Burcelin R, Sartori C et al (2001) Insulin resistance, hyperlipidemia, and hypertension in mice lacking endothelial nitric oxide synthase. Circulation 104:342–345
47. Matsuzawa Y, Funahashi T, Kihara S, Shimomura I (2004) Adiponectin and metabolic syndrome. Arterioscler Thromb Vasc Biol 24:29–33
48. Romeo GR, Lee J, Shoelson SE (2012) Metabolic syndrome, insulin resistance, and roles of inflammation - mechanisms and therapeutic targets. Arterioscler Thromb Vasc Biol 32:1771–1776
49. Ridaura VK, Faith JJ, Rey FE et al (2013) Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science 341:1241214

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BRIEF REPORT

Myositis-specific autoantibodies and their association with malignancy in Italian patients with polymyositis and dermatomyositis

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Abstract This study aims to characterize myositis-specific antibodies in a well-defined cohort of patients with idiopathic inflammatory myopathy and to determine their association with cancer. Sera from 40 patients with polymyositis, dermatomyositis, and controls were tested by protein and RNA immunoprecipitation to detect autoantibodies, and immunoprecipitation-Western blot was used for anti-MJ/NXP-2, anti-MDA5, and anti-TIF1γ/α identification. Medical records were re-evaluated with specific focus on cancer. Anti-MJ/NXP-2 and anti-TIF1γ/α were the most common antibodies in dermatomyositis. In six dermatomyositis cases, we found five solid forms of cancer and one Hodgkin's lymphoma in long-term remission. Among patients with cancer-associated dermatomyositis, three were positive for anti-TIF1γ/α, two for anti-Mi-2, and one for anti-MJ/NXP-2. The strongest positivity of anti-TIF1γ was seen in two active forms of cancer, and this antibody was either negative or positive at low titers in the absence of cancer or in the 7-year remission Hodgkin's lymphoma. Four out of twenty (20 %) patients with polymyositis had solid cancer, but no specific association with autoantibodies was identified; further, none of the four cases of antisynthetase syndrome had a history of cancer. No serum myositis-associated autoantibody was observed in control sera, resulting in positive predictive value 75 %, negative predictive value 78.5 %, sensitivity 50 %, specificity 92 %, and area under the ROC curve 0.7083 for the risk of paraneoplastic DM in anti-TIF1γ/α (+) patients. Myositis-specific autoantibodies can be identified thanks to the use of immunoprecipitation, and their association with cancer is particularly clear for anti-TIF1γ/α in dermatomyositis. This association should be evaluated in a prospective study by immunoprecipitation in clinical practice.

Keywords Biomarkers · Cancer · Idiopathic inflammatory myositis · Immunoprecipitation

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Introduction

Idiopathic inflammatory myopathy (IIM) is characterized by muscle inflammation, skin alterations, and internal organ involvement, resulting in muscle atrophy, skin microangiopathy, and tissue fibrosis [1]. IIMs are divided into several conditions with polymyositis (PM) and dermatomyositis (DM) as the most frequent forms despite being considered rare worldwide [2]. Beyond the clinical and histopathological differences, PM and DM can be further classified into subsets thanks to myositis-specific autoantibodies (MSA) which have diagnostic and prognostic roles [3, 4]. Some MSA have been known for decades, as for the anti-Jo-1 characterizing the antisynthetase syndrome or anti-Mi-2, peculiar for DM [5, 6]. Several MSA have been defined most recently by protein and RNA immunoprecipitation (IP). Paradigmatic MSA include DM-associated anti-MDA5, anti-MJ/NXP-2, and anti-TIF1γ/α which define specific clinical features and predict the

association with cancer [2, 6, 7], sometimes without independent confirmation [8].

To validate the proposed clinical associations, we utilized IP for MSA in a well-characterized cohort of patients with PM and DM from two clinical centers, with particular focus on the specificities identified in recent years and their association with cancer.

Materials and methods

Patients

The study included IIMs patients followed at the outpatient clinic at Humanitas Research Hospital (Rozzano, Milan, Italy) and Spedali Civili (Brescia, Italy) in the period 2013–2016. We included sera from 20 patients with PM, 2 with antisynthetase syndrome, 18 with DM, and controls represented by healthy subjects (NHS; $n = 12$) and patients with systemic sclerosis (SSc; $n = 79$), Behçet's disease (BD; $n = 45$), and psoriatic arthritis (PsA; $n = 145$). We used established criteria for the diagnosis of PM/DM, SSc, BD, and PsA and collected clinical and laboratory data at enrollment.

The study was approved by the Institutional Review Board of the hospitals and informed consent was obtained from all subjects.

Methods for autoantibody analysis

Patients' sera were isolated from whole blood through centrifugation at 2000*g* for 15 min, and then stored in −20 °C freezer until use. MSA were first screened by protein-IP using ^{35}S-methionine-labeled K562 cell extract followed by SDS-PAGE and autoradiography, and by RNA-IP using unlabeled K562 cell extract followed by urea-PAGE and silver staining [9, 10]. MSA were determined using reference sera obtained from the Autoantibody Standardization Committee (www.autoab.org) and from internal controls.

Candidates for anti-MJ/NXP-2 and anti-MDA5 were tested by IP-Western Blot (IP-WB) based on IP of a 140-kD protein, while candidates for anti-TIF1γ/α were selected based on bands at 155–140 kD by protein-IP. In detail, 8 μl of candidate sera were cross-linked with protein-A Sepharose beads and then immunoprecipitated with cell extract from 10^7 K562 cells. Proteins were then fractionated by 8 % SDS-PAGE and transferred to a nitrocellulose filter, probed with 1 μg/ml of anti-MORC3 mouse polyclonal antibody (Abnova, Taipey City, Taiwan) for MJ/NXP-2, followed by horseradish peroxidase (HRP) goat anti-mouse IgG (1:5000 dilution) (ThermoFisher, Waltham, MA, USA) and developed using Immobilon Western Chemiluminescent HRP substrate (Millipore, Darmstadt, Germany). The same procedure was used for anti-MDA5 antibodies using 1:1000 rabbit anti-MDA5 antibody (Millipore, Darmstadt, Germany) followed by 1:5000 HRP-conjugated goat anti-rabbit Ig light chain antibody (Jackson Immunoresearch, West Grove, PA, USA), and developed using Supersignal West Femto (ThermoFisher, Waltham, MA, USA). For TIF1γ IP-WB, we used 1:1000 mouse monoclonal anti-TIF1γ antibody (Abcam, Cambridge, UK), followed by 1:10.000 HRP goat anti-mouse IgG (ThermoFisher, Waltham, MA, USA), and developed using Immobilon Western Chemiluminescent HRP substrate (Millipore, Darmstadt, Germany).

Statistical analysis

All comparisons were performed by Mann-Whitney test and Pearson Chi square test using Stata 13.1 for Macintosh (StataCorp, 2013, CollegeStation, Texas, USA) and Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was accepted as $p < 0.05$.

Results

Through protein- and RNA-IP, we identified serum autoantibodies in IIMs as illustrated in Fig. 1a. None of our control sera were positive for MSA. Before using IP and IP-WB, only 6/18 (33 %) DM and 4/20 (20 %) PM cases had autoantibodies detected by routine autoimmunity tests, particularly anti-Ro/SSA, La/SSB, Mi-2, and -Jo-1. Thanks to IP analysis, we confirmed this positivity and identified additional MSA in patients with positive antinuclear antibodies (ANA) by indirect immunofluorescence but negative autoantibody for extractable nuclear antigens (ENA). We did not observe double autoantibody positivity in our cohort, except for the association of Ro/SSA, La/SSB, and Jo-1 as reported [11]. Protein- and RNA-IP confirmed two less common antisynthetase antibodies as anti-EJ (anti-glycyl tRNA synthetase) and anti-PL-12 (anti-alanyl tRNA synthetase) in one PM and one antisynthetase syndrome case, respectively (Fig. 1a). Using RNA-IP, we identified the 7SL RNA band characteristic of anti-SRP antibodies, in association with anti-Ro/SSA in one patient with PM (Fig. 1b), and in both cases necrotizing myositis was seen at muscle biopsy. Eight cases (1 DM and 7 PM) remain seronegative by IP, while in six DM cases, we identified bands at different molecular weight by protein-IP but their antigenic significance is still unknown (data not shown). Nine samples (5 IIMs and 4 SSc) had one band detectable around 140 kD by protein IP, and were tested by IP-WB for anti-MJ/NXP-2 and -MDA5 antibodies to identify the specificity corresponding to this band. In three DM cases, we

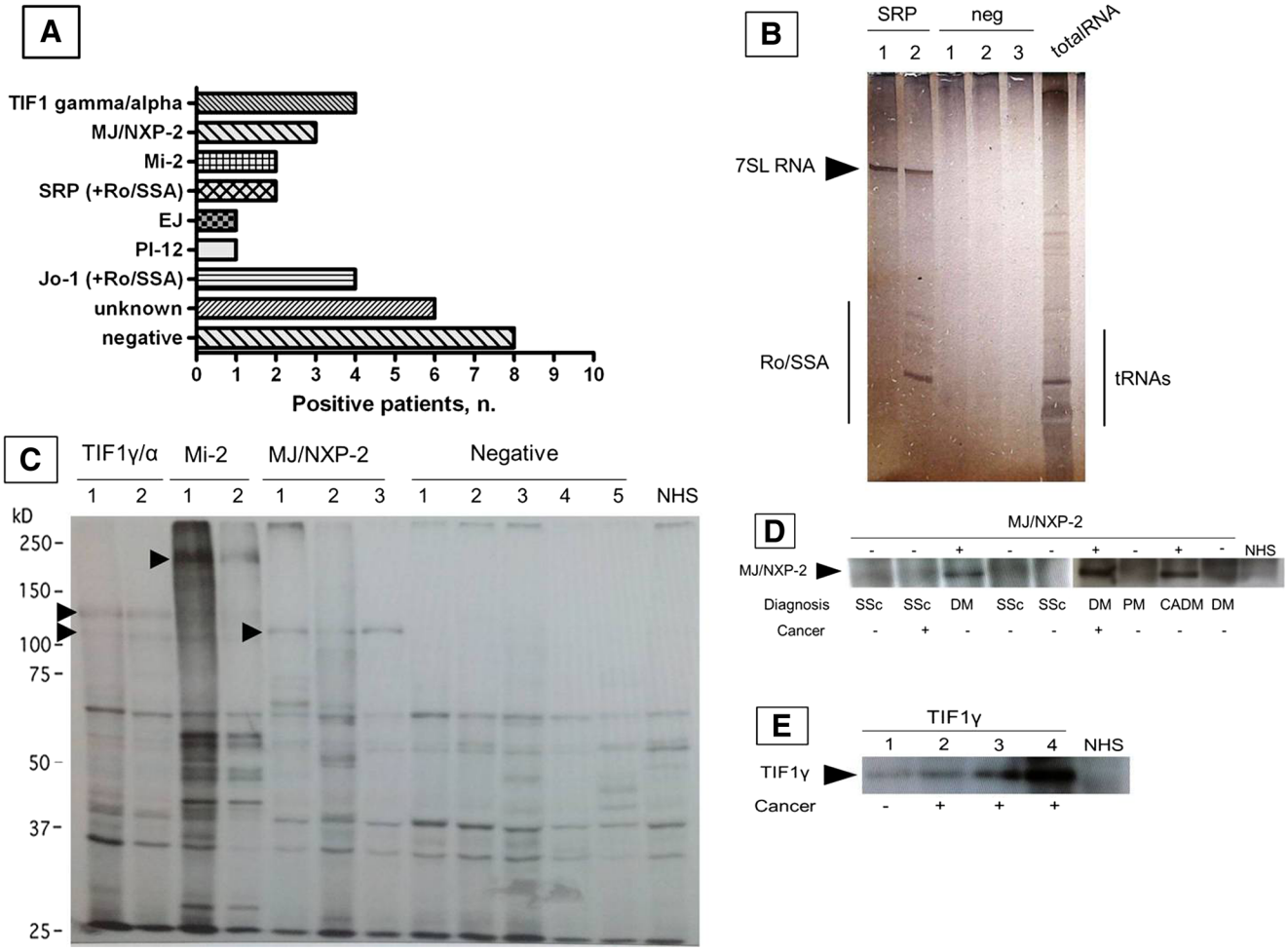

Fig. 1 MSA identified in our cohort of Italian PM/DM through the use of protein-IP, RNA-IP, and IP-WB. **a** Bar graph showing the specific autoantibodies and the number of corresponding cases identified in our cohort of PM/DM patients. **b** RNA-IP of two positive SRP samples, recognized by the band corresponding to 7SL RNA (*black arrow*). In one case, association with anti-Ro/SSA antibodies was identified (*black vertical line*) and supported by protein-IP (data not shown). The three negative RNA-IP samples shown are the anti-MJ/NXP-2 (+) patients reported in **c** and **d**, that typically do not show reactivity by RNA-IP. Total RNA was used as positive control. **c** Protein-IP (8 % SDS-PAGE gel) of representative PM/DM patients and corresponding autoantibodies: two anti-TIF1γ/α (+) cases shown by the bands at 155/140 kD (*black arrows*; two additional cases not shown in this protein-IP gel were identified based on the mobility of the same bands), two anti-Mi-2 (+) cases shown by the 240, 150, 72, 65, 63, 50, and 34 kD bands (*black arrow* for the 240 kD band), and the three anti-MJ/NXP-2 (+) cases identified by the 140 kD band (*black arrow*). Five DM cases negative for MSA are also shown, and one NHS (normal human serum) is present in the last lane. **d** IP-WB for anti-MJ/NXP-2 positive cases. The three anti-MJ/NXP-2 (+) cases shown in the protein-IP gel in **c** were tested together with other samples (myositis and SSc) that had 140 kD band at protein-IP; no SSc sample had positivity for MJ/NXP-2. This panel also represents in which cases an association with cancer was present, as described in Table 2. **e** IP-WB for anti-TIF1γ positive cases. The four anti-TIF1γ (+) cases identified by protein-IP through the detection of the 155/140 kD bands were positive by IP-WB as shown in this panel. The weakest sample (#1) was the only one not associated with cancer until the moment of evaluation of the patient, sample #2 had a diagnosis of Hodgkin's Lymphoma 7 years before DM onset and it is now considered in remission, sample#3 has active lung cancer, and sample#4 has advanced ovary cancer. One normal human serum (*NHS*) is represented in the last lane

confirmed the anti-MJ/NXP-2 positivity (Fig. 1c, d), while no MSA was detected in our control population.

The main clinical and laboratory features of patients are described in Table 1. The diagnosis of myositis was confirmed by muscle biopsy and/or electromyography only in 13/18 (72 %) of DM patients, coined clinically amyopathic DM. No significant difference was detected for organ involvement, laboratory tests abnormalities, and ongoing therapies in DM and PM patients, while the expression of anti-TIF1γ/α antibodies was significantly associated to DM patients (p = 0.04) as shown in Table 1. The ANA pattern reported by routine autoimmunity tests was very variable for titer and pattern, and in some cases also defined as "negative" (Tables 1 and 2), thus it was necessary to proceed with further testing by IP for the identification of MSA. Two anti-MJ/NXP-2 necessary to proceed with further testing by IP for the identification of MSA. Two anti-MJ/NXP-2 (+) DM patients had severe diffuse calcinosis that required surgical

Table 1 Main demographic and clinical features of our cohort of DM and PM patients, for which we performed serum IP analysis. Two anti-synthetase cases are not included

	DM (n = 18)	PM (n = 20)	p
Demographic features			
Female:Male	13:5	14:6	–
Mean age at enrollment, years (range)	49 (21–75)	59 (29–83)	0.05
Mean age at myositis onset, years (range)	42.5 (15–71)	53.5 (24–78)	ns
Clinical features			
Myositis (%)*	13 (72)	20 (100)	0.01
Raynaud's phenomenon (%)	7 (39)	4 (20)	ns
Arthritis (%)	5 (28)	5 (25)	ns
Interstitial lung disease (%)	2 (11)	5 (25)	ns
Dysphagia (%)	2 (11)	7 (35)	ns
Cancer (%)	6 (33)	4 (20)	ns
Use of steroid therapy (%)	17 (94)	19 (95)	ns
Use of immunosuppressants (%)	16 (89)	16 (80)	ns
Laboratory features			
Median CK at myositis onset, U/l (25th–75th percentile)	1605 (92–5160)	1400 (842–3295)	ns
ANA positive titer ≥1:320 (%)	9 (50)	11 (55)	ns
ENA identified by IP			
Anti-TIF1γ/α	4 (22)	0	0.04
Anti-MJ/NXP-2	3 (17)	0	ns
Anti-Mi-2	2 (11)	0	ns
Anti-SRP	1+Ro/SSA(5)	1 (5)	ns
Anti-Jo-1	0	4+Ro/SSA (20)	ns
Anti-EJ	0	1 (5)	ns
Anti-PL-12	0	1 (5)	ns
Anti-HMGCR	0	0	–
Anti-MDA5	0	0	–

ANA anti-nuclear antibodies, *CK* creatine kinase, *ENA* extractable nuclear antigen, *GI* gastro-intestinal, *IP* immunoprecipitation, *ns* not significant

*Confirmed by electromyography and/or muscle biopsy

removal in one case, and ongoing therapy with pamidronate infusions in one case of clinically amyopathic DM [12]. The association with cancer was present only in one DM case positive for this autoantibody (Fig. 1d). No serum was positive for anti-MDA5 antibodies, and in fact, no patient in our PM/DM cohort had symptoms such as rapidly progressive interstitial lung disease that are usually associated with this autoantibody [6]. The four anti-TIF1γ (+) sera have history of cancer in the three strongest positive cases (Fig. 1e), while the weakest positive case is the only one without cancer history until the moment of our clinical evaluation.

Cumulatively, we calculated the positive predictive value (75 %), negative predictive value (78.5 %), sensitivity (50 %), specificity (91.6 %), and area under the ROC curve (0.7083) for the risk of paraneoplastic DM in anti-TIF1γ/α (+) patients and these were compared to previous reports.

Discussion

The routine use of protein- and RNA-IP may increase the detection rate of rare autoantibodies in clinical practice, particularly in rare conditions such as PM and DM, thus maximizing the diagnostic and prognostic power of these biomarkers. In fact and despite the low incidence and prevalence worldwide, IIMs are characterized by wide clinical phenotype variability, mirrored by a significant number of MSA. We thus utilized the sensitive and specific IP to identify autoantibody prevalence and clinical significance in a well-defined cohort of Italian patients affected by PM/DM, with particular focus on cancer associations.

Our most relevant findings include that anti-MJ/NXP-2 and -TIF1γ/α antibodies are the two most frequent MSA in DM cases previously anti-ENA negative at routine tests.

Table 2 Main characteristics of the anti-MJ/NXP-2 (+) and TIF1γ/α (+) cases identified in our cohort of PM/DM patients. The cases described in this table are shown in Fig. 1d, e

	Anti-MJ/NXP-2 case 1	Anti-MJ/NXP-2 case 2	Anti-MJ/NXP-2 case 3	Anti-TIF1γ/α case 1	Anti-TIF1γ/α case 2	Anti-TIF1γ/α case 3	Anti-TIF1γ/α case 4
Demographic data							
Sex	Female	Female	Male	Male	Female	Female	Female
Age (years)	33	21	21	72	40	59	54
Diagnosis	DM	CADM	DM	DM	DM	CADM	DM
Age at onset (years)	19	15	18	67	22	58	52
Clinical data							
Skin lesions	+ (Gottron's papules, erythematosus rash)	+ (Gottron's papules, erythematosus rash)	+ (V-neck erythema)	+ (Gottron's papules, erythematosus rash)	+ (Gottron's papules, heliotrope rash)	+ (Gottron's papules, erythematosus rash)	+ (Gottron's papules, erythematous rash)
Calcinosis	+++	+++	−	−	−	−	−
Myositis	+	−	+	+	+	−	+
Arthritis	−	−	−	−	−	−	−
Raynaud's phenomenon	−	−	−	−	−	−	+
Interstitial lung disease	−	−	−	−	−	−	−
Cancer	+	−	−	−	+	+	+
Cancer location	Thyroid	−	−	−	Hodgkin's lymphoma	Lung adenocarcinoma	Ovary
Age at cancer onset (years)	31	−	−	−	15	56	52
Immunosuppressive therapy*	+ (PDN, HCQ, MTX, CsA, IV Ig, AZA)	+ (PLQ)	+ (PDN, PLQ, MTX, IV Ig, AZA, CsA)	+ (PDN, MMF)	+ (PDN, HCQ, CTX, AZA)	−	+ (PDN, MTX, IV Ig)
Laboratory data							
Increased CK at myositis onset	+	−	+	+	+	−	+
CK at last visit	+	Normal	Normal	Normal	Normal	Normal	+
ANA	1:640 nuclear dots	1:160 speckled	1:80 speckled	Negative	1:160 speckled	Negative	>1:640 speckled

ANA anti-nuclear antibodies, *AZA* azathioprine, *CADM* clinically amyopathic DM, *CK* creatine kinase, *CsA* cyclosporine, *CTX* cyclophosphamide, *DM* dermatomyositis, *HCQ* hydroxychloroquine, *IV Ig* intravenous immunoglobulins, *MMF* mycophenolate mofetil, *MTX* methotrexate, *PDN* prednisone

*The order of these therapies corresponds to the chronological order they were used by the patients

Accordingly, to what reported in the literature, our three anti-MJ/NXP-2 (+) DM cases have juvenile onset DM with typical skin DM features, no internal organ involvement, and the worst clinical manifestation is severe calcinosis [13]. All these cases required immunosuppressive therapy beyond steroids to control muscle inflammation, but in one case, DM was not completely controlled and this unresponsive patient had a diagnosis of papillary thyroid cancer 12 years after the onset of DM. In fact, cancer has been reported in adult anti-MJ/NXP-2(+) DM patients despite not being confirmed in our previous publication on a different Italian cohort [10, 14]. The identification of anti-MJ/NXP-2 antibodies was based on the first observation of a common band of 140 kD molecular weight by protein-IP, but it was then necessary to perform IP-WB to have a positive result for MJ/NXP-2. Anti-MDA5 antibodies also migrate in the same molecular weight range, but no sample tested positive by IP-WB and it was concordant with the clinical observation that these samples did not show the suggestive clinical features (i.e., rapidly progressive interstitial lung disease) that are commonly referred to anti-MDA5 positivity.

We detected serum anti-TIF1γ/α antibodies in four DM cases, only in one case there was no history of cancer despite extensive screening exams and it was the weakest positive case. All the other three cases have cancer history. In one case, this autoantibody was present in a DM patient with Hodgkin's

lymphoma diagnosed and treated 7 years prior to the onset of DM features, and considered in remission at the time of the blood draw. This is in contrast with previous reports of anti-TIF1γ/α antibodies not found in juvenile DM cases associated with cancer and of the highest associated risk of malignancy during the year prior to and the year after IIMs diagnosis [15]. The two strongest samples positive for anti-TIF1γ/α antibodies have active cancer unresponsive to treatment at the time of blood drawn. It is also important to highlight the fact that both our two anti-Mi-2(+) DM cases had a history of breast cancer prior to DM onset but no higher risk of cancer has been reported in association with this autoantibody [4]. Our estimate of the positive and negative predictive values, sensitivity, specificity, and area under the ROC curve for the risk of paraneoplastic DM in anti-TIF1γ/α (+) patients were concordant to previous reports [16–21].

In our PM cohort, four cases of cancer were reported, in three being diagnosed several years prior to and in one concomitant to the onset of PM; in all cases, solid forms affecting the thyroid, colon, breast, skin, and only two of them had a known autoantibody signature at routine tests represented by anti-Ro/SSA antibodies. No tumor was reported in the four cases affected by antisynthetase syndrome, and no MSA was identified in SSc, BD, PsA cases with or without a history of cancer. No PM case showed positivity for anti-3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) antibodies despite the onset of necrotizing myopathy after the use of statin [3]. Other autoantibodies that were reported by routine autoimmunity laboratories through techniques such as immunoblotting were not confirmed by IP, as for anti-PM/Scl (PM100) and PL-7.

We collected data relative to indirect immunofluorescence ANA patterns reported by routine laboratory tests, and we observed that the most frequent ANA pattern reported in our anti-MJ/NXP-2(+) and anti-TIF1γ/α(+) cases is speckled, and in one anti-MJ/NXP-2(+) case, the presence of nuclear dots suggestive for promyelocytic leukemia nuclear bodies was reported, thus needing further evaluation [10]. Despite the use of the most sensitive techniques, eight patients with IIMs remained negative for both ANA and ENA, while in six additional cases, we could identify bands by protein-IP, but no clear specificity [22]. These gaps underline the existing limitations in the identification of autoantibodies in rheumatic diseases such as PM/DM which are mainly due to lack of standardization for ANA and ENA, low number of positive cases studied for autoantibodies clinical association, identification of rare and new autoantibodies through time and labor-consuming techniques such as IP, and the lack of commercially available techniques that may help in the identification of rare autoantibodies in a clinical setting [23] and shed light on PM/DM pathogenesis [2]. We acknowledge that the efforts of international registries such as Euromyositis or the Autoantibody Standardization Committee are expected to minimize the frequency of seronegative cases and to provide a clear estimate of the prevalence of rare autoantibodies [24].

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Compliance with ethical standards

Disclosures None.

References

1. Lundberg IE et al (2016) Diagnosis and classification of idiopathic inflammatory myopathies. J Intern Med 280(1):39–51
2. Ceribelli A. et al. (2016) *The Immune Response and the Pathogenesis of Idiopathic Inflammatory Myositis: a Critical Review.* Clin Rev Allergy Immunol
3. Gunawardena H. (2015) *The Clinical Features of Myositis-Associated Autoantibodies: a Review.* Clin Rev Allergy Immunol
4. Satoh M. et al. (2015) *A Comprehensive Overview on Myositis-Specific Antibodies: New and Old Biomarkers in Idiopathic Inflammatory Myopathy.* Clin Rev Allergy Immunol
5. Targoff IN (1992) Autoantibodies in polymyositis. Rheum Dis Clin N Am 18(2):455–482
6. Ceribelli A et al (2014) Prevalence and clinical significance of anti-MDA5 antibodies in European patients with polymyositis/dermatomyositis. Clin Exp Rheumatol 32(6):891–897
7. Satoh M et al (2012) Autoantibodies to transcription intermediary factor TIF1beta associated with dermatomyositis. Arthritis Res Ther 14(2):R79
8. Fiorentino DF et al (2016) PUF60: a prominent new target of the autoimmune response in dermatomyositis and Sjogren's syndrome. Ann Rheum Dis 75(6):1145–1151
9. Ceribelli A et al (2010) Anti-Th/To are common antinucleolar autoantibodies in Italian patients with scleroderma. J Rheumatol 37(10):2071–2075
10. Ceribelli A et al (2012) Anti-MJ/NXP-2 autoantibody specificity in a cohort of adult Italian patients with polymyositis/dermatomyositis. Arthritis Res Ther 14(2):R97
11. Yamasaki Y et al (2016) Clinical subsets associated with different anti-aminoacyl transfer RNA synthetase antibodies and their association with coexisting anti-Ro52. Mod Rheumatol 26(3):403–409
12. Palaniappan P, Lionel AP, Kumar S (2014) Successful treatment of calcinosis cutis in juvenile dermatomyositis with pamidronate. J Clin Rheumatol 20(8):454–455
13. Rider LG et al (2013) The myositis autoantibody phenotypes of the juvenile idiopathic inflammatory myopathies. Medicine (Baltimore) 92(4):223–243
14. Ichimura Y et al (2012) Anti-NXP2 autoantibodies in adult patients with idiopathic inflammatory myopathies: possible association with malignancy. Ann Rheum Dis 71(5):710–713
15. Tiniakou, E. and A.L. Mammen, *Idiopathic Inflammatory Myopathies and Malignancy: a Comprehensive Review.* Clin Rev Allergy Immunol, 2015.
16. Selva-O'Callaghan A et al (2010) Malignancy and myositis: novel autoantibodies and new insights. Curr Opin Rheumatol 22(6):627–632
17. Targoff IN et al (2006) A novel autoantibody to a 155-kd protein is associated with dermatomyositis. Arthritis Rheum 54(11):3682–3689

18. Gunawardena H et al (2008) Clinical associations of autoantibodies to a p155/140 kDa doublet protein in juvenile dermatomyositis. Rheumatology (Oxford) 47(3):324–328
19. Kaji K et al (2007) Identification of a novel autoantibody reactive with 155 and 140 kDa nuclear proteins in patients with dermatomyositis: an association with malignancy. Rheumatology (Oxford) 46(1):25–28
20. Chinoy H et al (2007) The diagnostic utility of myositis autoantibody testing for predicting the risk of cancer-associated myositis. Ann Rheum Dis 66(10):1345–1349
21. Fujikawa K et al (2009) Association of distinct clinical subsets with myositis-specific autoantibodies towards anti-155/140-kDa polypeptides, anti-140-kDa polypeptides, and anti-aminoacyl tRNA synthetases in Japanese patients with dermatomyositis: a single-centre, cross-sectional study. Scand J Rheumatol 38(4):263–267
22. Selmi C et al (2016) Serum antinuclear and extractable nuclear antigen antibody prevalence and associated morbidity and mortality in the general population over 15 years. Autoimmun Rev 15(2): 162–166
23. Cavazzana I et al (2016) Testing for myositis specific autoantibodies: comparison between line blot and immunoprecipitation assays in 57 myositis sera. J Immunol Methods 433:1–5
24. Chan EK et al (2016) Report on the second International Consensus on ANA Pattern (ICAP) workshop in Dresden 2015. Lupus 25(8): 797–804

Calcinosis in poly-dermatomyositis: clinical and laboratory predictors and treatment options

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Abstract

Objective

We aimed to identify the possible clinical and laboratory predictors of calcinosis in a cohort of patients with a diagnosis of polymyositis (PM) and dermatomyositis (DM).

Methods

We carried out a retrospective analysis of a cohort of myositis patients attending our clinic between January 2013 and May 2014.

Results

74 patients (58 females, 16 males) with PM (30 cases), DM (30 cases), overlap syndrome (13 cases) and inclusion body myositis (1 case) were enrolled. Sixteen patients (21.6%) had calcinosis that occurred a mean of 43.7 months after diagnosis of PDM. At multivariate analysis, patients with calcinosis experienced longer follow-up duration (p=0.006), anti-PM/Scl (p=0.033) and anti-NXP2 (p=0.024) positivity compared to patients without calcinosis. Furthermore, anti-NXP-2 positive C+ showed a diffuse form of calcinosis from the beginning and lower frequency of respiratory tract involvement. No single drug or associations of drugs was found effective in the treatment of calcinosis.

Conclusion

A longer follow-up period of time, DM diagnosis and positivity for PM/Scl and NXP-2 could all be considered risk factors which foresee the development of calcinosis. Moreover, the positivity for antibodies to NXP-2 depicts a distinct phenotype of calcinosis with an early onset and quick widespread dissemination.

Key words

calcinosis, myositis, autoantibodies, anti-NXP-2, anti-PM/Scl

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Introduction

Calcinosis represents a severe complication of poly-dermatomyositis (PDM): it could develop in about 44–70% of children with juvenile dermatomyositis (JDM), especially when a delay of diagnosis or a poor control of the disease occurs (1). Calcinosis can also arise in adult cases of polymyositis (PM) and dermatomyositis (DM) respectively in 5 and 10%, and recent data reported up to 20% prevalence of calcinosis in adult DM (2). Calcinosis is defined as a clinical condition characterised by the deposition of insoluble salts of calcium in skin, subcutaneous tissues and muscle, which may be responsible for pain and functional disability.

In JDM, cutaneous calcinosis typically occurs between 2–3 years after the onset of the disease, much earlier than in other connective tissue diseases and in adult PDM, in which it appears about 8 years after diagnosis. Calcinosis is usually localised in damaged tissues (due to local trauma or inflammatory process), without significant changes of calcium and phosphate serum levels (3). Several pathogenetic hypotheses are proposed, including inflammatory infiltrates in calcific deposits (4, 5); a local vascular ischaemia (6); a dysregulation of mechanisms controlling the deposit/solubility of calcium and phosphate (7, 8) and mitochondrial damage of muscle cells during DM (9).

Predictive parameters of the development of calcinosis in JDM were identified in early onset of DM, long disease duration, poor response to conventional drugs (1, 10-12). In patients with systemic sclerosis (SSc) an association of calcinosis with arthritis and digital ulcers was noticed (6).

Recently a new myositis-specific antibody, named anti-MJ/NXP-2, has been reported in association with cutaneous calcinosis in JDM (13) as well as in adult DM (14, 15). So far, in adult patients, a clinical profile of risk factors for calcinosis has not been defined. In addition, although many therapeutic aids have been proposed, calcinosis of the adult seems particularly refractory and currently there are no known treatments of proven efficacy (2).

The aim of this study is to identify possible clinical and/or immunological prognostic factors and treatment options in a monocentric cohort of patients with a diagnosis of PDM and calcinosis.

Materials and methods

Patients

Patients affected by PM, DM (16), inclusion body myositis (IBM) (17) and overlap syndromes (OS) with myositis such as systemic lupus erythematosus (SLE) (18), SSc (19), rheumatoid arthritis (RA) (20) and Sjögren syndrome (SSj) (21) attending our outpatient clinic between January 2013 and May 2014 were enrolled in the study: all cases with calcinosis (C+) and, as a control group, patients without calcinosis (C-) were considered. Clinical, serological and treatment data were retrospectively collected from clinical charts.

Calcinosis was defined as the presence of calcium deposition in the skin, subcutaneous tissues or muscle on physical examination and with plain radiographs in every patient. The therapy effect was assessed at the end of treatment and defined as "yes" when calcinosis improved, "partial" when stable and "no" when calcinosis worsened based on subjective reporting by a patient and on clinical evaluation by trained rheumatologists according to Galimberti *et al.* (22).

Methods

Antinuclear antibodies (ANA) were tested by indirect immunofluorescence (IIF) on HEp-2 cells and considered positive at titre ≥1:160 (BioRad, Hercules, CA, USA). Myositis-specific and associated autoantibodies (MSA and MAA) were detected by counterimmunoelectrophoresis (23), immunoprecipitation (IP) using 35-S-methionine-labeled K562 cell extract, RNA components analysis of immunoprecipitates by silver staining and anti-Jo-1 ELISA as previously described (24).

Ethics

The study was approved by the Institutional Review Board of the Hospital. The patients' written consent was obtained according to the Declaration of Helsinki, and the study was conducted

in compliance with the standards currently applied in our country.

Statistical analysis

Comparison between patients with and without calcinosis was performed with student's 2-tailed *t*-test for continuous variables and Chi-square or Fisher's exact tests for categorical variables. A multivariate analysis was conducted by a logistical regression model (Statview); *p*-values less than 0.05 were considered significant. Odds ratio (OR) and 95% confidence interval (CI 95%) were also calculated.

Results

Demographic and clinical data

Seventy-four patients (58 females, 16 males) with PM (30 cases), DM (30 cases), OS (13 cases) and inclusion body myositis (1 case) were enrolled. The majority of them were Caucasian (72; 97.3%), one African and one Asian. Mean age at disease onset was 43±17.4 years, and the average follow-up was 56 months (range 1 to 288 months). Calcinosis was found in 16 out of 74 patients (21.6%): 11/16 with DM (68.7%), 4/16 OS (25%) and 1/16 PM (6.2%). The prevalence of calcinosis was 36.7% in DM (11/30) 30.8% among the OS (4/13) and 3.3% (1/30) in PM cases. Calcinosis occurred a mean of 43.7 months (± 71) after the diagnosis; in 4 cases the onset was concomitant and for 1 patient it occurred before the diagnosis.

At onset 69% of calcinosis was isolated, while after one year more than 60% of our patients had multiple sites involved. Pelvic girdle, hands and extremities were the most frequently affected sites (50%, 37.5% and 31% respectively). About a quarter of cases showed calcinosis at root of upper limb; elbow, trunk and face were more rarely involved. Pain was associated in 50% of cases, superinfection in 38%, ulceration in 25%. About a third of cases were asymptomatic.

Comparison between patients with (C^+) and without calcinosis (C^-)

Clinical and serological features of patients with (C^+) and without calcinosis (C-) are listed in Table I. C^+ patients showed a quite longer follow-up (201.8 *vs*. 111.9 months, p=0.001), a higher percentage of DM (p=0.026, OR 4.5, CI 95%=1.16–19.38) and less prevalence of PM (p=0.001, OR 0.067, CI 95%=0.03–0.54). OS was more frequently detected in C^+ group, without statistical significance. Concerning clinical data, C^+ cases more frequently showed Gottron's lesions (p=0.021, OR 4.27, CI 95%=1.14–16.4), a low frequency of high CK elevation (p=0.012, OR 0.19, CI 95%=0.04–0.79), but no other statistical differences were found between the two groups. Fingertip ulcers were detected only in a few patients, more frequently in C^+ than in C- without statistical difference. Two out of the three patients with calcinosis and fingertips ulcers were affected by scleromyositis with antibodies to PM/Scl. Only one out of the 6 patients with calcinosis of the hands concomitantly had fingertips ulcers. Furthermore, no differences were found in the frequency of other systemic sclerosis features. Regarding cancer occurrence, no differences between the two groups were recorded.

Table I. Clinical and laboratory data of 16 patients with calcinosis (C^+) and 58 patients without calcinosis (C^-) at the univariate analysis.

	C^+, n. 16 (%)	C^-, n. 58 (%)	*p*-value	OR (CI 95%)
Follow-up, months; mean ± SD	201.8 ± 118.7	111.9 ± 87.4	0.001	
DM	11 (68.7)	19 (32.7)	0.021	4.5 (1.2-17.7)
PM	1 (6.2)	30 (51.7)	0.003	0.06 (0.003-0.54)
Overlap syndrome	4 (25)	9 (15.5)	NS	
IBM	0 (0)	1 (2)	NS	
F:M ratio	13/3	45/13	NS	
Fever	7 (44)	18 (31)	NS	
Fatigue	15 (94)	55 (95)	NS	
Facial erythema	9 (56)	17 (29)	NS	
Heliotrope rash	9 (56)	17 (29)	NS	
Gottron's papules	8 (59)	11 (19)	0.02	4.27 (1.14-16.4)
Raynaud's phenomenon	10 (63)	29 (50)	NS	
Arthritis	5 (31)	27 (47)	NS	
Myositis	15 (94)	51 (88)	NS	
Fingertip ulcers	3 (18.75)	2 (6.7)	NS	
CK elevation	8/15 (53)	48/56 (86)	0.012	0.19 (0.04-0.79)
Dyspnea	12 (75)	48 (83)	NS	
Dysphagia	10 (63)	33 (57)	NS	
Heart involvement	1 (6)	7 (12)	NS	
ILD	8 (50)	20 (34)	NS	
Alveolitis	2 (13)	7 (12)	NS	
Respiratory muscle defect	5 (31)	20 (34)	NS	
DLCO reduction (< 70% predicted)	6/14 (43)	29/55 (53)	NS	
FVC reduction (<70% predicted)	0/14 (0)	10/55 (18)	NS	
Altered capillaroscopy	9/10 (90)	31/38 (82)	NS	
Scleroderma pattern	7/10 (70)	15/38 (39)	NS	
Electromyography alterations	13/13 (100)	43/55 (78)	NS	
Muscle biopsy alteration	6/6 (100)	31/36 (86)	NS	
Skin biopsy alteration	5/5 (100)	12/13 (92)	NS	

Autoantibody analysis

ANA were globally found in 52 patients (70.3%), with 5 cytoplasmic pattern; anti-ENA in 72.9% of cases. Anti-ENA were more frequently detected in C^+ group (94% *vs*. 67%), with a nearly significant *p*-value (0.053), mostly represented by anti-Jo1 (23%), anti-Ro/SSA (23%), anti-NXP-2 (10.8%) and anti-Ku (9.4%). No differences between C^+ and C- groups were reported, except for anti-PM/Scl which were found more frequently in C^+ cases (p=0.0068, OR 19, CI 95%=1.7–490), probably due to a higher number of overlap cases in these patients. Anti-NXP-2 antibodies seem to be more frequent in C^+ cases, although the difference is not statistically significant (Table II).

Anti-NXP-2 + patients with calcinosis

Four patients with calcinosis showed anti-NXP-2 antibodies: all of them showed DM and 3 out of 4 NXP-2 positive patients presented multiple site involvement since the onset of the disease, representing 60% of patients with early diffuse calcinosis (three out

Table II. Autoantibodies distribution in 16 patients with calcinosis (C^+) and 58 patients without calcinosis (C^-).

	C^+ n. 16 (%)	C- n. 58 (%)	*p*-value	OR (95% CI)
ANA	14 (88)	38 (66)	NS	
ENA positivity	15 (94)	39 (67)	0.053	
MSA	7 (43.7)	24 (41.4)	NS	
MAA	9 (56.2)	26 (44.8)	NS	
Jo-1	3 (19)	14 (24)	NS	
Ku	0 (0)	7 (12)	NS	
U1RNP	0 (0)	3 (5)	NS	
PM/Scl	4 (25)	1 (2)	0.0068	19 (1.7-490)
NXP-2	4 (25)	4 (7)	0.061	
Mi2	0 (0)	2 (3)	NS	
Ro/SSA (60 and/or 52 KD)	3 (19)	14 (24)	NS	
Ro/SSA+La/SSB	2 (13)	1 (2)	NS	
MDA-5	2 (13)	3 (5)	NS	
SRP	0 (0)	1 (2)	NS	
SMN	0 (0)	1 (2)	NS	

Table III. Treatment options used in 16 patients with calcinosis.

	n. (%)	Mean duration, months (SD)	Treatment effect
Calcium-channel blockers (CCHB)	2 (13)	36 (17)	no
Diltiazem	7 (43.7)	32.2 (14.5)	no
Bisphosphonates	11 (69)	61 (39.8)	no
Warfarin	2 (13)	77 (77)	no
Intravenous immunoglobulin (IVIg) 2gr/kg every 4 weeks	7 (44)	6 months	Yes in 1 case
Colchicine	9 (56)	35.7 (37.8)	Yes in 1 case
Rituximab	2 (13)	1.5 cycle	Yes in 1 case
Infliximab 5mg/kg every 4 weeks	2 (13)	8.5 (6.3)	no
Sodium thiosulphate ointment	5 (31)	7.6 (6)	Partial in 1 case
Surgery	6 (38)	-	Yes in 3

of five). By comparing 4 anti-NXP-2+ with calcinosis with other 12 patients with calcinosis, we recorded a lower frequency of dyspnea (p=0.027;OR: 0.03; CI 95%=0.0–0.9), and higher values of DLCO (mean 92%±17.4% *vs*. 64%±15.3%; p=0.02).

Overlap syndromes

An OS of PDM and other systemic connective tissue disease was diagnosed in 13 of our patients (17.6%), respectively 4 cases among C^+ and 9 among the C^- groups. Overlap SSc-myositis was the most common OS (7 cases, 53.8%), followed by the overlap of PDM-SLE (4 cases, 30.8%), PDM-SSj (2 cases, 15.4%) and PDM-RA (1 case, 7.7%). By comparing the antibody profile of patients with the OS to the PDM alone we reported a higher frequency of anti-PM/Scl (p=0.016, OR18, CI 95%=1.14-500) and anti-Ku antibodies (p=0.16, OR8.59, CI 95%=1.31–60.8) in patients with the OS. Moreover, antibodies to PM/Scl were more frequent in scleromyositis with calcinosis (p=0.029, OR infinite, CI 95% 0.8-infinite) *versus* those without calcinosis.

Multivariate analysis

MSA define clusters of patients with distinctive clinical features, therefore it is possible that a number of clinical and laboratory features might not be independently associated with calcinosis. For the multivariate analysis we included the features associated to the univariate analysis plus the anti-NXP-2 positivity, because during the first analysis this antibody almost reached the significant threshold. By the multivariate analysis, most of the previous associations persisted: calcinosis resulted independently associated with longer follow-up duration (p=0.006, OR 0.006, CI 95%=1.004–1.022), anti-PM/Scl (p=0.33, OR 20.9, CI 95%=1.28–340) and inversely associated with CK elevation at disease onset (p=0.05, OR 0.97, CI 95%=0.009–1.003). Consequently our hypothesis concerning anti-NXP-2 antibody has been confirmed, and this antibody (p=0.024, OR 21.9, CI 95%=1.5–319) could be considered as an independent predictive risk factor for calcinosis development.

Treatment

All patients received oral steroid upon diagnosis, and in both groups the most common dosage prescribed was at 0.5–1 mg/kg/day without any statistical differences (56% in C^+ and 60% in C^- patients respectively); during follow-up prednisone was mostly given at 5–10 mg/daily. Methotrexate and azathioprine were the immunosuppressant most frequently recommended, followed by cyclosporine-A, mycophenolate mofetil, cyclophosphamide and hydroxychloroquine. By comparing the use of immunosuppressant in patients C^+ and C^-, no differences were noted regarding the number of immunosuppressant used or concerning the doses of steroids except for the higher use of azathioprine among patients with calcinosis (69% *vs* .17%, p<0.001 OR 10.56, CI 95%=2.6–45.8). Different calcinosis-specific treatments were settled: calcium-channel blockers (*i.e*. diltiazem 90 to 240 mg/day or nifedipine 30 mg/day), bisphosphonates (oral alendronic acid in 8 cases, oral risedronic acid in 2, *i.m*. clodronate in 1 case), warfarin (anticoagulant dose), intravenous Ig (2 g/kg/month for 6 months), colchicine, rituximab (500 mg/week for 4 consecutive weeks), infliximab (3 mg/kg/6 weeks), sodium thiosulphate ointment (3% then 10% concentration) (Table III). None of these treatments allowed the reduction of calcinosis or the prevention of new sites involved, however for few cases there was a subjective improvement in associated symptoms (*i.e*. pain reduction).

Surgical removal was performed in 6 cases: in 3 patients a relapse of calcinosis occurred, while 3 cases showed a total remission. Two patients presented infective complication, locally in one case and evolved in sepsis in the other case.

Discussion

In our study the prevalence of calcinosis in DM reached 36.7% while in OS

it stretched to 30.8%. These figures are higher if compared to most recent paper (14) but are in line with other previous papers (25, 26). A possible explanation for these discrepancies could be that our study considered every consecutive patient seen in our outpatient clinic, satisfying the classification criteria for idiopathic myopathies (16) and therefore a number of patients diagnosed in our Institution and presently in remission were not included in the analysed cohort.

In the present study, clinical predictors of development of calcinosis were identified in DM diagnosis, Gottron's papules and a longer follow-up. The duration of follow-up could be considered as a surrogate marker of the disease duration, it can subsist due to the persistence of active clinical problem, as unresolved calcinosis, while the longer follow-up in C^+ patients is consistent with what reported in previous studies (14). However, despite a follow-up of more than 15 years, in 5 out of 16 (30%) of our cases, calcinosis was detected at the diagnosis of DM or before. In the remaining 70% of cases calcinosis appears relatively early during the follow-up, with a mean of 4 years since the onset of PDM. In addition, while at the onset only a few patients presented diffuse calcinosis, after one year more than 60% reported multiple sites involved. The rapid onset after diagnosis and the rapid spread of locations makes it more likely that the onset of calcinosis represents a specific pattern of disease rather than the consequence of a chronically active disease (27). No other clinical manifestations seem to differ between C^+ and C^- cases and our results confirm what has been recently published by Valenzuela (14), except for the distribution of calcinosis that in 37.5% of our patients involved the hands with the absence of fingertip ulcers that are instead prominent in Valenzuela's experience (14). Furthermore, in previous researches an between fingertip ulcers and antibodies to MDA-5 was reported (14, 28), while in our cohort the association was not confirmed, both in our present and past studies (24). This lack of association could exist due to the low number of cases considered.

It is well known that calcinosis is more related to DM than PM, as in JDM, and this epidemiological association has been confirmed in our population. Conversely, CK levels were significantly higher in C^- compared to C^+ at the onset of disease.

Even though not statistically significant, a trend to more ANA and anti-ENA positivity was recorded in C+ group. Laboratory predictors of onset of calcinosis in this study could be found in anti-NXP-2/MJ and PM/Scl. The association with anti-PM/Scl is justified by the inclusion of OS in our study in which this antibody is over-represented ($p<0.0152$) and associated with calcinosis ($p<0.003$) as already reported in the literature (26, 29). On the contrary, anti-Ku associated with OS ($p=0.0178$) seems to protect from the appearance of calcinosis, even though this result is not statistically significant. Concerning MSA, anti-NXP-2/MJ antibodies were significantly associated to C^+ by multivariate analysis and therefore they represent the main serological marker of calcinosis in DM, in adults as well as in JDM. Furthermore, since the beginning of the study, anti-$NXP2^+$ C^+ subgroup showed a particular phenotype characterised by lower prevalence of respiratory muscle involvement, and diffuse distribution of calcinosis, representing a marker of more severe calcinosis in adults (30), as well as in JDM (13). Anti-NXP-2 antibody has been recently reported to associate with cancer in IIM patients (31, 32). However, this is a controversial topic since in none of our previous experiences, as well in that of others (33), neither in previously 10 anti-NXP-2 positive patients (15), nor in the present study we have detected cancer-associated myositis despite the long follow-up (mean 117.6 months).

In the past, calcinosis was attributed to a persistently active disease (27) measured by a number of prescribed immunosuppressant drugs. No difference between the number of immunosuppressants and steroid dose used in each group of patients was observed in our series. Nevertheless, azathioprine was more frequently used in C^+ cases, probably in order to obtain a better control of calcinosis deposition because of the ineffectiveness of the previously prescribed drugs. We also prescribed many drugs commonly and specifically devoted to contrast the onset and/or the diffusion of calcinosis (34). Unfortunately, no single drug or association of drugs was found effective in the treatment of calcinosis. Even the use of infliximab, a biologic agent blocking the TNF-α, that is considered a possible inducer of calcium deposition, was ineffective in our experience as well as in the others (35), while it has been reported to be effective in JDM (36). Surgical removal seems to be effective in some patients: unfortunately 3 out of 6 patients (50%) who underwent excision of calcinosis developed serious infective complications.

Our study has some limitations. The most important one regards the small number of patients enrolled in the study. Nevertheless, a recent paper (14) with a similar number of patients has been published showing similar results especially regarding the length of follow-up and the relevance of anti-NXP-2/MJ antibody as marker of calcinosis. Another issue can be identified in the empirical evaluation of calcinosis in response to treatment which is based only on subjective clinical reporting. However, as far as we know, there are currently no validated outcome measures to assess calcinosis in myositis.

In conclusion, in this study, predictors of development of calcinosis could be identified in a longer follow-up, DM diagnosis and positivity for PM/Scl and NXP-2. Moreover, the positivity for antibodies to NXP-2 depicts a distinct phenotype of calcinosis with an early onset and quick widespread dissemination and lower prevalence of muscle respiratory tract involvement. Persistence of calcinosis resistant to any pharmacological treatment can justify further research on calcinosis in PDM patients comparing C^+ *versus* C- with myositis.

References

1. LOWRY CA, PILKINGTON CA: Juvenile dermatomyositis: extramuscular manifestations and their management. *Curr Opin Rheumatol* 2009; 21: 575-80.
2. GUTIERREZ A JR, WETTER DA: Calcinosis cutis in autoimmune connective tissue dis-

eases. *Dermatol Ther* 2012; 25: 195-206.
3. BOULMAN N, SLOBODIN G, ROZENBAUM M, ROSNER I: Calcinosis in rheumatic diseases. *Sem Arthritis Rheum* 2005; 34: 805-12.
4. SHIMIZU M, UENO K, ISHIKAWA S, KASAHARA Y, YACHIE A: Role of activated macrophage and inflammatory cytokines in the development of calcinosis in juvenile dermatomyositis. *Rheumatology* (Oxford) 2014; 53: 766-7.
5. MAMYROVA G, O'HANLON TP, SILLERS L *et al*.: Cytokine gene polymorphisms as risk and severity factors for juvenile dermatomyositis. *Arthritis Rheum* 2008; 58: 3941-50.
6. AVOUAC J, GUERINI H, WIPFF J *et al*.: Radiological hand involvement in systemic sclerosis. *Ann Rheum* 2006; 65: 1088-92.
7. FARZANEH-FAR A, PROUDFOOT D, WEISSBERG PL, SHANAHA CM: Matrix gla protein is regulated by a mechanism functionally related to the calcium-sensing receptor. *Biochem Biophys Res Commun* 2000; 277: 736-40.
8. VAN SUMMERER MJH, SPLIET WG, VAN ROYEN-KERKOF A *et al*.: Calcinosis in juvenile dermatomyositis: a possible role for the vitamin K-dependent protein matrix gla protein. *Rheumatology* 2008; 47: 267-71.
9. WEINEL S, CALLEN JP: Calcinosis cutis complicating adult-onset dermatomyositis. *Arch Dermatol* 2004; 140: 365-6.
10. BOWER SL, BLANE CE, SULLIVA DB, CASSIDY JT: Childhood dermatomyositis: factors predicting functional outcome and development of dystrophic calcification. *J Pediatrics* 1983; 103: 882-8.
11. PACHMANLM, HAYFORD JR, CHUNG A *et al*.: Juvenile dermatomyositis at diagnosis: clinical characteristics of 79 children. *J Rheum* 1998, 25: 1198-204.
12. SALLUM AM, PIVATO FC, DOIA-FIHLO U *et al*.: Risk factors associated with calcinosis of juvenile dermatomyositis. *J Pediatr* (Rio J) 2008; 84: 68-74.
13. GUNAWARDENA H, WEDDERBURN LR, CHINOY H *et al*.: Autoantibodies to a 140-kd protein in juvenile dermatomyositis are associated with calcinosis. *Arthritis Rheum* 2009; 60: 1807-14.
14. VALENZUELA A, CHUNG L, CASCIOLA-ROSEN L, FIORENTINO DF: Identification of clinical features and autoantibodies associated with calcinosis in dermatomyositis. *JAMA Dermatol* 2014; 150: 724-9.
15. CERIBELLI A, FREDI M, TARABORELLI M *et al*.: Anti-MJ/NXP-2 autoantibody specificity in a cohort of adult Italian patients with polymyositis/dermatomyositis. *Arthritis Res Ther* 2012; 14: R97.
16. BOHAN A, PETER JB: Polymyositis and dermatomyositis. *New Engl J Med* 1975; 13: 344-6.
17. GRIGGS RC, ASKANAS V, DIMAURO S *et al*.: Inclusion body myositis and myopathies. *Ann Neurol* 1995; 38: 705-13.
18. PETRI M, ORBAI AM, ALARCÓN GS *et al*.: Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. *Arthritis Rheum* 2012; 64: 2677-86.
19. VAN DEN HOOGEN F, KHANNA D, FRANSEN J *et al*.: 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League against Rheumatism collaborative initiative. *Arthritis Rheum* 2013; 65: 2737-47.
20. ALETAHA D, NEOGI T, SILMAN AJ *et al*.: 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum* 2010; 62: 2569-81.
21. VITALI C, BOMBARDIERI S, JONSSON R *et al*.: Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Ann Rheum Dis* 2002. 61: 554-8.
22. GALIMBERTI F, LI Y, FERNANDEZ AP: Intravenous immunoglobulin for treatment of dermatomyositis-associated dystrophic calcinosis. *J Am Acad Dermatol* 2015; 73: 174-6.
23. BERNSTEIN RM, BUNN CC, HUGHES GRV: Identification of autoantibodies to acidic antigens by couterimmunoelectrophoresis. *Ann Rheum Dis* 1982; 41: 554-5.
24. CERIBELLI A, FREDI M, TARABORELLI M *et al*.: Prevalence and clinical significance of anti-MDA-5 antibodies in European patients with polymyositis/dermatomyositis. *Clin Exp Rheumatol* 2014; 32: 891-7.
25. BALIN SJ, WETTER DA, ANDERSEN LK, DAVIS MD: Calcinosis cutis occurring in association with autoimmune connective tissue disease: the Mayo Clinic experience with 78 patients, 1996-2009. *Arch Dermatol* 2012; 148: 455-62.
26. MARGUERIE C, BUNN CC, COPIER J *et al*.: The clinical and immunogenetic features of patients with autoantibodies to the nucleolar antigen PM-Scl. *Medicine* (Baltimore) 1992; 71: 327-36.
27. MARVI U, CHUNG L, FIORENTINO D: Clinical presentation and evaluation of dermatomyositis. *Indian J Dermatol* 2012; 57: 375-81.
28. FIORENTINO D, CHUNG L, ZWERNER J, ROSEN A, CASCIOLA-ROSEN L: The mucocutaneous and systemic phenotype of dermatomyositis patients with antibodies to MDA5 (CADM-140): a retrospective study. *J Am Acad Dermatol* 2011; 65: 25-34.
29. D'AOUST J, HUDSON M, TATIBOUET S *et al*.: Clinical and serological correlates of anti-PM/Scl antibodies in systemic sclerosis: a multicenter study of 763 patients. *Arthritis Rheum* 2014; 66: 1608-15.
30. SUGIURA K, MURO Y, AKIYAMA M: Autoantibodies to nuclear matrix protein2/MJ in adult-onset dermatomyositis with severe calcinosis. *J Am Acad Dermatol* 2012; 67: e167-8.
31. ICHIMURA Y, MATSUSHITA T, HAMAGUCHI Y *et al*.: Anti-NXP2 autoantibodies in adult patients with idiopathic inflammatory myopathies: possible association with malignancy. *Ann Rheum Dis* 2012; 71: 710-3.
32. FIORENTINO DF, CHUNG LS, CHRISTOPHERSTINE L *et al*.: Most patients with cancer-associated dermatomyositis have antibodies to nuclear matrix protein NXP-2 or transcription intermediary factor 1γ. *Arthritis Rheum* 2013; 65: 2954-62.
33. TROYANOV Y, TARGOFF IN, PAYETTE M-P *et al*.: Redefining Dermatomyositis: a description of new diagnostic criteria that differentiate pure dermatomyositis from overlap myositis with dermatomyositis features. *Medicine* 2014; 93: 318-32.
34. GUTIERREZ A JR, WETTER DA: Calcinosis cutis in autoimmune connective tissue diseases. *Dermatologic therapy* 2012; 25: 195-206.
35. SIDDIQUI NS, ALTOROK N: Diffuse soft-tissue calcinosis. *N Eng J Med* 2015; 373: 173.
36. RILEY P, MCCANN LJ, MAILLARD SM, WOO P, MURRAY KJ, PILKINGTON CA: Effectiveness of infliximab in the treatment of refractory juvenile dermatomyositis with calcinosis. *Rheumatology* (Oxford) 2008; 47: 877-80.

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B Cell Tolerance to Deiminated Histones in BALB/c, C57BL/6, and Autoimmune-Prone Mouse Strains

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Deimination, a posttranslational modification of arginine to citrulline carried out by peptidylarginine deiminases, may compromise tolerance of self-antigens. Patients with connective tissue autoimmunity, particularly rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), or Felty's syndrome, present with autoantibodies to deiminated histones (dH), which thus form a category of antibodies to citrullinated protein antigens (ACPA). In general, ACPA are a sensitive diagnostic for RA and may form in response to the release of nuclear chromatin (DNA plus dH) from granulocytes, usually referred to as neutrophil extracellular traps. The aim of this study was to examine spontaneously autoimmune mice for autoantibodies and T cell responses to dH. We compared IgG binding to deiminated and non-deiminated histones (nH) by ELISA and Western blotting in spontaneously autoimmune strains of (NZB × NZW) F_1 and NZM2410 together with their derivative congenic strains, C57BL/6.*Sle1* and C57BL/6.*Sle1.Sle3*, which display profound autoreactivity against nuclear self-antigens. The splenocyte proliferation against the two antigens was determined in the spontaneously autoimmune (NZB × NZW) F_1 strain from which other autoimmune strains used in the study were derived. Immunizations with dH and nH were attempted in BALB/c mice to assess their splenocyte response. Splenocytes from BALB/c mice and from autoimmune mice at the time of conversion to autoimmunity proliferated strongly in response to dH, yet serum IgG from autoimmune (NZB × NZW) F_1, NZM2410, and C57BL/6.*Sle1.Sle3* mice displayed a remarkable bias against binding to dH. At the time of seroconversion, the antibodies already exhibited preference for nH, and only nH were recovered from circulating immune complexes. Analysis of histone deimination showed constitutive deimination in thymic extracts from C57BL/6 and C57BL/6.*Sle1.Sle2.Sle3* triply congenic mice and in spleens of autoimmune triply congenic mice. Our study demonstrates that tolerance mechanisms against dH are intact in BALB/c and C57BL/6 mice and continue to be effective in mice with overt autoimmunity to nH. We conclude that, in contrast to human RA and SLE patients, where we frequently observe autoantibodies against dH, autoimmune mice maintain strong tolerance mechanisms to prevent the development of autoantibodies to dH.

Keywords: autoimmunity, antibodies to citrullinated protein antigens, citrullines, B cells, lupus erythematosus, rheumatoid arthritis, autophagy, tolerance

INTRODUCTION

Antibodies to citrullinated protein antigens (ACPA) are diagnostic markers for rheumatoid arthritis (RA) (1) and also arise in other human autoimmune disorders such as systemic lupus erythematosus (SLE) and Felty's syndrome (2, 3). Citrullines are introduced into proteins by peptidylarginine deiminase (PAD) family of enzymes (4), and much effort has been devoted to learning the circumstances that activate PADs and lead to the PAD-mediated conversion of arginine residues into citrulline residues (5, 6). Several citrullinated antigens have been identified in RA, and a common mechanism has been proposed to account for the generation of citrullinated autoantigens (7–9). The proposed mechanism places particular importance on PAD2 and PAD4, enzymes that are expressed in cells of the innate and adaptive immune system (10, 11). These calcium-dependent enzymes are activated under inflammatory conditions (5). Direct stimuli of PADs include microbial pathogens and pro-inflammatory chemokines and cytokines (5). The enzymes are also activated by sterile inflammatory stimuli, such as cholesterol and urate crystals (8). In fact, it has been argued that any perforation to the plasma membrane could lead to the activation of PADs (12).

One particularly relevant event that is linked to PAD activation and may contribute to the induction of ACPA is a form of granulocyte cell death, which is induced by microbes and inflammatory stimuli and results in the release of nuclear chromatin (5, 13). Such neutrophil extracellular traps (NETs) are considered an innate antimicrobial response because the externalized chromatin is associated with neutrophil granule components such as myeloperoxidase and elastase, which, together with histones themselves, assist in bacterial killing and microbial entrapment (14). In the process of NET release, termed NETosis, PADs gain access to multiple intracellular and extracellular substrates such as histones, filaggrin, fibrinogen, and collagen, which are frequently targeted by ACPA (15). So, it is a prevalent hypothesis that NETosis provides conditions that lead to the production of deiminated (citrullinated) autoantigens that may stimulate cells of the adaptive immune system in the context of an inflammatory response. Moreover, the structural components of NETs, DNA, and histones, also become externalized during NETosis and, in an infection, may become entangled with bacteria and activate the immune system. Interestingly, dendritic cells respond to NETs with the production of interferons and other pro-inflammatory cytokines (16, 17). Other forms of cell death may also have consequences for the induction of autoantibodies, as autoantibodies bind to acetylated histones, a modification of apoptotic chromatin that may elicit autoantibody responses in mice and humans (18, 19).

To provide a mouse model for the study of ACPA, we sought to identify spontaneous mouse models of systemic autoimmunity that would produce autoantibodies to citrullinated histones. Although PAD4 expression parallels the severity of the inflammatory process in mouse models of RA (20), PAD4's contribution to the production of ACPA has been more difficult to ascertain (21, 22). Because ACPA are difficult to induce in most strains of mice (23), questions have been raised whether mouse ACPA participate in RA pathogenesis at all (22). Human ACPA often react with deiminated histones (dH), and antibody binding to citrullinated histone peptides is a sensitive diagnostic test for RA (2, 24). Because histones are the major substrates of PADs in neutrophils, and dH are built into NETs, we expected that spontaneous anti-histone autoantibodies would preferentially bind to PAD-modified histones. However, we observed that mouse autoantibodies from (NZB × NZW) F_1 (NZB/W) mice and their recombinant derivative strains, including NZM2410 and C57BL/6J.*Sle1* (B6.*Sle1*) or C57BL/6J.*Sle1*.*Sle3* (B6.*Sle1*.*Sle3*) congenics, showed strong preference for non-deiminated histones (nH) over dH by ELISA and Western blot. Thus, even after tolerance to histones was broken and autoantibodies to nH were expressed, autoimmune-prone congenic strains retained B cell tolerance toward dH. B cell binding to dH was repressed, whereas autoantibody binding focused instead on PAD4 substrate arginines. The B cell bias against dH argues that dH remain effective tolerogens in autoimmune mice. In support of this possibility, we observed elevated levels of dH in thymus extracts from B6 and B6.*Sle1*.*Sle2*.*Sle3* (B6.TC mice) and spleens of autoimmune B6.TC mice. Our observations suggest that, even in overtly autoimmune lupus mice, central (thymic) tolerance inhibits B cells that react with a deiminated variant of an important nuclear autoantigen. These results point to unexpected intricacies in the murine immune response to deiminated autoantigens. We interpret these results as possible outcomes of PAD expression in antigen-presenting cells.

MATERIALS AND METHODS

Mice

Sera were obtained from B6 mice, as well as from NZB/W, NZM2410, B6.*Sle1*, and B6.*Sle1*.*Sle3* mice at 6–8 months of age. Tissues were prepared from groups of matched B6 and B6.TC mice of 4–6 months of age. Splenocytes were isolated from 6 BALB/c mice of 4 months of age and 13 NZB/W F1 female mice that were divided into 3 age groups: 6–10 weeks of age, 20–21 weeks of age, and 25–30 weeks of age. The treatment and care of animals were in accordance with the guidelines of the Office of Research, UTHSC, the University of Florida and the Norwegian Ethical and Welfare Board, and the study overall was approved by UTHSC Institutional Animal Care and Use Committee under the protocol #11-164.

ELISA

For binding assays, we treated purified calf-thymus histones with recombinant PAD4 *in vitro*, as described previously (2, 3). To assess the extent of deimination, we analyzed the progress of the reaction by colorimetry of citrullines and testing the resulting dH by Western blot with an antibody to citrullinated histone H3 (Abcam, ab#5103). The results of this analysis are shown in **Figure 1**.

Flat bottom, 96 well microtiter plates (Immulon 4HBX; Thermo Electron Corp.) were coated overnight with 5 μg/ml of nH, poly L-lusine, bovine serum albumin (BSA) (Sigma), ovalbumin (OVA) (Sigma), protamine sulfate (Sigma), or dH, as previously described (25). Plates were washed three times with

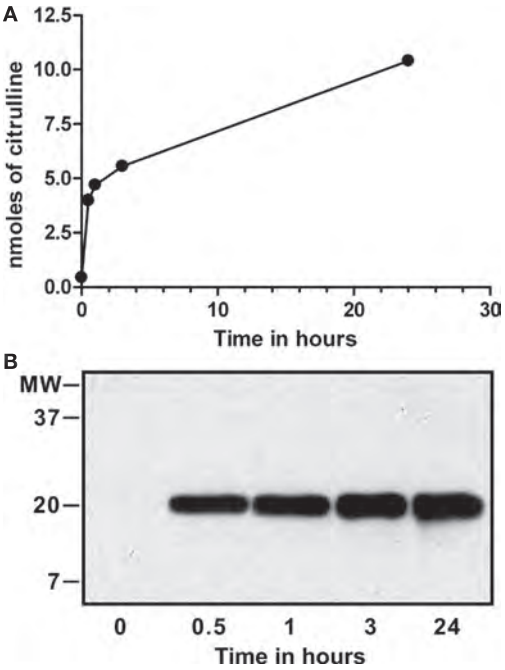

FIGURE 1 | ***In vitro*** **deimination of histones by peptidylarginine deiminase (PAD) 4**. Calf-thymus histones (0.1 mM) were incubated with 0.2 μM of recombinant PAD4 for up to 24 h, and nanomoles of citrulline produced were determined by colorimetry at 595 nm using citrulline standard solution **(A)**. Calf-thymus histones from time points tested above were probed on Western blot with a commercial antibody that reacts against the amino terminus of histone H3 with citrullines at positions 2, 8, and 17 (Abcam, ab#5103). Deimination was detected at each time point except at time = 0 and increased from 0.5 to 24 h **(B)**. The reaction reached a plateau by 24 h, and we calculated that 1.3 citrullines were present, on average, per histone H3 molecule.

0.05% Tween-20 in PBS and blocked with 2.5% BSA in 0.02% NaN_3 and PBS for 2 h. A 1:100 initial dilution of primary sera along with threefold serial dilutions in 1.6% Tween-20 and 1% BSA in PBS were incubated for 1 h in the plates. Then, serum dilutions were removed, and wells were washed with 0.1% Tween-20 in PBS. Alkaline phosphate-conjugated goat anti-mouse kappa (Southern Biotech) was added at 1:1,000 dilution in 1% BSA with 0.05% Tween-20 in PBS for 1 h. Phosphatase substrate (Sigma) was used to develop the ELISA, and OD values were read at 405 nm on a Multiscan Plus plate reader (Labsystems).

Serum antibodies against dsDNA were detected by ELISA exactly as described (26, 27). In short, calf-thymus dsDNA (10 μg/ml in PBS) was coated on microtiter plates (MaxiSorb; Nunc, Copenhagen, Denmark). Sera from mice were diluted twofold from 1:100 to 1:3,200 in PBS containing 0.02% Tween-20 and incubated in wells. ELISA readings were obtained with peroxidase-conjugated rabbit anti-mouse Fc-γ antibodies at 405 nm.

Ex Vivo Tissue Lysate Preparation

Seven-month-old B6.TC autoimmune female mice and age-matched control B6 IgH^a were dissected to recover a portion of spleen, bone marrow, kidney, and liver. Thymi from 4- to 6-month-old mice were similarly obtained. Tissue was cut, minced with scissors, and crushed between two sterile frosted glass slides. Dissociated tissues were washed in PBS (without Ca^{++}) and centrifuged at 5,000 × *g* for 5 min to pellet cells. Cell pellets were mixed with lysis buffer (65mM Tris pH 7.2, 2%SDS, 10% glycerol), containing protease inhibitors. To test for dH in tissue lysates, equal amounts of total protein were analyzed by Western blotting, as described below.

Western Blot

For Western blot analysis, proteins were resolved on 15% SDS-PAGE and transferred to nitrocellulose. Membranes were blocked in 5% BSA in 0.1% Tween-20 in TBS (TBST) overnight at 4°C. Subsequently, the membranes were incubated with sera at 1:500 dilution in TBS containing 2.5% BSA, 1% NP-40, and 0.1% SDS. After 2 h of incubation, membranes were washed with 1% NP-40 in TBS. Anti-mouse IgG–HRP was used for detection at 1:20,000 dilution in TBST for 1 h, and blots were developed using chemiluminescence (PerkinElmer).

Peptide inhibition assays included a preceding step, in which 3 μg of 20-mer peptides, both matching the amino terminus of H3 but either containing arginines or citrullines at positions 2, 8, and 17, were incubated with 1:300 dilutions of mouse sera for 1 h prior to use in binding to dH and nH on the membrane. Results of these Western blots were quantitated by infrared emission of secondary anti-mouse IgG antibodies (Odyssey). Separately, autoimmune sera were treated with DNase1 prior to Western blotting. Briefly, 400 μl of a 1:200 dilution of sera were incubated with 20 units of DNase1 (New England Biolabs) for 1 h at room temperature to limit the possibility that DNA–anti-DNA complexes present in sera contribute to the observed histone binding. Following this incubation, the sera were diluted 1:500 in Western blot binding buffer.

To probe for deiminated histone H3 (dH3) in B6.TC and B6 mice, equal amounts of tissue lysates were resolved on 12% SDS-PAGE and transferred to nitrocellulose. Membranes were blocked with 5% BSA in TBST for 30 min and incubated with anti-dH3 anti-citrullinated histone H3 rabbit antibodies (Abcam, ab#5103) overnight at 4°C. Membranes were washed and incubated with HRP-conjugated goat-anti-rabbit secondary IgG antibody for 1 h at room temperature, washed three times in TBST, and twice in TBS alone. The HRP activity was detected as above.

Splenocyte Proliferation Assay

BALB/c mice were boosted twice with 100 μg of total histones (dH or nH) or 100 μg OVA, 14 and 2 days prior to the splenocyte proliferation assay. Ninety-six well tissue culture plates (Corning Incorporated) were filled with 100 μl aliquots of 100 μg/ml (or threefold serial dilutions) of dH, nH, or OVA in RPMI 1640 (Mediatech Inc.) supplemented with 10% FBS. Splenocytes were isolated and resuspended in RPMI with 10% FBS at 1×10^6 cells/ml. One hundred microliters of cell suspension was added to each well, and plates were incubated at 37°C in 5% CO_2 for 72 to 96 h. Tritiated thymidine (1 μCi/well) was added for the last 17 h of incubation. Plates were harvested onto glass fiber filters, and thymidine incorporation was assessed by scintillation counting. Splenocyte proliferation assays were also performed using

female NZB/W mice purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Splenocytes were collected from NZB/W mice of different ages. Following red blood cell lysis, the splenocytes were resuspended in DMEM-10 media with 10% FCS and 10,000 U/ml penicillin and 10 mg/ml streptomycin. One hundred thousand cells were incubated with dH or nH (20 μg/ml of protein) in triplicate wells. Tritiated thymidine incorporation (1 μCi/well) was measured after 20 h, 3 or 6 days by liquid scintillation, as described (28, 29).

RESULTS

Spontaneously Arising Anti-Histone Autoantibodies

To assess the production of anti-nH/dH autoantibodies in mice that spontaneously develop antinuclear autoantibodies, we tested sera from NZB/W and their recombinant inbred derivative strain NZM2410 for binding to nH and dH. In addition, we tested the contribution of lupus-predisposing genetic intervals *Sle1* and *Sle3* that were back-crossed from the NZM2410 onto the B6 background (B6.*Sle1* and B6.*Sle1*.*Sle3*). *Sle1* is a locus that breaks tolerance to chromatin, whereas *Sle3* affects functions of myeloid cells (30). The parental strains, NZB and NZW, have distinct MHC, H-2^d and H-2^z, respectively. The lupus-predisposing H-2^z was maintained in the NZM2410 congenics, whereas the *Sle1* and *Sle3* congenics have the H-2^b from B6. Antibody binding to dH and nH was assessed by ELISA (**Figure 2**) and Western blot (**Figure 3**). As controls, sera from age- and sex-matched B6 mice were used.

By ELISA, NZB/W (**Figure 2A**), B6.*Sle1* (**Figure 2B**), B6.*Sle1*.*Sle3* (**Figure 2C**), and NZM2410 (**Figure 2D**) sera showed preference for nH over dH. This preferential binding was statistically significant for NZB/W, NZM2410, and B6.*Sle1*.*Sle3*, as assessed by paired, one-tailed *T*-test. Binding differed for different mice and dilutions but, in general, binding to nH was stronger than the binding to dH. We also confirmed the additive effect of *Sle1* and *Sle3* loci, as the B6.*Sle1*.*Sle3* combination resulted in greater absorbance values relative to the B6.*Sle1* mice. In parallel assays, sera from B6 mice (**Figure 2E**) showed no reactivity to histones. Both dH and nH were present in equivalent concentrations on the plates, as shown by the nearly identical binding of the LG2.2 monoclonal antibody (**Figure 2F**) whose epitope, the first 13 amino acid residues of histone H2B, is identical between nH and dH (31).

To examine the possibility that antibodies to dH arise first but are replaced by antibodies to nH, we collected mouse sera over time to identify mice during the conversion to anti-histone autoimmunity. In **Figure 2G**, we show that binding preference to nH over dH was present at an early time when anti-histone reactivity first appeared. This result indicates the two reactivities arise jointly, rather than in succession, as might be predicted by epitope spreading.

To dissect the preferential binding to nH, we used Western blotting. The stringency of binding was increased by including both SDS (0.05%) and NP-40 (0.5%) in the binding buffer. Indeed, under these conditions, the binding of serum antibodies from NZB/W, NZM2410 B6.*Sle1*, and B6.*Sle1*.*Sle3* mice to dH was weaker relative to the binding to nH, such that many of the sera bound exclusively to nH (**Figure 3A**). A variety of binding patterns were observed, including exclusive binding to one or two core histones. Binding to dH3 was observed most often, whereas binding to deiminated H4 or H2A was rare. In addition, binding to a band with the mobility of the deiminated linker histone H1 was observed in several blots. Overall, binding was more biased toward nH over dH, although some IgG dH was also observed in individual NZM2410 and B6.*Sle1*.*Sle3* mice. In no instance did binding to nH exceed binding to dH.

To examine the possibility that the binding to dH represented a truly separate population of antibodies, we conducted inhibition experiments using 20-mer peptides that matched the amino terminus of histone H3 and either contained arginines or citrullines at positions 2, 8, and 17 (**Figure 3B**). We observed that the arginine-containing peptide (orange bars) was a more effective inhibitor of binding to both nH and dH, relative to the citrulline-containing peptide (blue bars), suggesting that the antibody binding to either antigen reflects antibody specificity for nH and that the binding to dH likely represents cross-reactivity due to shared epitope structure.

Splenocyte Proliferation

We asked whether T cells from autoimmune mice also recognize dH by using NZB/W mice that spontaneously develop an autoimmune response against nuclear Ags, including DNA and histones (32). Splenocytes from NZB/W mice of different ages were tested for proliferation in the presence of dH or nH. At 6–10 weeks of age, prior to any measurable anti-DNA reactivity, the splenocytes did not proliferate in response to either form of histone (**Figures 4A,B**). At 20–21 weeks of age, anti-DNA autoantibodies could be detected in the sera of some NZB/W mice (indicated at the top of each panel), and splenocytes from these mice showed low levels of proliferation in response to nH and dH (**Figures 4C,D**). Thus, splenocyte responses to histones arose in parallel with, or slightly prior to, humoral responses to DNA.

Twenty-five-week-old NZB/W mice with established autoimmunity showed splenocyte proliferation in response to dH and nH (**Figures 4E,F**), suggesting the presence of histone-reactive T cells in the spleens of autoimmune mice. Although some mice showed a tendency to preferentially respond to dH, others preferred nH, as shown by data from two of the analyzed mice. Notably, preference could switch, depending on the length of stimulation (**Figures 4E,F**), suggesting the presence of a limited number of T cell clones with distinct specificities and growth characteristics. Because proliferation generally showed a bias for dH or nH rather than being equal, we infer that epitopes containing arginines or citrullines were both presented by the MHC and recognized by T cells in splenocytes.

To examine the ability of dH to drive a T cell response, we examined T cell proliferation *in vitro*. Splenocytes from BALB/c mice immunized with dH proliferated during incubation with dH (**Figure 4G**) to comparable extent as splenocytes from mice

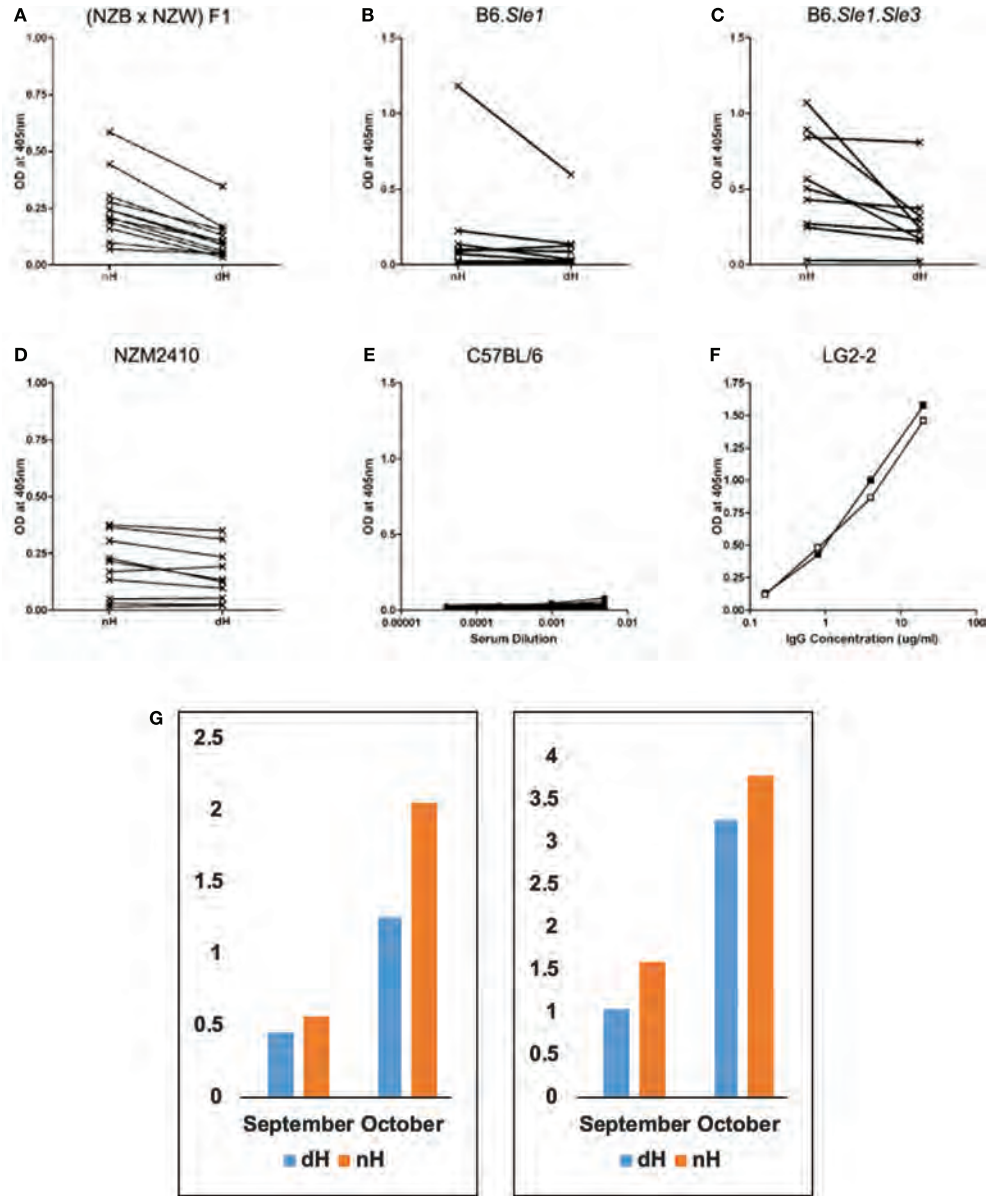

FIGURE 2 | **Binding of IgG from autoimmune-prone and control mice to non-deiminated histones (nH) and deiminated histones (dH)**. Sera from NZB/W **(A)**, B6.*Sle1* **(B)**, B6.*Sle1*.*Sle3* **(C)**, NZM2410 **(D)**, and control B6 **(E)** mice were tested for IgG binding to nH and dH. Complete binding curves were obtained, and OD values for a single dilution were plotted in panels **(A–D)**. We plotted data from 1:1,000 dilutions in panels **(A–C)** and from the 1:200 dilution in panel **(D)**. Absorbance values for each serum corresponding to IgG binding to dH and nH are shown and are connected by a line indicating the pairs of data for the binding of each serum to the two antigens. Significance of the readings was tested by paired, one-tailed *T*-test. Binding to dH was significantly less than to nH for NZB/W ($p < 0.0001$), NZM2410 ($p < 0.016$), and B6.*Sle1*.*Sle3* ($p < 0.016$). The binding of B6.*Sle1* IgG tended to be lower to dH ($p < 0.10$). IgG from mice with systemic lupus erythematosus susceptibility genes showed preferential binding to nH. In comparison, control B6 mice showed negligible binding to either form of histones **(E)**. As control for equal coating of Ags, we used LG2-2, a mouse anti-histone H2B mAb **(F)**, whose epitope does not include any residues that are substrates for deimination (31). Thus, the binding curves for dH (filled symbols) and nH (open symbols) are nearly superimposable. Individual mice were followed over time **(G)**, to observe the initial development of anti-histone autoreactivity. Two NZB/W mice that first showed anti-histone reactivity at 4 months of age (September) reacted more strongly to nH than to dH, and the preferential antibody binding was maintained at 5 months of age (October). The sera were diluted 1:300, and the measurements were performed three times with consistent results. The Y-axis displays values of optical density measured by ELISA.

immunized with OVA and incubated with OVA (**Figure 4I**). By contrast, splenocytes from BALB/c mice immunized with nH showed no enhanced proliferation regardless of whether they were incubated with dH, nH, or media alone (**Figure 4H**).

Spleens of Autoimmune Mice Have Increased Levels of dH

To test for the presence of dH *in vivo*, we prepared tissue lysates of bone marrow, spleen, liver, kidney, and thymus from B6.TC

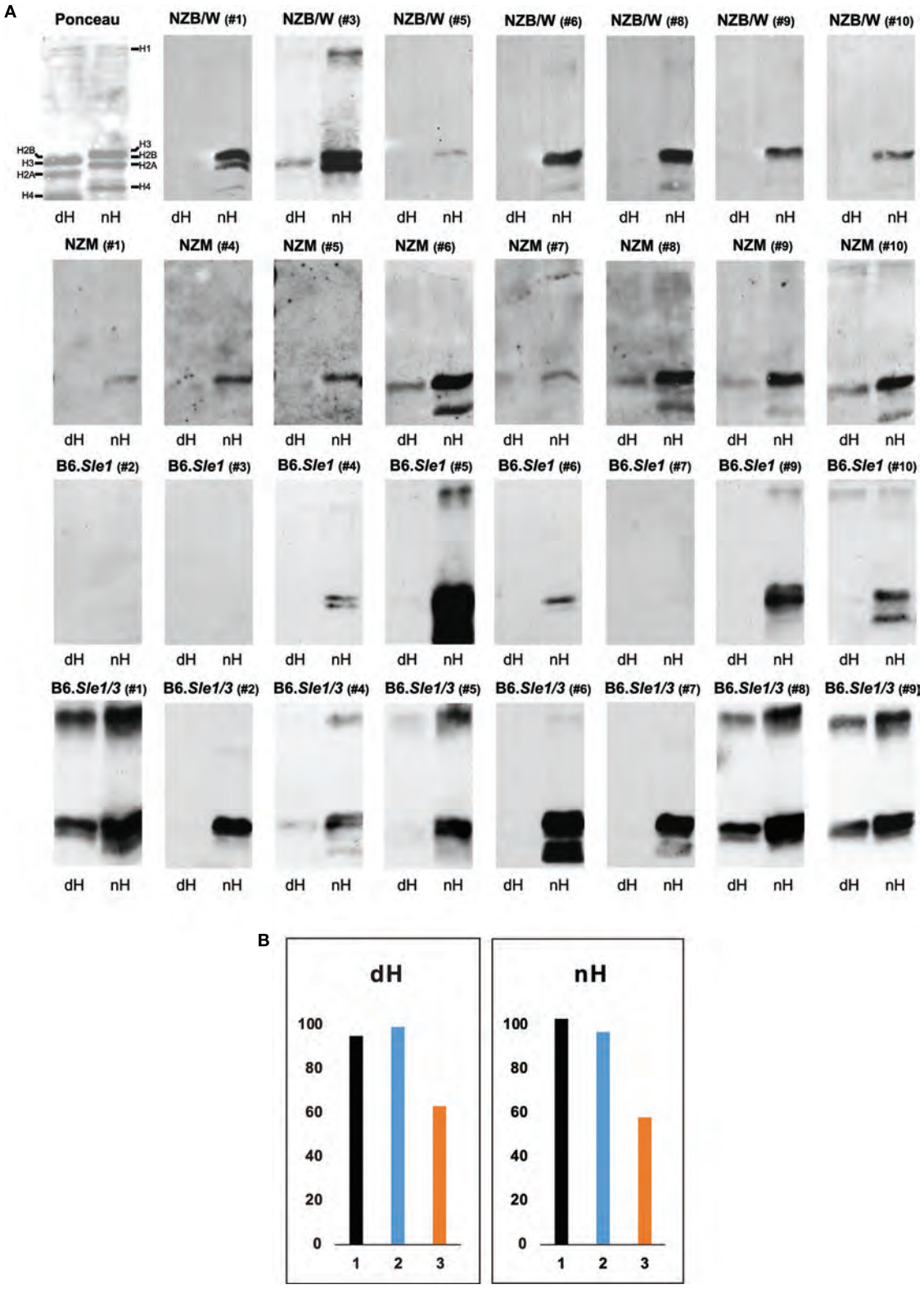

FIGURE 3 | **Continued**

FIGURE 3 | **Continued**

Western blot of IgG to deiminated histones (dH) and non-deiminated histones (nH). Equal amounts of dH and nH were resolved on SDS-PAGE, transferred to nitrocellulose, and stained with Ponceau red (upper left panel). The stained bands migrating with the mobilities of core and linker histones are indicated along the margins. Note that due to the replacement of the positively charged arginine by the neutral citrulline, the electrophoretic mobility of certain core histones is increased in the dH sample, indicating nearly complete modification by peptidylarginine deiminase 4. **(A)** Strips of membrane containing nH or dH were probed with NZB/W, NZM2410, B6.*Sle1*, and B6.*Sle1*.*Sle3* sera at 1:500 dilution and developed with anti-mouse IgG-horseradish peroxidase. Autoimmune-prone mouse sera bound nH in preference to dH. The experiments were performed at least three times with consistent results. **(B)** To explore whether the binding to histones on the membranes is equally sensitive to inhibition by 20-mer peptides matching the H3 amino terminus and containing arginine residues (nH peptide; orange bars) versus citrulline residues (dH peptide; blue bars) at positions 2, 8, and 17, we preincubated an NZB/W serum that showed binding to both nH and dH (black bars) with either peptide, as described in Section "Materials and Methods," and carried out the Western blots. The nH peptide was a more effective inhibitor of binding to both histones than the dH peptide. The *Y*-axis indicates binding intensities in units of infrared emission (IE).

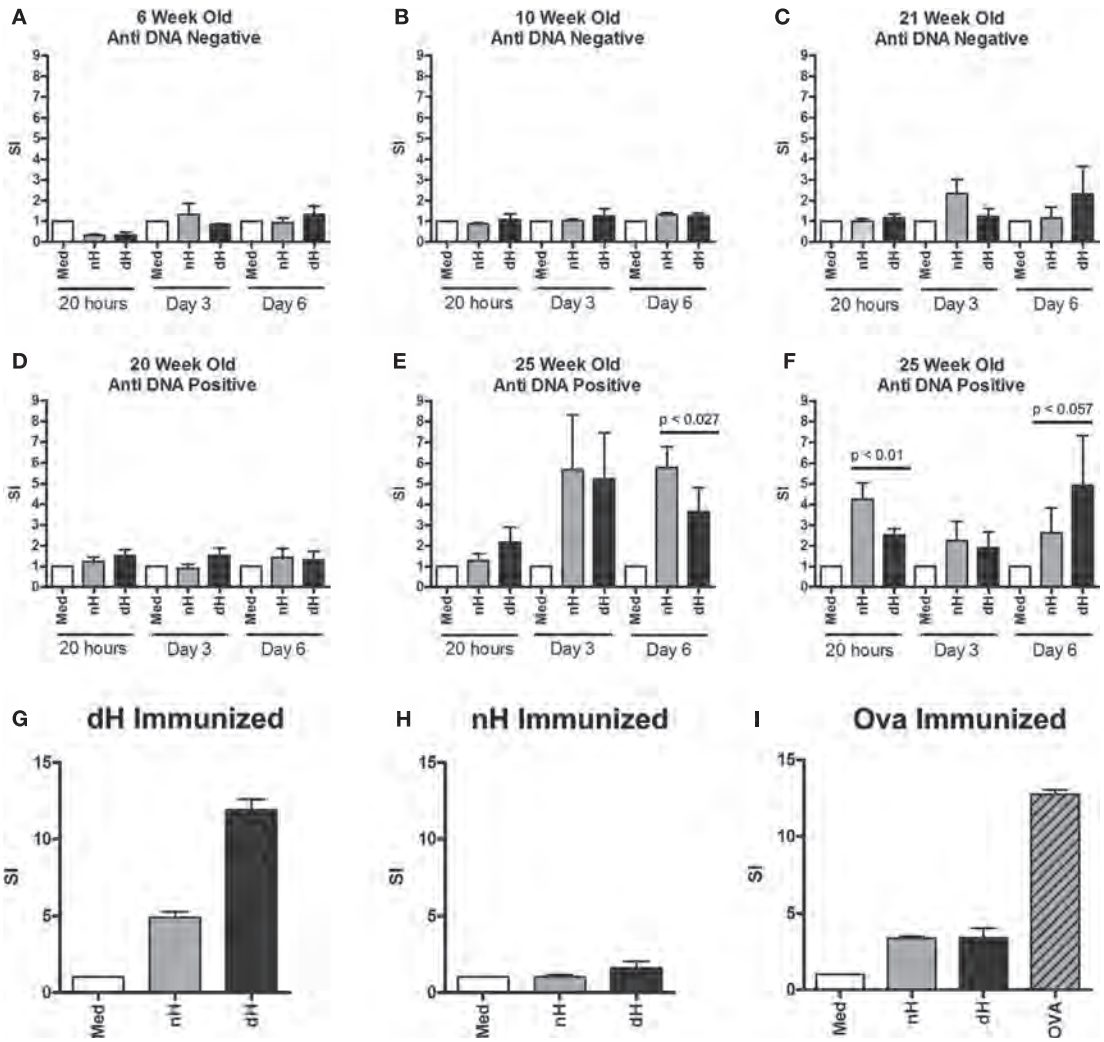

FIGURE 4 | **Proliferation of splenocytes from NZB/W and immunized BALB/c mice**. Splenocytes derived from NZB/W mice of different ages were tested for dsDNA binding by ELISA **(A–F)**. In parallel, splenocytes were stimulated with deiminated histones (dH) or non-deiminated histones (nH) *in vitro*, as indicated. Proliferation was assessed following 4 h, 3 and 6 days in culture, and the response was determined by [^{3}H] thymidine incorporation. In addition, splenocytes from mice immunized with dH **(G)** or nH **(H)** were tested for proliferation in response to dH or nH, or ovalbumin **(I)** as control. The results are presented as stimulation indices (SI) that were calculated from mean cpm values of triplicate wells. The significance of differences between samples was determined by unpaired *T*-test, and the *p* values are indicated.

mice and probed them with anti-dH by Western blot. The bone marrow lysates of autoimmune B6.TC mice and control mice at 7 months of age did not appreciably react with antibodies to dH3 (**Figure 5A**). By contrast, spleens of B6.TC mice had clearly increased levels of dH3 as compared to B6 controls or lysates of the Jurkat lymphoma cells (**Figure 5B**). These results provide a

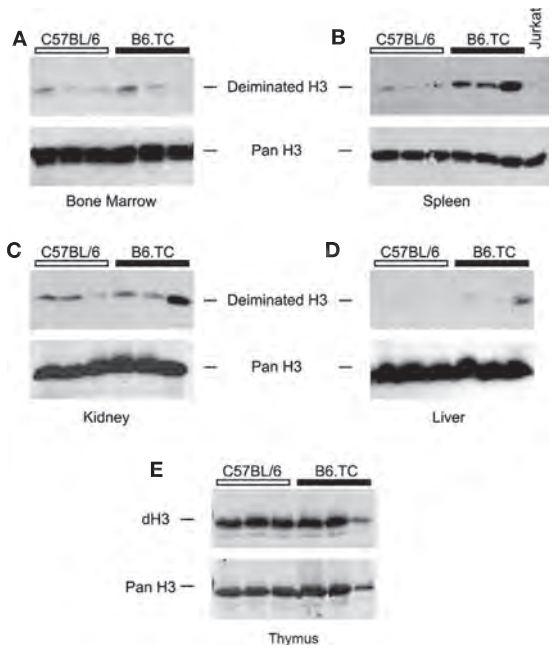

FIGURE 5 | Western blot detection of deiminated histone H3 (dH3) in mouse tissues. Equal amounts of freshly prepared lysates from bone marrow **(A)**, spleen **(B)**, kidney **(C)**, liver **(D)**, and thymus **(E)** of autoimmune B6.TC and control B6 mice were blotted onto nitrocellulose membranes and probed with anti-dH3 antibody. Tissues from three mice were tested in each group. As control, Jurkat lymphoma cells were used **(B)**. The same blots were also re-probed with an antibody that recognizes total histone H3 (Pan H3) to confirm equal loading of lysates.

qualitative estimate rather than a precise measure of deimination. This is by necessity, as the cellular composition and disease process may affect histone deimination in a complex manner in a tissue such as the spleen. The overall amounts of histone H3 were similar in all samples, as indicated by the equivalent immunoreactivity of an anti-H3 antibody. Therefore, the spleens of autoimmune B6.TC mice contained increased quantities of dH3. In addition, the kidneys and the liver from individual autoimmune mice exhibited increased levels of dH3 (**Figures 5C,D**). Importantly, thymic extracts from 4- to 6-month-old B6 and B6.TC mice clearly showed constitutively elevated levels of dH (**Figure 5E**).

DISCUSSION

In this study, we observed that autoimmune mice exhibit a striking B cell bias toward binding nH over dH, a bias that is present at the earliest stages of anti-histone autoimmunity (**Figure 2G**). In 28 of 28 autoimmune NZB/W, NZM2410, B6.*Sle1*, and B6.*Sle1*. *Sle3* mice that made IgG anti-histone antibodies, preference was invariably in favor of nH (**Figure 3A**). Even more strikingly, in 18 animals that produced antibodies to nH, immunoblotting could not detect antibodies to dH. To exclude the possibility that anti-dH antibodies are only transiently expressed, or ensconced in immune complexes, we carried out longitudinal antibody-binding assays that consistently revealed preferential or exclusive binding to nH (**Figure 2G**). These results indicate that, even in mouse strains that spontaneously convert to autoimmunity, deimination reduces IgG binding to histones, and autoantibody binding is focused on arginine-containing epitopes that are absent from dH. It follows that dH remain effective tolerogens even after development of autoimmunity in the tested mouse strains. We conclude this is an important characteristic of autoimmune mice, and we propose that a more detailed comparison with autoimmune disease patients may shed light on the induction of autoimmunity. Moreover, we argue that the specific regulation of PAD4 underlies these results.

Peptidylarginine deiminase 4 is most abundant in granulocytes and other cells of the innate immune system. However, PAD4 is also expressed in another immunologically relevant context. In elegant studies, Ireland and Unanue described the fact that antigen-presenting cells in mice express PAD4 and PAD2 in a compartment that is regulated by proteins in the autophagy pathway (33). The deiminase activity is constitutively expressed in dendritic cells and macrophages, whereas it is inducible in B cells by stress or stimulation through the Ig antigen receptor (33). The authors reported that arginine residues in antigenic peptides are converted to citrullines, and that T cells respond to target antigenic epitopes containing citrulline. This mechanism was demonstrated by using foreign antigens, in their case, hen egg lysozyme that was administered in a conventional immunization. The resulting T cell clones bound preferentially, or even exclusively citrullinated lysozyme peptides. Our results support this mechanism because splenocytes from BALB/c mice proliferated as vigorously against dH as against OVA (**Figure 4G** versus **Figure 4I**). By contrast, splenocytes from NZB/W mice, after these mice had converted to autoimmunity, showed comparable proliferation to either antigen (**Figures 4E,F**). This fundamental difference in outcome points to differences in immunized versus spontaneous autoimmune responses to nH.

As further shown by Ireland and Unanue, autophagy induction in B cells is necessary for their inducible expression of PAD4 activity associated with antigen processing (33). Conversely, a transient or sustained impairment of autophagy in B cells could provide conditions that would support the presentation of histone epitopes lacking citrullines. Under these conditions, B cells would express peptide epitopes that could act as neo-antigens for T cells and solicit T cell help. Autophagy has been linked by genetics to autoimmunity (27). A contribution of the autophagic processes to autoimmunity is consistent with the deficient or impaired functions of ATG5 (and other components of non-canonical autophagy) in SLE, but the mechanism for this relationship is unclear (27, 28). Our data suggest that effective autophagy may be required to maintain certain aspects of immune tolerance in mice.

However, an unanswered question is whether deiminated peptide presentation also occurs during thymic development, and whether tolerizing peptides expressed by thymic antigen-presenting cells are also deiminated. In support of this possibility, we found that thymus lysates from B6 and B6.TC mice show abundant histone deimination (**Figure 5E**), a result that suggests antigen presentation in the mouse thymus is tightly linked to

deimination. If so, citrulline-containing epitopes of autoantigens such as histones may induce powerful tolerance in mice. In that scenario, only B cells that bound to nH and presented non-deiminated epitopes would break tolerance and receive T cell help. Consequently, autoimmunity might initially be directed against non-deiminated peptides, provided that B cells, at an early stage of autoimmune pathogenesis, suspend or shut off the deimination of processed epitopes. Support for this alternative comes from the consistent anti-nH response that we observed in numerous autoimmune mice from different autoimmune strains (**Figures 1** and **2**). Thus, only B cells that no longer expressed PAD4 activity in their antigen processing compartment may escape tolerance. Our hypothesis of the key dependence of self-tolerance on the adequate function of autophagy for the presentation of dH peptides is in line with the remarkable preference of autoimmune mouse antibodies for nH. As corollary, a steady-state balance between nH and dH may be maintained under pre-autoimmune conditions, but an imbalance between the supply, processing, or recognition of nH, likely coincident with a disturbance of autophagic antigen processing in B cells, may result in an antigen-specific response to nH that may break immune tolerance and result in a sustained autoimmune response to nH.

To conclude, we briefly address the difference between mice and humans in their ability to express antibodies to dH. As we and others have shown, autoantibodies in various human autoimmune conditions preferentially bind dH (3, 24, 34), in striking contrast with the opposite bias in mice. Again, the key may be in the expression of PAD4 activity in B cells. Our tissue expression results indicate the increased presence of dH in the spleens of autoimmune mice (**Figure 5B**). By contrast, healthy human B cells appear incapable of expressing PAD4, as indicated by data in the Human Protein Atlas (35). There, evidence suggests that human B cells, even after B cell antigen engagement in the white pulp, fail to express detectable PAD4. Thus, expression of dH epitopes on human B cells may not be intrinsic to the B cells, and presentation of dH epitopes may not engender tolerance that it is as effective as it is in mice. Consequently, B cell presentation of deiminated peptides in humans may be more likely to break tolerance and lead to ACPA generation.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and approved the final version to be published. MR had full access to all the data in the study and took responsibility for the integrity of the data and the accuracy of data analysis. Study conception and design: ND, MS, LM, OR, and MR. Acquisition of data: ND, AH, YZ, IN, and MR. Analysis and interpretation of data: ND, AH, YZ, IN, MS, LM, OR, and MR.

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REFERENCES

1. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO III, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Ann Rheum Dis* (2010) 69(9):1580–8. doi:10.1136/ard.2010.138461
2. Dwivedi N, Upadhyay J, Neeli I, Khan S, Pattanaik D, Myers L, et al. Felty's syndrome autoantibodies bind to deiminated histones and neutrophil extracellular traps. *Arthritis Rheum* (2012) 64(4):982–92. doi:10.1002/art.33432
3. Dwivedi N, Neeli I, Schall N, Wan H, Desiderio DM, Csernok E, et al. Deimination of linker histones links neutrophil extracellular trap release with autoantibodies in systemic autoimmunity. *FASEB J* (2014) 28(7):2840–51. doi:10.1096/fj.13-247254
4. Vossenaar ER, Zendman AJ, van Venrooij WJ, Pruijn GJ. PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. *Bioessays* (2003) 25(11):1106–18. doi:10.1002/bies.10357
5. Neeli I, Khan SN, Radic M. Histone deimination as a response to inflammatory stimuli in neutrophils. *J Immunol* (2008) 180(3):1895–902. doi:10.4049/jimmunol.180.3.1895
6. Chang HH, Dwivedi N, Nicholas AP, Ho IC. The W620 polymorphism in PTPN22 disrupts its interaction with peptidylarginine deiminase type 4 and enhances citrullination and NETosis. *Arthritis Rheumatol* (2015) 67(9):2323–34. doi:10.1002/art.39215
7. Muller S, Radic M. Citrullinated autoantigens: from diagnostic markers to pathogenetic mechanisms. *Clin Rev Allergy Immunol* (2015) 49(2):232–9. doi:10.1007/s12016-014-8459-2
8. Dwivedi N, Radic M. Citrullination of autoantigens implicates NETosis in the induction of autoimmunity. *Ann Rheum Dis* (2014) 73(3):483–91. doi:10.1136/annrheumdis-2013-203844
9. Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, Gizinski A, Yalavarthi S, Knight JS, et al. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci Transl Med* (2013) 5(178):178ra40. doi:10.1126/scitranslmed.3005580
10. Spengler J, Lugonja B, Ytterberg AJ, Zubarev RA, Creese AJ, Pearson MJ, et al. Release of active peptidyl arginine deiminases by neutrophils can explain production of extracellular citrullinated autoantigens in rheumatoid arthritis synovial fluid. *Arthritis Rheumatol* (2015) 67(12):3135–45. doi:10.1002/art.39313
11. Darrah E, Rosen A, Giles JT, Andrade F. Peptidylarginine deiminase 2, 3 and 4 have distinct specificities against cellular substrates: novel insights into autoantigen selection in rheumatoid arthritis. *Ann Rheum Dis* (2012) 71(1):92–8. doi:10.1136/ard.2011.151712
12. Romero V, Fert-Bober J, Nigrovic PA, Darrah E, Haque UJ, Lee DM, et al. Immune-mediated pore-forming pathways induce cellular hypercitrullination and generate citrullinated autoantigens in rheumatoid arthritis. *Sci Transl Med* (2013) 5(209):209ra150. doi:10.1126/scitranslmed.3006869
13. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science* (2004) 303(5663):1532–5. doi:10.1126/science.1092385
14. Brinkmann V, Zychlinsky A. Beneficial suicide: why neutrophils die to make NETs. *Nat Rev Microbiol* (2007) 5(8):577–82. doi:10.1038/nrmicro1710
15. Pratesi F, Panza F, Paolini I, Petrelli F, Puxeddu I, Casigliani-Rabl S, et al. Fingerprinting of anti-citrullinated protein antibodies (ACPA): specificity, isotypes and subclasses. *Lupus* (2015) 24(4–5):433–41. doi:10.1177/0961203314560005
16. Lande R, Ganguly D, Facchinetti V, Frasca L, Conrad C, Gregorio J, et al. Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. *Sci Transl Med* (2011) 3(73):73ra19. doi:10.1126/scitranslmed.3001180

17. Garcia-Romo GS, Caielli S, Vega B, Connolly J, Allantaz F, Xu Z, et al. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. *Sci Transl Med* (2011) 3(73):73ra20. doi:10.1126/scitranslmed.3001201
18. van Bavel CC, Dieker J, Muller S, Briand JP, Monestier M, Berden JH, et al. Apoptosis-associated acetylation on histone H2B is an epitope for lupus autoantibodies. *Mol Immunol* (2009) 47(2–3):511–6. doi:10.1016/j.molimm.2009.08.009
19. Liu CL, Tangsombatvisit S, Rosenberg JM, Mandelbaum G, Gillespie EC, Gozani OP, et al. Specific post-translational histone modifications of neutrophil extracellular traps as immunogens and potential targets of lupus autoantibodies. *Arthritis Res Ther* (2012) 14(1):R25. doi:10.1186/ar3707
20. Seri Y, Shoda H, Suzuki A, Matsumoto I, Sumida T, Fujio K, et al. Peptidylarginine deiminase type 4 deficiency reduced arthritis severity in a glucose-6-phosphate isomerase-induced arthritis model. *Sci Rep* (2015) 5:13041. doi:10.1038/srep13041
21. Shelef MA, Sokolove J, Lahey LJ, Wagner CA, Sackmann EK, Warner TF, et al. Peptidylarginine deiminase 4 contributes to tumor necrosis factor alpha-induced inflammatory arthritis. *Arthritis Rheumatol* (2014) 66(6):1482–91. doi:10.1002/art.38393
22. Konig MF, Darrah E, Andrade F. Insights into the significance of peptidylarginine deiminase 4 and antibodies against citrullinated antigens in the absence of "true ACPAs" in an experimental model of arthritis: comment on the article by Shelef et al. *Arthritis Rheumatol* (2014) 66(9):2642–4. doi:10.1002/art.38719
23. Cantaert T, Teitsma C, Tak PP, Baeten D. Presence and role of anti-citrullinated protein antibodies in experimental arthritis models. *Arthritis Rheum* (2013) 65(4):939–48. doi:10.1002/art.37839
24. Pratesi F, Dioni I, Tommasi C, Alcaro MC, Paolini I, Barbetti F, et al. Antibodies from patients with rheumatoid arthritis target citrullinated histone 4 contained in neutrophils extracellular traps. *Ann Rheum Dis* (2014) 73(7):1414–22. doi:10.1136/annrheumdis-2012-202765
25. Kearney PL, Bhatia M, Jones NG, Yuan L, Glascock MC, Catchings KL, et al. Kinetic characterization of protein arginine deiminase 4: a transcriptional corepressor implicated in the onset and progression of rheumatoid arthritis. *Biochemistry* (2005) 44(31):10570–82. doi:10.1021/bi050292m
26. Moens U, Seternes OM, Hey AW, Silsand Y, Traavik T, Johansen B, et al. In vivo expression of a single viral DNA-binding protein generates systemic lupus erythematosus-related autoimmunity to double-stranded DNA and histones. *Proc Natl Acad Sci U S A* (1995) 92(26):12393–7. doi:10.1073/pnas.92.26.12393
27. Kalaaji M, Sturfelt G, Mjelle JE, Nossent H, Rekvig OP. Critical comparative analyses of anti-alpha-actinin and glomerulus-bound antibodies in human and murine lupus nephritis. *Arthritis Rheum* (2006) 54(3):914–26. doi:10.1002/art.21622
28. Andreassen K, Bredholt G, Moens U, Bendiksen S, Kauric G, Rekvig OP. T cell lines specific for polyomavirus T-antigen recognize T-antigen complexed with nucleosomes: a molecular basis for anti-DNA antibody production. *Eur J Immunol* (1999) 29(9):2715–28. doi:10.1002/(SICI)1521-4141(199909)29:09<2715::AID-IMMU2715>3.3.CO;2-R
29. Bredholt G, Rekvig OP, Andreassen K, Moens U, Marion TN. Differences in the reactivity of CD4+ T-cell lines generated against free versus nucleosome-bound SV40 large T antigen. *Scand J Immunol* (2001) 53(4):372–80. doi:10.1046/j.1365-3083.2001.00873.x
30. Mehrad B, Park SJ, Akangire G, Standiford TJ, Wu T, Zhu J, et al. The lupus-susceptibility locus, Sle3, mediates enhanced resistance to bacterial infections. *J Immunol* (2006) 176(5):3233–9. doi:10.4049/jimmunol.176.5.3233
31. Monestier M, Fasy TM, Losman MJ, Novick KE, Muller S. Structure and binding properties of monoclonal antibodies to core histones from autoimmune mice. *Mol Immunol* (1993) 30(12):1069–75. doi:10.1016/0161-5890(93)90153-3
32. Lambert PH, Dixon FJ. Pathogenesis of the glomerulonephritis of NZB/W mice. *J Exp Med* (1968) 127(3):507–22. doi:10.1084/jem.127.3.507
33. Ireland JM, Unanue ER. Autophagy in antigen-presenting cells results in presentation of citrullinated peptides to CD4 T cells. *J Exp Med* (2011) 208(13):2625–32. doi:10.1084/jem.20110640
34. Dwivedi N, Upadhyay J, Neeli I, Khan S, Pattanaik D, Myers L, et al. Felty's syndrome autoantibodies bind to deiminated histones and neutrophil extracellular chromatin traps. *Arthritis Rheum* (2012) 64(4):982–92. doi:10.1002/art.33432
35. Uhlen M, Oksvold P, Fagerberg L, Lundberg E, Jonasson K, Forsberg M, et al. Towards a knowledge-based human protein atlas. *Nat Biotechnol* (2010) 28(12):1248–50. doi:10.1038/nbt1210-1248

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Autoantibodies to Su/Argonaute 2 in Japanese patients with inflammatory myopathy

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ABSTRACT

Background: Anti-Su antibodies are found in 5–20% of cases of various systemic autoimmune rheumatic diseases and in 5–10% of dermatomyositis (DM)/polymyositis (PM) patients. In 2006, the 100 kDa Su antigen was identified as argonaute2 (Ago2), and it was found to play a major role in RNA interference. However, immunoprecipitation (IP) remains the main method for detecting anti-Su and the clinical significance of the antibodies is uncertain.

Methods: Sera from patients with DM/PM (n = 224) were screened by an ELISA that uses recombinant biotinylated Ago2 protein. Some serum samples were tested by IP and by indirect immunofluorescence (IIF) analysis.

Results: Seventeen (7.5%, 17/224) sera from DM/PM were positive in ELISA. Of the 33 IP-tested sera (17 ELISA-positive and 16 ELISA-negative with high background), 13 were found to be anti-Ago2/Su-positive in IP and ELISA. Only one IP-positive serum was judged to be ELISA-negative. Among the 13 patients with anti-Su/Ago2, 7 cases also had myositis-specific autoantibodies. Six sera that were positive by both IP and ELISA showed the GW body pattern in IIF. Interestingly, one ELISA-positive serum with an inconclusive result in IP also showed the GW body pattern.

Conclusion: Our novel ELISA appears to be useful for screening anti-Su/Ago2 antibodies (sensitivity: 93%, specificity: 79%).

1. Introduction

Anti-Su antibodies were first detected by double immunodiffusion using calf thymus extract in sera from patients with systemic lupus erythematosus (SLE) [1]. MRL/*lpr* mice and pristane-induced SLE model mice also produce anti-Su antibodies [2,3]. In 1994, target antigens of anti-Su antibodies were characterized by immunoprecipitation (IP) as 100/102 kDa doublet proteins often accompanied by a 200 kDa protein [4]. In 2006, the 100 kDa component of the Su antigen was identified as Argonaute 2 (Ago2), a key component in RNA interference machinery [5]. Ago2 also plays important roles in the regulation of microRNA expression, function, maturation and stability [6]. Recently, the roles of microRNAs in the pathogenesis of autoimmune diseases have been studied extensively [7].

In addition to being reported in SLE, anti-Su antibodies have been reported in many other systemic autoimmune rheumatic diseases, including systemic sclerosis (SSc), dermatomyositis/polymyositis (DM/PM), and rheumatoid arthritis (RA). The prevalence is relatively high in SLE (17–24%) and SSc (17–32%), but lower in RA and DM/PM (7–9%), based on limited studies [1,4,8–10]. The data on anti-Su in DM/PM patients in the previous studies were from small cohorts; only 17 to 43 serum samples from DM/PM patients were examined [1,8,10]. The clinical features of anti-Su antibody-positive DM/PM are not well characterized. One reason for this is because detection has been done by IP or by insensitive, rarely used double immunodiffusion. Thus, in this study, we aimed to establish a simple method for screening anti-Su/Ago2 antibodies and to analyze clinical features associated with DM/PM patients with these antibodies. We have already accumulated data of more than 200 patients with idiopathic inflammatory myopathies in our previous studies [11,12].

2. Material and methods

2.1. Patients and sera

Two hundred twenty-four Japanese patients (68 males, 156 females) were enrolled in the study. Demographic and medical information were collected from chart reviews. One hundred fifty-four of

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the 224 sera were from patients who had visited the clinic of the Department of Dermatology at Nagoya University Graduate School of Medicine between 1994 and 2015. Sera from 70 other patients were sent to our laboratory from collaborating hospitals to test for myositis-specific autoantibodies. One hundred fifty-nine patients fulfilled the criteria of Bohan and Peter for DM/PM [13], and the remaining 65 met the Sontheimer criteria for clinically amyopathic DM (CADM) [14]. Of the 224 patients, 90 patients had classical DM, 65 had clinically amyopathic DM, 25 had cancer-associated DM, 17 had juvenile DM, 20 had PM and 7 had myositis overlap syndrome. The ages at disease onset were 3 to 85 years (mean = 52 ± 18).

Age-appropriate cancer screening and computed tomography of the chest for the evaluation of interstitial lung disease (ILD) were performed. Patients were classified as having cancer-associated DM if a diagnosis of a malignancy was made within 3 years prior to the development of the DM-associated symptoms or after the onset of the first DM symptoms. This study was approved by the ethics committees of the Nagoya University Graduate School of Medicine and University Hospital.

2.2. Laboratory tests and serologic assays

Serum samples were screened for antibodies against SS-A/Ro60 and U1-RNP using commercial enzyme-linked immunosorbent assay (ELISA) kits (MBL®, Nagoya, Japan). In addition, anti-Mi-2, anti-TIF1γ, anti-MDA-5, anti-NXP-2, anti-HMG-CoA, anti-Ku70/80, anti-SRP54, anti-PM/Scl-75/100 and anti-SAE1/2 were tested by ELISA using biotinylated recombinant proteins [15]. When the results obtained by anti-aminoacyl-transfer RNA synthetase (anti-ARS) ELISA kit (MBL®) were positive, the individual anti-ARS of EJ, Jo-1, KS, PL-7 and PL-12 were tested by the in-house ELISA [15].

2.3. Recombinant protein

The full-length cDNA clone of human Ago2 in pDEST17 vector was a gift from Dr. Edward Chan (University of Florida) [16]. Biotinylated recombinant protein was produced from the cDNA using the T7 Quick Coupled Transcription/Translation System (Promega®, Madison, WI, USA) [15].

2.4. Enzyme-linked immunosorbent assay (ELISA)

All sera were tested by ELISA for antibodies to Ago2, following our previously published protocols [15]. A 96-well Immobilizer Streptavidin plate (Thermo Scientific Nunc®, Roskilde, Denmark) was incubated with 1 μl of biotinylated recombinant Ago2 protein/50 μl PBS containing 0.05% Tween20/well. The wells were then incubated with diluted patient sera (1:1000 dilution), followed by incubation with horse radish peroxidase-conjugated goat anti-human IgG antibody (1:30,000 dilution; Dako®, Grostrup, Denmark). Anti-Ago2 reactivity was determined by incubation with substrate, and relative luminescence units (RLU) were read. The RLU of the samples was converted into units using a standard curve created with a serially diluted prototype positive serum.

2.5. Immunoprecipitation (IP)

Autoantibodies in sera were tested by IP of ^{35}S-methionine radiolabeled K562 cell extract, SDS-PAGE, and autoradiography. Anti-Su antibodies were identified by the presence of the characteristic 100/102-kDa doublet Su antigens [4].

2.6. Indirect immunofluorescence (IIF)

Sera were tested by IIF with two different commercial kits using HEp-2 cells (Fluoro HEPANA Test: MBL®, and Premmune HEp20-10: EUROIMMUN®, Luebeck, Germany) according to their protocols.

2.7. Statistical analysis

The results were analyzed by Fisher's exact test, Mann-Whitney *U* test, or log rank test, as appropriate, using SPSS version 22 (IBM, Armonk, NY, USA). *P* values less than 0.05 were considered significant.

3. Results

3.1. Detection of anti-Su/Ago2 antibodies by ELISA and immunoprecipitation

An in-house ELISA was used to detect anti-Ago2 antibodies in sera. The cutoff value was defined as a mean plus 5 standard deviations of the ELISA unit (= 2.7 units) as obtained from the 40 healthy controls. All healthy control sera were anti-Ago2-antibody-negative in our ELISA. Of the 224 DM/PM patients' sera, 17 (7.6%) were ELISA-positive.

Next, 33 sera (including 17 ELISA-positive sera and 16 ELISA-negative sera that had units close to the cutoff value) were tested by IP for the presence of 100/102 kDa Su proteins. Anti-Su/Ago2 was confirmed by IP in 13 of 17 ELISA-positive sera. One of the 17 ELISA-positive sera strongly immunoprecipitated a protein that migrated close to the 100/102kD Su antigen, and whether the serum had anti-Su or not was inconclusive (Fig. 1a, lane 15). In contrast, only one of 16 ELISA-negative sera that showed relatively high reactivity but was below the cutoff in the anti-Ago2 ELISA was positive for anti-Su/Ago2 by IP (Fig. 1a, lane 14). From these data, our ELISA showed a sensitivity of 93% (13/14) and a specificity of 79% (15/19), compared to anti-Su/Ago2 antibody detection by IP as the gold standard.

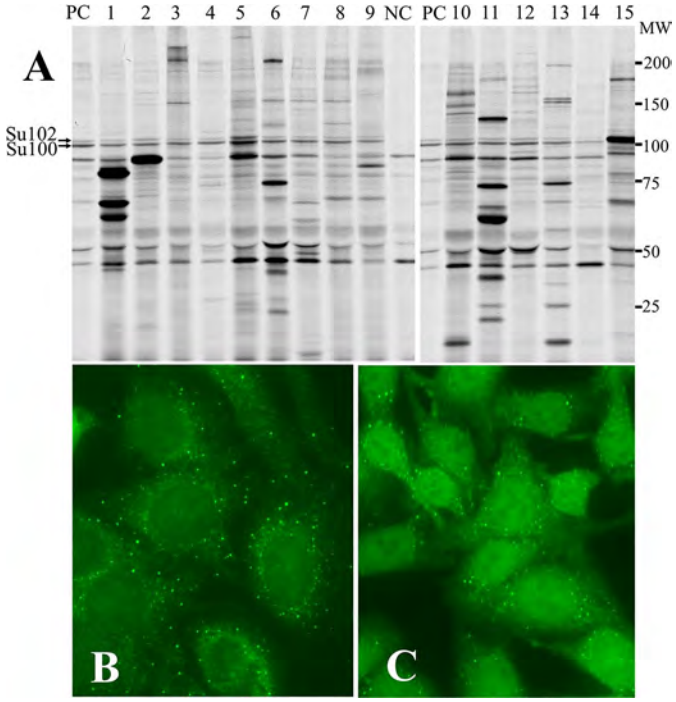

Fig. 1. Immunoprecipitation and indirect immunofluorescence analysis of anti-Su/Ago2-positive sera.

A. Immunoprecipitation for anti-Su/Ago2 antibodies. Lanes 1-13: Anti-Su/Ago2-positive sera in ELISA/IP analysis, lane 14: anti-Su-positive serum in IP analysis, lane 15: anti-Ago2-positive serum in ELISA analysis. Lanes PC and NC: positive control and negative control sera. MWM: molecular weight marker. Lane numbers correspond to the case numbers in Table 2.

B and C. GW body staining pattern by an anti-Ago2/Su antibody-positive serum from a patient with clinically amyopathic dermatomyositis using two indirect immunofluorescence (IIF) kits. B: IIF using EUROIMMUN® kit, C: IIF using MBL® kit.

Table 1
Clinical and serological feature of the anti-Su/Ago2-positive and -negative idiopathic inflammatory myopathy patients.

	Anti-Su positive % (N = 13)	Anti-Su negative % (N = 211)	P value
Male:female	3:10	65:146	0.75
Mean age (mean ± SD)	48.0 ± 14.6	52.2 ± 18.1	0.5
Myositis-specific autoantibodies			
MDA5	23 (3)	23 (50)	1
TIF1γ	7 (1)	17 (36)	0.69
ARS	23 (3)	15 (32)	1
Negative	46 (6)	33 (70)	1
Clinical diagnosis			
Classical DM	30 (4)	40 (86)	0.4
JDM	7 (1)	7 (16)	0.99
CADM	38 (5)	28 (60)	0.5
Cancer-associated DM	0 (0)	11(25)	0.8
PM	15 (2)	8 (18)	0.3
Overlap	7 (1)	3 (6)	0.33

ARS: aminoacyl tRNA synthetases, CADM: clinically amyopathic dermatomyositis, DM: dermatomyositis, JDM: juvenile dermatomyositis, MDA5: melanoma differentiation associated gene 5, PM: polymyositis, TIF1γ: transcriptional intermediary factor 1γ.

3.2. Clinical and laboratory profiles of patients with anti-Su/Ago2 antibodies

There was no statistically significant difference in prevalence of myositis subsets, age, sex or coexisting autoantibodies between the anti-Su/Ago2 antibody-positive group and the anti-Su/Ago2 antibody-negative groups (Table 1). Six of the 8 patients who were anti-Su/Ago2 antibody-positive had ILD. None of the anti-Su/Ago2 antibody-positive patients had internal malignancy. In our study, 177 of 224 patients with myositis were available for the evaluation of ILD. Six of 8 (75%) anti-Su-positive patients had ILD, whereas 104 of 177 (61%) anti-Su-negative myositis patients had ILD. The difference was not statistically significant (P = 0.7). Moreover, malignancy in anti-Su-positive and -negative patients was 0 of 13 (0%) and 25 of 211 (11%) respectively; no statistical difference was found (Table 1).

3.3. IIF analysis of anti-Su/Ago2-positive sera

Twenty-seven sera (17 anti-Ago2 ELISA-positive and 10 below the cutoff value described above) were tested by IIF using 2 different IIF kits to evaluate characteristic GW body (GWB) staining (cytoplasmic discrete foci pattern) [5] for anti-Su (Figs. 1b, c). Seven sera showed the GWB pattern by both kits, 6 of which were positive by both IP and ELISA (Table 2). The remaining one serum was ELISA-positive, but the IP result was inconclusive because of protein bands that migrate close to the 100/102kD Su antigen (Fig. 1a, lane 15). Ten ELISA-negative sera showed no GWB staining.

We compared the clinical characteristics of GW body positive-staining vs. negative-staining anti-Su patients (Table 3). Levels of anti-Ago2 determined by ELISA appear higher in the GW body positive-staining group, but the difference is not statistically significant.

Table 3
Clinical and serological features of GW body positive-staining and negative-staining PM/DM patients.

	GW body staining (+) N = 6	GW body staining (−) N = 7	P value
Age	53.7 ± 10.6	43.57 ± 17.7	0.67
Anti-Ago2 ELISA (unit)	84.6 ± 118	18.13 ± 14.4	0.277
Creatine kinase (IU/L)	1722.5 ± 2928	534 ± 719	0.41
Interstitial lung disease (%)	2 (33%)	4 (57%)	0.59

4. Discussion

To our knowledge, this is the first study on anti-Su/Ago2 antibodies using anti-Ago2 ELISA and it is the largest myositis cohort. The IP and ELISA results showed very good agreement, and we proved that our ELISA system is effective in screening anti-Su/Ago2 antibodies. In previous studies, anti-Su antibodies were associated with anti-Ku antibodies in overlap syndrome [4], lower levels of IgG anti-β2 glycoprotein I antibodies in primary anti-phospholipid syndromes [17], or the absence of other SLE-related antibodies [1]. In our DM/PM cohort, no specific clinical features associated with anti-Su/Ago2 antibodies were found.

Anti-Su/Ago2 antibodies are known to show a unique staining pattern in IIF. Even though Ago2 localizes to GWB, the GWB pattern in IIF is positive for only ~1/3 cases with anti-Su/Ago2 antibodies [5]. There are several hypotheses to explain the inconsistent IIF results. It is possible that exposed Ago2 epitopes are affected by post-translational modification or proteins interacting with Ago2. Similarly, it is likely that Ago2 epitopes in relatively native liquid-phase molecules in IP

Table 2
Clinical and serological characteristics of anti-Su/Ago2 antibody-positive PM/DM patients.

Case	Age	Sex	Diagnosis	Concomitant antibodies	ELISA$^+$ (units)	GWB IIF pattern	CK max (IU/L)	Malignancy	Interstitial lung disease
1	47	M	DM	EJ	4.9	+	582	−	−
2	54	F	DM	PL-7	8.0	+	NA	−	NA
3	50	F	DM	−	25	−	500	−	−
4	50	M	DM	−	17	−	28	−	NA
5	73	M	CADM	ACA	80	+	NA	−	NA
6	57	F	CADM	MDA5	92	+	42	−	+
7	49	F	CADM	MDA5	3.2	−	30	−	+
8	46	F	CADM	MDA5	9.8	+	165	−	+
9	23	F	CADM	−	6.3	−	165	−	+
10	15	F	JDM	TIF1γ, SS-A	4.4	−	24	−	NA
11	54	F	DM + RA	Jo-1, SS-A	40	−	1915	−	+
12	64	F	PM	−	31	−	1076	−	+
13	45	F	PM	U1RNP	313	+	6101	−	NA
14	51	F	Cancer-a. DM	−	1.8	−	NA	+	−
15	74	M	PM	−	4.8	+	1670	+	+

Cases 1–13; Anti-Su/Ago2-positive cases by both immunoprecipitation and ELISA. Case 14; Anti-Su-positive case by immunoprecipitation. Case 15; Anti-Ago2-positive case by ELISA. ACA: anti-centromere antibody, CADM: clinically amyopathic dermatomyositis, Cancer-a.: cancer-associated, CK: creatine kinase, DM: dermatomyositis, GWB: GW bodies, IIF: indirect immunofluorescence, JDM: juvenile dermatomyositis, MDA5: melanoma differentiation associated gene 5, NA: not available, RA: rheumatoid arthritis, −: negative, +: positive.
$^+$ Cut-off value of ELISA: 2.7 unit.

differ from solid-phase epitopes in IIF slide or recombinant proteins used in ELISA. In the present study, 6 sera that were positive for anti-Su/Ago2 by both IP and ELISA showed a GWB pattern by the 2 different IIF kits, and the densities of GWB seemed similar in both kits. We obtained good concordance in the IIF results using two different HEp-2 kits.

Case 14 showed 1.8 units in ELISA, which was higher than the mean + 2SD of healthy controls but did not reach the ELISA cutoff value. The serum from this patient showed typical IP patterns of anti-Su antibody (Figure line 14, Table 2). This might have been the result of differences in antibody affinities to the cell extract (used in IP) and recombinant proteins (used in ELISA) due to the Ago2-epitope divergences discussed above.

There was one anti-Ago2 ELISA-positive serum that showed a GWB pattern in IIF. The serum from this patient immunoprecipitated a strong protein band around 100 kDa that comigrates with the characteristic Su/Ago2 bands in IP (Table 1); thus, it was not possible to conclude whether this serum had anti-Su/Ago2. Su/Ago2 is a component of cytoplasmic GWB; however, there are many other autoantigens that are components of GWB. Thus, GWB staining alone is not enough to classify this serum as anti-Su/Ago2-positive. Although IP is considered the gold standard for detecting anti-Su antibodies, our ELISA could sometimes be useful for detecting anti-Su/Ago2 in cases like this. Our newly developed ELISA can be used to detect anti-Su/Ago2 antibodies. To elucidate the clinical features of anti-Su/Ago2 antibody-positive patients with various diseases, future studies in larger cohorts will be needed.

Competing interests

None declared.

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References

[1] E.L. Treadwell, M.A. Alspaugh, G.C. Sharp, Characterization of a new antigen-antibody system (Su) in patients with systemic lupus erythematosus, Arthritis Rheum. 27 (1984) 1263–1271.
[2] E.L. Treadwell, P. Cohen, D. Williams, K. O'brien, A. Volkman, R. Eisenberg, MRL mice produce anti-Su autoantibody, a specificity associated with systemic lupus erythematosus, J. Immunol. 150 (1993) 695–699.
[3] M. Satoh, W.H. Reeves, Induction of lupus-associated autoantibodies in BALB/c mice by intraperitoneal injection of pristine, J. Exp. Med. 180 (1994) 2341–2346.
[4] M. Satoh, J.J. Langdon, C.H. Chou, et al., Characterization of the Su antigen, a macromolecular complex of 100/102 and 200-kDa proteins recognized by autoantibodies in systemic rheumatic diseases, Clin. Immunol. Immunopathol. 73 (1994) 132–141.
[5] A. Jakymiw, K. Ikeda, M.J. Fritzler, W.H. Reeves, M. Satoh, E.K. Chan, Autoimmune targeting of key components of RNA interference, Arthritis Res. Ther. 8 (2006) R87.
[6] E. Bernstein, A.A. Caudy, S.M. Hammond, Role for a bidentate ribonuclease in the initiation step of RNA interference, Nature 409 (2001) 363–366.
[7] G. Wang, L.S. Tam, E.K. Li, et al., Serum and urinary cell-free MiR-146a and MiR-155 in patients with systemic lupus erythematosus, J. Rheumatol. 37 (2010) 2516–2522.
[8] Y. Yamasaki, S. Narain, H. Yoshida, et al., Autoantibodies to RNA helicase A: a new serologic marker of early lupus, Arthritis Rheum. 56 (2007) 596–604.
[9] C.A. Palafox Sánchez, M. Satoh, et al., Reduced IgG anti-small nuclear ribonucleoprotein autoantibody production in systemic lupus erythematosus patients with positive IgM anti-cytomegalovirus antibodies, Arthritis Res. Ther. 11 (2009) R27.
[10] Vázquez-Del Mercado M, Palafox-Sánchez et al., High prevalence of autoantibodies to RNA helicase A in Mexican patients with systemic lupus erythematosus, Arthritis Res. Ther. 2010; 12(2010) R6.
[11] Y. Muro, K. Sugiura, M. Nara, et al., High incidence of cancer in anti-small ubiquitin-like modifier activating enzyme antibody-positive dermatomyositis, Rheumatology (Oxford) 54 (2015) 1745–1747.
[12] Y. Muro, R. Nakashima, Y. Hosono, et al., Autoantibodies to DNA mismatch repair enzymes in polymyositis/dermatomyositis and other autoimmune diseases: a possible marker of favorable prognosis, Arthritis Rheum. 66 (2014) 3457–3462.
[13] A. Bohan, J.B. Peter, Polymyositis and dermatomyositis (first of two parts), N. Engl. J. Med. 292 (1975) 344–347.
[14] R.D. Sontheimer, Would a new name hasten the acceptance of amyopathic dermatomyositis (dermatomyositis siné myositis) as a distinctive subset within the idiopathic inflammatory dermatomyopathies spectrum of clinical illness? J. Am. Acad. Dermatol. 46 (2002) 626–636.
[15] Y. Muro, K. Sugiura, M. Akiyama, A new ELISA for dermatomyositis autoantibodies: rapid introduction of autoantigen cDNA to recombinant assays for autoantibody measurement, Clin. Dev. Immunol. 2013 (2013) 856815.
[16] A. Jakymiw, S. Lian, T. Eystathioy, S. Li, et al., Disruption of GW bodies impairs mammalian RNA interference, Nat. Cell Biol. 7 (2005) 1267–1274.
[17] A. Ceribelli, A. Tincani, I. Cavazzana, et al., Anti-argonaute2 (Ago2/Su) and -Ro antibodies identified by immunoprecipitation in primary anti-phospholipid syndrome (PAPS), Autoimmunity 44 (2011) 90–97.

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A Comprehensive Overview on Myositis-Specific Antibodies: New and Old Biomarkers in Idiopathic Inflammatory Myopathy

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Abstract Autoantibodies specific for idiopathic inflammatory myopathy (myositis-specific autoantibodies (MSAs)) are clinically useful biomarkers to help the diagnosis of polymyositis/dermatomyositis (PM/DM). Many of these are also associated with a unique clinical subset of PM/DM, making them useful in predicting and monitoring certain clinical manifestations. Classic MSAs known for over 30 years include antibodies to Jo-1 (histidyl transfer RNA (tRNA) synthetase) and other aminoacyl tRNA synthetases (ARS), anti-Mi-2, and anti-signal recognition particle (SRP). Anti-Jo-1 is the first autoantibodies to ARS detected in 15–25 % of patients. In addition to anti-Jo-1, antibodies to seven other aminoacyl tRNA synthetases (ARS) have been reported with prevalence, usually 1–5 % or lower. Patients with any anti-ARS antibodies are associated with anti-synthetase syndrome characterized by myositis, interstitial lung disease (ILD), arthritis, Raynaud's phenomenon, and others. Several recent studies suggested heterogeneity in clinical features among different anti-ARS antibody-positive patients and anti-ARS may also be found in idiopathic ILD without myositis. Anti-Mi-2 is a classic marker for DM and associated with good response to steroid treatment and good prognosis. Anti-SRP is specific for PM and associated with treatment-resistant myopathy histologically characterized as necrotizing myopathy. In addition to classic MSAs, several new autoantibodies with strong clinical significance have been described in DM. Antibodies to transcription intermediary factor 1γ/α (TIF1γ/α, p155/140) are frequently found in DM associated with malignancy while anti-melanoma differentiation-associated gene 5 (MDA5; CADM140) are associated with clinically amyopathic DM (CADM) complicated by rapidly progressive ILD. Also, anti-MJ/nuclear matrix protein 2 (NXP-2) and anti-small ubiquitin-like modifier-1 (SUMO-1) activating enzyme (SAE) are recognized as new DM-specific autoantibodies. Addition of these new antibodies to clinical practice in the future will help in making earlier and more accurate diagnoses and better management for patients.

Keywords Autoantibodies · Anti-nuclear antibodies · Polymyositis · Dermatomyositis · Inflammatory myopathy

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Introduction

Specific autoantibodies in systemic autoimmune rheumatic diseases (SARD) are clinically useful biomarkers associated with a particular disease and/or clinical manifestations. Some of them, such as anti-Sm and dsDNA antibodies in systemic lupus erythematosus (SLE), anti-topoisomerase I (topo I; Scl-70) and RNA polymerase III in scleroderma (SSc; systemic sclerosis), and anti-Jo-1 in polymyositis/dermatomyositis (PM/DM) are highly specific for a particular diagnosis and called disease marker antibodies and included in classification

criteria of each disease [1, 2]. These autoantibodies are detectable years before clinical manifestation or diagnosis and thus have predictive value for the development of the disease [3]. A majority of disease-associated autoantibodies in SLE and SSc have been known for decades and there are a few classic PM/DM-specific autoantibodies such as anti-Jo-1 [4] and Mi-2 [5, 6]. In recent years, there has not been any identification of new autoantibodies in SLE and SSc with significant impact on clinical medicine although many known classic autoantibodies are continuingly used as standard clinical tests. In contrast, several new and important autoantibody specificities with strong clinical impact, such as antibodies to transcription intermediary factor 1γ (TIF1γ) that are frequently found in cancer-associated DM [7, 8] and anti-melanoma differentiation-associated gene 5 (MDA5) associated with clinically amyopathic DM (CADM) with rapidly progressive interstitial lung disease (ILD) [9–12], have been identified in PM/DM. Autoantibodies that are found in PM/DM are often classified into myositis-specific autoantibodies (MSA) and myositis-associated autoantibodies (MAA) [6, 13, 14]. MSAs are almost exclusively found in PM/DM among SARD, although some antibodies such as anti-aminoacyl transfer RNA (tRNA) synthetases (ARS) are also found in patients classified as idiopathic ILD [15–17]. In this article, we will focus on recent updates on clinical significance and discuss other issues related to MSA.

Myositis-Specific Antibodies and Myositis-Associated Antibodies

Autoantibodies found in patients with PM/DM have been classified into MSA and MAA [6, 13, 18, 19] (Table 1). These are clinically relevant well-accepted concepts. However, there are differences in opinion on antibodies to be included in MSA vs. MAA, in particular to the latter. MSA is defined as autoantibody specificities that are considered relatively specific for PM/DM [6, 13, 18, 21]. The disease specificity is usually defined based on comparison of the prevalence of autoantibodies within various SARD and data on screening of MSA in non-SARD patients are often limited. Detection of certain anti-ARS, such as antibodies to PL-12 [15] and KS [16] in patients with idiopathic ILD, has been reported. One recent study reported that 10 % of patients with idiopathic ILD had anti-ARS [17]. This is not a surprise because the anti-ARS has a strong association with ILD and ILD may precede myositis in some cases. Nevertheless, some patients appear to stay as idiopathic ILD for many years and may not develop myositis. Despite detection of some MSA in non-PM/DM patients, their specificity for PM/DM among SARD is consistent and has clear clinical significance.

There are several classic MSA known for years including antibodies to Jo-1, PL-7, PL-12, EJ, OJ, Mi-2, and SRP [6]. Perhaps, there is not much disagreement to classify them as MSA as they are specific for PM/DM among SARD. In addition, each of them is associated with certain clinical manifestation such as anti-ARS with anti-synthetase syndrome and anti-SRP with treatment-resistant necrotizing myopathy. "Which new autoantibodies should be classified as MSA" is not well established and arguable. Among new antibodies, perhaps anti-TIF1γ/α and anti-MDA5/CADM-140 are the two that have the best evidence to be considered MSA and also both are associated with distinct clinical subsets of DM; anti-TIF1γ/α with cancer-associated DM [7, 8, 22] and anti-MDA5 with ADM or CADM often complicated by rapidly progressive ILD (RPILD) [9, 10]. Other antibodies such as anti-MJ/nuclear matrix protein 2 (NXP-2) and small ubiquitin-like modifier-1 (SUMO-1) activating enzyme (SAE) are less prevalent and limited data are available [14].

Definition of MAA is more vaguely defined than MSA as "autoantibody specificity found in PM/DM but not specific

Table 1 Myositis-specific and myositis-associated autoantibodies

Type of autoantibodies	Myositis-specific antibodies (MSA)	Myositis-associated antibodies (MAA)	Other autoantibodies often found in myositis
Autoantibody specificities	Classic MSA: Jo-1, PL-7, PL-12, EJ, OJ, Mi-2, SRP	PM-Scl, Ku, U1RNP, U1/U2RNP, U3RNP	Ro52, Ro60, Su/Ago2
	New antibodies that can be considered MSA: KS, TIF1γ/α, TIF1β, MJ/NXP-2, MDA5/CADM-140, SAE		
Association with SARD	PM/DM, PM/DM-overlap syndrome	PM/DM, PM/DM-overlap syndrome, SSc, SLE	Various SARD
Detection in non-PM/DM	Uncommon (anti-ARS can be in overlap syndrome and idiopathic ILD)	Not uncommon	Often
Association with myopathy when found in non-PM/DM	Yes	Yes	No or not established
Prevalence In general population [20]	Almost none	PM-Scl, Ku, U1/U2RNP—almost none; U1RNP, ~0.1 %	Relatively common (0.5–1 %)

SARD systemic autoimmune rheumatic diseases, *PM* polymyositis, *DM* dermatomyositis, *SSc* scleroderma, systemic sclerosis, *SLE* systemic lupus erythematosus, *ILD* interstitial lung disease

for this diagnosis and may be found in other SARD" [21]. MAA include anti-PM-Scl, anti-Ku, anti-U1ribonucleoprotein (RNP), and U1/U2RNP, which are all associated with a subset of PM/DM-overlap syndrome. They also are associated with muscular involvement in SLE and SSc even if they are not considered PM/DM-overlap syndrome. Anti-Ro52 is classified as MAA in some studies [14]; however, the significance of anti-Ro52 appears to be different from others in this category. Anti-Ro52 is frequently detected in patients with anti-Jo-1, PL-7, PL-12, and others but also found frequently in patients with SLE, SSc, SjS, and other diseases [23].

Anti-Ro60 and anti-La [24–26] and anti-U3RNP [27, 28] are also included in MAA in some articles. Significance of anti-U3RNP may be somewhat similar to that of anti-Ku and PM-Scl as it is sometimes found in SSc-PM-overlap syndrome.

Distribution of anti-Ro52 appears to be more similar to that of anti-Ro60 and anti-Su/Argonaute2 (Ago2) [29] and its association with a subset of PM/DM or muscle involvement is not clear other than the data that it often coexists with anti-ARS [23]. Classifying these antibodies as MAA may be confusing because these three antibodies are also commonly found in SSc and SLE. If these antibodies are classified as MSA, they also may need to be called scleroderma-associated and SLE-associated autoantibodies and would be confusing. In addition, anti-Ro60, Ro52, and anti-Su/Ago2 are the specificities found in unselected populations or healthy individuals relatively frequently (~0.5–1 %) in contrast to rare occurrence (<0.1 %) of MSA and other MAA that is associated with overlap syndrome [20].

Spectrum of Muscle and Skin Involvement in Inflammatory Myopathy and Myositis-Specific Autoantibodies

Spectrum of muscle and skin involvements in inflammatory myopathy varies from muscle disease without skin disease (Fig. 1, PM, left), involvement of both muscle and skin with different degree of each (DM, middle) to skin disease with minimal muscle involvement (CADM), or no muscle involvement (ADM). Anti-ARS is detected in both PM and DM and occasionally in ADM. Most anti-SRP-positive patients have PM and a majority of anti-Mi-2, TIF1γ/α, MJ/NXP-2, and SAE are found in DM. Anti-MDA5 is mainly found in CADM/ADM and prominent muscle disease is uncommon. Anti-Jo-1 and other ARS, anti-SRP, and anti-Mi-2 are considered classic MSA. The several new MSA described recently including anti-TIF1γ/α, anti-MJ/NXP-2, anti-SAE, and anti-MDA5, are all mainly detected in DM and each of them is associated with a unique subset. Although all MSA are specific for PM or DM, presence of more than one MSA in each patient is uncommon for unknown reasons [6, 14]. Non-MSA autoantibodies associated with PM/DM overlap syndrome such as anti-Ku, PM-Scl, U1RNP, and U1/U2RNP are not shown here, but they can be found in both PM and DM

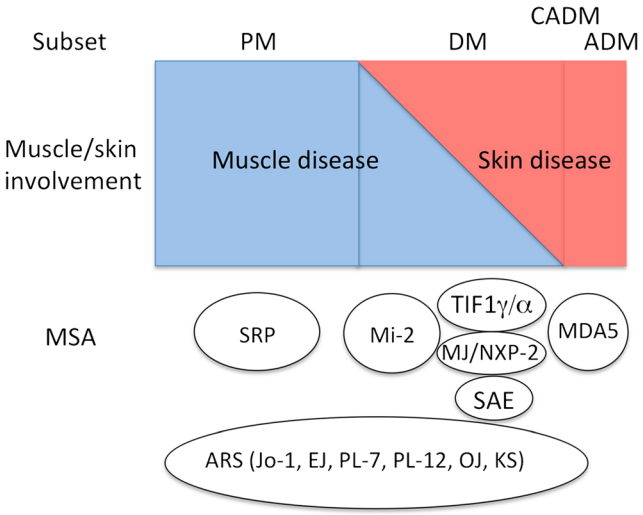

Fig. 1 A summary of the association of myositis-specific autoantibodies with the spectrum of muscle and skin involvements in different subsets of PM/DM

(Table 1). Coexistence of anti-U1RNP with anti-Ku or anti-ARS is relatively common.

Myositis-Specific Antibodies

MSA specificities and characteristics of target autoantigens are summarized in Table 2. Prevalence and association with subset of PM/DM and clinical association are summarized in Table 3. This section will mainly focus on clinical significance of MSA in adult PM/DM patients because the prevalence and clinical association of MSA are quite different in juvenile DM (JDM).

Anti-ARS

Anti-ARS are a group of autoantibodies that recognize the cytoplasmic amino acid-charging enzymes, aminoacyl tRNA synthetases. So far, autoantibodies to eight of them including histidyl (Jo-1), threonyl (PL-7), alanyl (PL-12), glycyl (EJ), isoleucyl (OJ), asparaginyl (KS), phenylalanyl (ZO), and tyrosyl (YRS/HA) tRNA synthetases have been reported [14]. Anti-Jo-1 antibodies are the first MSA and anti-ARS described in 1980, defined by DID using calf thymus extract as antigen [4]. Anti-Jo-1 was detected in 30.8 % (8/26) of PM, 4.5 % each of DM (1/22), and overlap syndrome (1/22, 1/11 of PM-SSc overlap) but not in SLE, SSc, rheumatoid arthritis (RA), and other diseases. Arnett et al. confirmed disease specificity of anti-Jo-1 (30 % (6/20) in PM and 13 % (2/16) of DM) but did not find clinical features associated with anti-Jo-1 [31]. Yoshida et al. confirmed disease specificity of anti-Jo-1 in Japanese patients, finding in 28 % of PM/DM (9/32, 8 PM and 1 DM) and 2/28 overlap syndrome but none in SLE, SSc, and RA [32]. Importantly, they for the first time noted unique clinical features associated with anti-Jo-1, later known as anti-synthetase syndrome. All nine cases of anti-Jo-1 (+) PM/DM

Table 2 Target autoantigens of myositis-specific autoantibodies

Autoantibodies	Target molecule	Function	Protein	RNA
Aminoacyl tRNA synthetase				
Jo-1	Histidyl tRNA synthetase	Incorporate histidine into proteins	50 kD	$tRNA^{his}$
PL-7	Threonyl tRNA synthetase	Incorporate threonine into proteins	80 kD	$tRNA^{thr}$
PL-12	Alanyl tRNA synthetase	Alanine and aspartate biosynthesis and alanine incorporation into proteins	110 kD	$tRNA^{ala}$
EJ	Glycyl tRNA synthetase	Glycine, serine and threonine metabolism, and aminoacyl tRNA biosynthesis	75 kD	$tRNA^{gly}$
OJ	Isoleucyl tRNA synthetase	Incorporate isoleucine into proteins	150 kD, (multienzyme complex, 170, 130, 75 kD)	$tRNA^{iso}$
KS	Asparaginyl tRNA synthetase	Glutamate, alanine and aspartate metabolism	65 kD	$tRNA^{asp}$
ZO	Phenylalanyl tRNA synthetase	Incorporate phenylalanine into proteins	60/70 kD	$tRNA^{phen}$
YRS (HA)	Tyrosyl tRNA synthetase	Incorporate tyrosine into proteins	59 kD	$tRNA^{tyr}$
SRP	Signal Recognition Particle	Protein maturation in the ribosome	72, 68, 54, 19, 14, 9 kD	7SL RNA
Mi2	Helicase protein	Transcriptional regulation	240, 150, 72, 65, 63, 50 and 34 kD	–
MDA5 (CADM140)	MDA5 (melanoma differentiation-associated gene 5)	RNA-specific helicase that mediates the antiviral response	140 kD	–
TIF1γ/α (p155/140, TRIM33/TRIM24)	TIF1γ/α	Transcription and RNA metabolism	155 and 140 kD	–
TIF1β (TRIM28)	TIF1β	Transcription and RNA metabolism	120 kD	–
MJ/NXP-2	NXP2 (MORC3)	Transcriptional regulation and activation of the tumor suppressor p53	140 kD	–
SAE	Small ubiquitin-like modifier 1 (SUMO-1) activating enzyme	Post-translational modifications	90 and 40 kD	–

Modified from [30]

had ILD and polyarthritis, and hypocomplementemia and rheumatoid factor were also more common compared with anti-Jo-1-negative patients.

Following studies confirmed an association of anti-Jo-1 antibodies with a unique clinical subset characterized by myositis, ILD, arthritis, mechanic's hands, and Raynaud's

Table 3 Prevalence and clinical association of myositis autoantibodies

Autoantibodies	Prevalence (%)	Disease association	Clinical association/significance
Aminoacyl tRNA synthetases			
Jo-1	15–30	PM, DM	Anti-synthetase syndrome (myositis, ILD, polyarthritis, Raynaud's phenomenon, mechanic's hands)
PL-7	<5	PM, DM	Anti-synthetase syndrome
PL-12	<5	PM, DM, CADM, ILD	Anti-synthetase syndrome, ILD, CADM
EJ	<5	PM, DM	Anti-synthetase syndrome
OJ	<5	PM, DM	Anti-synthetase syndrome, ILD
KS	<1	PM, DM, ILD	ILD
ZO	Rare		Myositis
YRS (HA)	Rare		Myositis
SRP	5	PM	Myositis (necrotizing)
Mi2	10	DM	DM with typical skin lesions and mild myositis
MDA5/CADM140	15–20	CADM/ADM	CADM, rapidly progressive ILD, severe skin manifestations
TIF1γ/α	10–15	DM,	Malignancy-associated DM
MJ/NXP2	1–5	DM	Adult and juvenile DM with severe skin disease
SAE	1	DM	DM

Modified from [30]

PM polymyositis, *DM* dermatomyositis, *ILD* interstitial lung disease, *CADM* clinically amyopathic dermatomyositis, *ADM* amyopathic dermatomyositis

phenomenon [13]. Autoantibodies to the other seven ARS have been described using immunoprecipitation (IP) technique [14]. Interestingly, autoantibodies to all other aminoacyl tRNA synthetases are associated with the same syndrome, which has been designated anti-synthetase syndrome [13]. Anti-Jo-1 antibodies, usually found in 15–25 % of PM/DM patients, are by far the most common among anti-ARS antibodies; all others are usually found only in 0.5–6 % of patients [13, 18, 33–36]. The reported frequency of various anti-synthetase antibodies is very similar in all previous studies regardless of the race, ethnicity, or nationality of the subjects [18, 34–36], except for a report suggesting clustering of anti-PL-12 in southern USA [37] and high prevalence of anti-PL-7 in a Japanese cohort [38]. Therefore, whether there is any difference in clinical manifestations between patients with different anti-ARS autoantibodies has not been studied extensively. Nevertheless, some differences in clinical manifestations between different anti-synthetase antibodies have been described. Reports on anti-PL-12 and anti-KS suggested that they are common in ILD without myositis [15, 16, 37].

A high frequency (5/7, 71 %) of PM/DM-SSc overlap in anti-PL-7-positive Japanese patients was reported [39]. However, it was not confirmed by another Japanese study [38] and also was unusual in other studies [33]. Yamasaki et al. found anti-PL-7 in 17 % of their PM/DM cohort and reported an association with milder muscular involvement [38]. More recently, several studies reporting on differences in clinical features of different anti-ARS have been published. Kalluri et al. analyzed clinical features of 31 anti-PL-12-positive patients and reported that anti-PL-12 is strongly associated with ILD but less so with myositis and arthritis and 3/31 cases were idiopathic ILD [40], consistent with an earlier study [15, 37]. Labirua-Iturburu et al. reviewed their 18 cases of anti-PL-7-positive patients and found 50 % of patients had pericardial effusion, in addition to common features of anti-synthetase syndrome [41]. Marie et al. compared 75 anti-Jo-1 (+) patients vs. 20 anti-PL-7 (n=15)/PL-12 (n=5) (+) patients and reported that the latter had milder muscle involvement and less recurrence of muscle disease [42, 43]. In contrast, anti-PL-7/PL-12 was associated with early and severe ILD and gastrointestinal manifestations. Aggarwal et al. compared 122 anti-Jo-1 vs. 80 non-Jo-1 anti-ARS and reported that anti-Jo-1 (+) patients had more myositis (83 vs. 40 %) and less overlap/undifferentiated connective tissue disease (UCTD; 17 vs. 47.5 %) [44]. The non-Jo-1 group had delayed diagnosis and low survival rate. Hamaguchi et al. compared clinical features of patients with different anti-ARS and reported that while most patients with anti-Jo-1, EJ, and PL-7 had a diagnosis of PM/DM, it was CADM or ILD for anti-PL-12 and ILD for anti-KS and anti-OJ [45].

In summary, patients with any anti-ARS have a similar clinical syndrome known as anti-synthetase syndrome; however, several recent studies suggest that antibodies to non-Jo-1 ARS are associated with earlier and more severe ILD and poor prognosis compared with anti-Jo-1 (+) patients. Also, non-Jo-1 anti-ARS (+) patients are more likely to have ILD without typical myositis.

Anti-SRP

SRP antigen is a complex of 7SL RNA and several proteins including 72, 68, 54, 19, 14, and 9 kD, playing a role in regulating the translocation of proteins across the endoplasmic reticulum. Anti-SRP antibodies were originally described by IP in a patient with PM [46]. Another study also identified anti-SRP antibodies in two patients (a Japanese patient with typical PM and a patient with non-destructive arthritis). The former serum was described in an earlier study as immunoprecipitating 7.5S RNA [47], which was identified as 7SL RNA of the SRP complex [48]. The first large case series of anti-SRP-positive cases by Targoff et al. reported 4 % in PM/DM and 18 % of PM/DM patients with anti-cytoplasmic antibodies other than anti-Jo-1 from the analysis of 265 cases of PM/DM [49]. All 13 cases were PM and anti-SRP was associated with classic PM and some were unusually severe and/or rapid onset. Low prevalence of ILD, arthritis, and Raynaud's phenomenon were associated with this subset [49]. Hirakata et al. found anti-SRP in 4 % (3/52) of PM/DM and a case was DM [36]. Kao et al. reported 19 cases of anti-SRP from analysis of 134 PM, 129 DM, and other SARD (predominantly SSc, n=790). Anti-SRP was found in 16 cases of pure PM (12 % in PM, 16/134) and not in DM (0/129); however, three were without myositis (two with SSc and one with anti-synthetase syndrome) [50]. Hengstman et al. described 23 anti-SRP-positive cases, 20 PM and 3 DM. Muscle pathology of anti-SRP-positive patients was characterized by the presence of necrotic muscle fibers and no inflammatory infiltrates [51] similar to the findings by Miller et al. [52]. Takada et al. analyzed 23 anti-SRP-positive patients, 21 were PM (2 with RA), 3 DM, and 2 RA without myositis [53]. Clinical and pathological characteristics were consistent with other studies. Benveniste et al. reported correlation of anti-SRP antibody levels determined by a newly developed addressable laser bead assay (ALBIA) to one of the SRP components SRP54 and creatine kinase (CK) levels [54]. Other studies validated anti-SRP54 enzyme-linked immunosorbent assay (ELISA) vs. IP and showed a parallel change of anti-SRP54 levels and serum CK levels [55, 56]. Since anti-SRP antibody itself is not considered directly pathogenic, it is possible to consider the simple result of treatment with steroid, rituximab, and immunosuppressive agents for myositis have reduced both CK levels and anti-SRP antibody levels.

Although coexistence of more than one MSA in each patient is uncommon, there are a few reports on coexistence of anti-SRP and anti-ARS; two cases of anti-SRP with anti-Jo-1 [57, 58] and cases with anti-PL-12 were reported [50, 59].

MSA generally recognize protein components of the target antigen; however, one study reported that 50 % (5/10) of Japanese and 5 % (1/22) of North American patients with anti-SRP had antibodies directed against 7SL RNA [60].

Several recent studies focused on the association of anti-SRP antibodies with a unique histological subset of PM/DM, necrotizing myopathy [51, 55, 56]. Prevalence of necrotizing myopathy in patients with anti-SRP antibodies in the literature is summarized (Table 4). Suzuki et al. analyzed clinical features of 100 anti-SRP antibody-positive cases, selected based on IP of 7SL RNA [56]. Eighteen of them were IP positive but negative for anti-SRP54 ELISA. Histologically, 84 % had necrotizing myopathy while 14 % had non-specific myositis and one each had PM or DM pathology, supporting findings in other studies that a majority of anti-SRP-positive cases had necrotizing myopathy.

Necrotizing myopathy is a heterogeneous pathological category including autoimmune (autoantibody associated), drug-induced, paraneoplastic, viral infections, and others. A few studies started from necrotizing myopathy and examined the sensitivity of anti-SRP antibodies [52, 55, 61, 62]. Prevalence of anti-SRP antibodies in patients with necrotizing myopathy is summarized (Table 5). Anti-SRP antibody was the most frequent etiology that 53 % (34/64) of patients with necrotizing myopathy had [63]. In contrast, none of sera from 23 patients with necrotizing myopathy were tested positive for anti-SRP by line immunoassay (LIA) in another study [61]. In Chinese patients with PM/DM, 16/123 cases (13 %) with necrotizing myopathy had anti-SRP antibodies detected by LIA [62]. Thus, two studies using IP to detect anti-SRP antibodies showed prevalence of 41–52 % [55, 63] while the other two studies using LIA showed low prevalence of 0–13 % [62, 64] in patients with necrotizing myopathy. Whether the difference is due to genetic or environmental factors, selection bias of patients, or different immunoassay remains to be clarified.

In summary, the majority of literature support that anti-SRP is specific for PM and associated with treatment-resistant severe myopathy, which is histologically characterized by necrotizing myopathy [50–53, 62].

Table 5 Prevalence of anti-SRP antibodies in patients with necrotizing myopathy

Author	Country	Year	Necrotizing myopathy (*N*)	Prevalence of anti-SRP (%)	Method
Ellis [61]	Australia	2012	23	0	LIA
Wang [62]	China	2014	123	13	LIA
Suzuki [63]	Japan	2014	64	54	IP
Aggarwal [55]	USA	2015	64	41	IP

Anti-Mi-2

Autoantibodies to Mi-2 were originally defined by DID using calf thymus extract as antigen and reported as the first specific serologic marker of DM [5]. Eleven of 52 DM patients were positive by DID but none detected in PM though a few more sera were detected positive by ELISA in other diseases [5]. Love et al. reported anti-Mi-2 in 13 % (10/79) DM and 8 % (1/13) cancer-associated myositis but again none in PM, confirming specificity for DM [18]. Anti-Mi-2 was associated with classic DM skin rash, good response to steroid, and good prognosis. Components of Mi-2 antigen were characterized by IP and western blot (WB) [65] and later identified as nucleosome remodeling deacetylase complex (NuRD) [66].

All previous studies confirmed that anti-Mi-2 antibodies are nearly specific for DM when tested by DID or IP, though positive sera in PM may also be found in particular by ELISA [5, 67, 68] (Table 6). Clinical studies are consistent in showing that anti-Mi-2 is associated with classic features of DM including Gottron's papules, heliotrope rash, shawl sign, and V-sign, but a risk to develop clinically significant ILD is low and cancer is uncommon [5, 18, 68, 69]. This subset of patients responds well to steroid therapy and has a good prognosis [18]. However, clinical characteristics associated with anti-Mi-2 have not been studied extensively due to limited availability of the immunoassays and relatively low prevalence in PM/DM. One study from Mexico compared clinical features of anti-Mi-2 positive patients vs. others as the prevalence of anti-Mi-2 in this cohort was high (35 % in PM/DM, 45 % in DM) [77]. High CK level before treatment was noted

Table 4 Prevalence of necrotizing myopathy in anti-SRP-positive PM/DM

Author	Country	Year	Anti-SRP test method	*N*=(anti-SRP+)	Necrotizing myopathy (%)
Miller [52]	UK	2002	IP	7	100
Hengstman [51]	European countries	2006	IP/dot blot for 7SL RNA	15	(73?)
Takada [53]	Japan	2009	IP	11	(82?)
Aggarwal [55]	USA	2015	IP anti-SRP54 ELISA (12 % were negative)	26	92
Suzuki [56]	Japan	2015	IP anti-SRP54 ELISA (18 % were negative)	100	84

SRP signal recognition particle, *IP* immunoprecipitation, *ELISA* enzyme-linked immunosorbent assay

Table 6 Prevalence of anti-Mi-2 antibodies in adult PM/DM

Author	Country	Method	DM	PM
Targoff [5]	USA	DID	21 % (11/52)	0 % (0/58)
		ELISA	21 % (11/52)	3 % (2/58)
Love [18]	USA	DID, IP	13 % (10/79)	0 % (0/58)
Hausmanowa-Petrusewicz [69]	Poland	DID, IP	19 % (4/21)	0 % (0/19)
Roux [67]	France	ELISA	30 % (4/13)	10 % (1/10)
Brouwer [68]	Europe	ELISA	21 % (38/181)	9 % (17/198)
Komura [70]	Japan	IP	19 % (5/26)	0/9
Ghirardello [71]	Italy	ELISA	27 % (6/22)	0 % (0/21)
Rönnelid [72]	Sweden	LIA	8 % (4/50)	1 % (1/89)
Ghirardello [73]	Italy	LIA	12 % (8/65)	1 % (1/100)
		IP, WB	21 % (14/65)	1 % (1/100)
Hamaguchi [74]	Japan	IP	2 % (9/376)	0 % (0/34)
Ceribelli [75]	Italy	IP	5 % (1/27)	0 % (0/25)
Muro [76]	Japan	IP of TnT/TnT-ELISA	4 % (5/124)	NA
Petri [77]	Mexico	IP	45 % (27/61) (MX 59 %; GDL 12 %)	10 % (3/29)

DID double immunodiffusion, *ELISA* enzyme-linked immunosorbent assay, *IP* immunoprecipitation, *LIA* line immunoassay, *WB* western blot, *TnT* in vitro transcription/translation system, *MX* Mexico City, *GDL* Guadalajara, *NA* not available

in anti-Mi-2 (+) DM vs. anti-Mi-2 (−) DM (initial CK, >1000 IU/L, 100 vs. 52 %, $P<0.0001$; initial CK, >5000 IU/L, 54 vs. 14 %, $P<0.005$). However, anti-Mi-2 (+) patients responded well to steroid therapy and prevalence of normal CK at last visit was comparable between groups (45 vs. 69 %).

Although all studies showed the specificity of anti-Mi-2 for DM, reported prevalence of anti-Mi-2 in different studies is quite different even in the same country [77] as summarized in Table 6. Prevalence of anti-Mi-2 in DM varies 5–27 % in Italy and 2–19 % in Japan. In a study that examined the role of environmental factors in the production of anti-Mi-2, prevalence was as low as 3.2 % (Montreal, Canada), 3.7 % (Warsaw, Poland), and 5.4 % (Bethesda, USA) in some areas while it was 60 % (Guatemala City, Guatemala), 36.1 % (Mexico City, Mexico), and 23.1 % (Santiago, Chile) in Central and South American countries [78]. Another study also showed 59 % prevalence of anti-Mi-2 in DM patients in Mexico City, but it was only 12 % in Guadalajara [77], suggesting a role of factors other than ultraviolet (UV). Correlation of UV radiation of the area with development of DM and production of anti-Mi-2 antibodies was suggested [78, 79]. However, roles of genetic vs. environmental factors responsible for the production of anti-Mi-2 will need further studies because the majority of countries with very high prevalence of anti-Mi-2 are in Central and South America [77, 78].

Anti-MDA5/CADM140

It has been known for years that a subset of DM patients may have typical DM skin rash but have little or no muscle involvement, and these patients are called ADM or CADM [80, 81]. A subset of CADM patients may also develop RPILD, resistant to treatment and with poor prognosis. DM patients with these uncommon features did not express the classic MSAs or marker antibodies until Sato et al. described a new autoantibody called anti-CADM140 that is associated with CADM and ILD [10]. In this report, Sato et al. described 53 % (8/15) of Japanese CADM patients with anti-CADM140 antibodies, but none in 61 PM, 27 classic DM, other SARD, or idiopathic pulmonary fibrosis, thus this antibody is considered specific for CADM. Furthermore, 50 % (4/8) of anti-CADM140-positive DM had RPILD vs. 6 % (2/34) in anti-CADM140-negative DM. The target antigen was later identified as a cytoplasmic viral double-stranded RNA (dsRNA) receptor involved in innate immune response, called MDA5 or interferon induced with helicase C domain 1 (IFIH1) [9]. The possible mechanism leading to the autoimmune response is the binding of the viral dsRNA to MDA5 and the consequent induction of type I interferon responses. Another study from different institutes in Japan confirmed the strong association of anti-MDA5/CADM140 with CADM, RPILD, poor prognosis, high prevalence of liver dysfunction, and increased serum levels of ferritin [11, 12, 82–84]. In particular, Gono et al. reported that high ferritin levels are associated with poor prognosis for ferritin levels >1600 ng/ml [83]. No overlap with other SARD is present in DM patients with anti-MDA5/CADM140-positive antibodies, as shown by Hoshino et al., who reported that 65 % (20/31) of anti-MDA5/CADM140 positives were CADM and only one had another diagnosis (SSc) [11].

Anti-MDA5/CADM140 was also associated with ILD in JDM [85], and a few cases of apparent myositis were reported in anti-MDA5/CADM140-positive patients [12, 85].

Overall, studies on anti-MDA5/CADM140 in DM showed a prevalence ranging from 3 to 58 %, and this percentage increases up to 100 % when only CADM patients are considered. Moreover, prevalence and specificity of autoantibodies in PM/DM are quite different between studies from different countries or even within the same countries [38, 77], and this is true also for anti-MDA5/CADM140 antibodies. All earlier reports on anti-MDA5/CADM140 antibodies were on cohorts of DM patients in Asian countries, mainly Japan and South Korea [9–12, 83, 84, 86–88], except for some recent studies from the USA [89, 90]. In one of the two reports on anti-MDA5/CADM140 antibodies in cohorts of American DM patients, the authors also identified a unique cutaneous phenotype characterized by skin ulcerations, tender palmar papules, or both, and by severe arthritis [89]. In another US study, 6.9 % (11/160) of DM had anti-MDA5/CADM140 but 6/11 had overt clinical myopathy and 8/11 had ILD [90]. Their anti-MDA5/CADM140 patients were similar to anti-synthetase syndrome and were not associated with RPILD, in contrast to Asian studies.

There have been two recent studies from Europe. In a study from Italy, anti-MDA5/CADM140 antibodies were detected in 7 % (5/76) of adult European Caucasian patients with PM/DM, and they were the second most frequent specificity after anti-MJ antibodies (8/76, 11 %) [91]. All five anti-MDA5/CADM140 (+) patients had a diagnosis of DM, with CADM and normal CPK levels similar to reports from Asian countries [9], and a significantly higher prevalence of ILD compared with anti-MDA5/CADM140 (−) DM patients. Another study from Spain reported 12 % (14/117, 8 were CADM) prevalence of anti-MDA5/CADM140 in 117 DM patients [92]. Eight of 14 anti-MDA5/CADM140-positive patients had RPILD, similar to Asian cohorts [10] but different from US cohorts [90]. Anti-MDA5-positive patients with ILD had lower survival rates vs. anti-ARS-positive ILD. Among cutaneous manifestations, only panniculitis was significantly associated with anti-MDA5/CADM140. These differences in the prevalence and clinical features associated with anti-MDA5/CADM140 suggest the importance of accumulating data on prevalence and clinical association of MSAs from different ethnicities.

Muro et al. showed that anti-MDA5/CADM140 antibody levels can be related to disease activity, as they decrease and become negative by ELISA in nine of ten patients considered in remission after treatment, whereas the level of control anti-diphtheria toxoid DT antibodies did not change [87]. Sato et al. reported that anti-MDA5/CADM140 antibody levels in patients who responded to therapy and survived was significantly lower than the patients who did not respond and died [93]. These are very interesting findings as they suggest that anti-MDA5/CADM140 antibody levels may be used as a biomarker of disease activity and to predict response to therapy.

In summary, most reports describe that anti-MDA5/CADM140 antibodies are specific for DM and a majority of patients have CADM and high prevalence of RPILD leading to poor prognosis [9–11].

Anti-TIF1γ/α and β

Targoff et al. identified a new autoantibody called anti-p155/140 that immunoprecipitated a set of 155 and 140 kD proteins and has a striking association with cancer-associated DM [7]. P155 was identified as TIF1γ and published in an abstract [94]. Fujimoto et al. later confirmed p155 as TIF1γ and identified p140 as TIF1α [8] and anti-p155/140 has now been called anti-TIF1γ/α. In addition, anti-TIF1β was also identified in combination with anti-TIF1γ/α in some cases [8, 95]. TIF1α, TIF1β, and TIF1γ belong to the TIF family of transcription cofactors and are part of a tripartite motif superfamily (TRIM24, TRIM28, and TRIM33, respectively) [96].

In the original study, anti-p155/140 was found in 75 % (6/8) of patients with cancer-associated myositis though it was also detected in 29 % of JDM, 33 % of overlap syndrome, and 21 % of adult DM [7]. When the clinical features of anti-p155/140 patients were compared with those with anti-ARS, prevalence of fever, Raynaud's phenomenon, arthritis, ILD, and mechanic's hand was lower, in particular none of the 16 anti-p155/140 patients had ILD. V-sign rash, shawl sign rash, and malignancy was significantly higher in anti-p155/140 patients [94]. The association of anti-TIF1γ/α with DM, in particular with cancer-associated DM has been confirmed in many reports from the USA [7], UK [97], Spain [98], Japan [8, 11, 82, 99], and South Korea [100]. Almost all anti-TIF1γ/α-positive patients have DM with typical skin rash but low prevalence of ILD. Prevalence of anti-TIF1γ/α antibodies in adult inflammatory myopathy (Table 7) and association of anti-TIF1γ/α antibodies with malignancy (Table 8) in the literature are summarized. Prevalence of anti-TIF1γ/α in cancer-associated DM is 22–100 %, and all reported statistically significant associations of anti-TIF1γ/α with malignancy in DM (Table 7) except one study [101] that reported high prevalence of malignancy in overall DM. Prevalence of malignancy in anti-TIF1γ/α antibody-positive patients was 42–100 % (Table 8). Meta-analysis indicated sensitivity of anti-TIF1γ/α for cancer-associated DM as 0.50 to 1.00, combined 0.78 (95 % confidence interval (CI), 0.45–0.94), whereas specificity was 0.79–1.00, combined 0.89 (95 % CI, 0.82–0.93) [22]. However, it should be noted that association of anti-TIF1γ/α with cancer does not seem to apply to children [97] or young adults affected by DM [8].

Table 7 Prevalence of anti-TIF1γ/α antibodies in adult inflammatory myopathy

Author	Country (year)	DM (%)	PM (%)	Cancer-associated DM (%)	Overlap with other CTD (%)
Targoff [7]	USA (2006)	21	0	100	15
Kaji [99]	Japan (2007)	13	0	50	
Chinoy [27]	UK (2007)	18	0	53	0
Gunawardena [97]	UK (2008)	30	0	100	
Fujikawa [82]	Japan (2009)	17	NA	100	
Kang [100]	South Korea (2010)	21	0	56	
Trallero-Araguas [98]	Spain (2010)	23	5	71	
Hamaguchi [74]	Japan (2011)	7	0	44	0
Ikeda [101]	Japan (2011)	16	NA	22	
Fujimoto [8]	Japan (2012)	17			
Ceribelli [75]	Italy (2012)	7	0		17
Petri [77]	Mexico (2013)	16	0		

DM dermatomyositis, *PM* polymyositis, *CTD* connective tissue disease, *NA* not available

Anti-MJ/NXP-2

Anti-MJ antibodies recognize a ~140-kD nuclear protein called nuclear matrix protein 2 (NXP-2; also known as MORC3) [102–104], which plays an important role in diverse nuclear functions such as RNA metabolism and maintenance of nuclear architecture [102]. NXP-2 localizes in the promyelocytic leukemia (PML) nuclear bodies, where it recruits and activates p53 to induce cellular senescence [103, 105]. Anti-MJ/NXP-2 antibodies were originally described in 1997 in a subset of patients with JDM, who were characterized by severe refractory DM with polyarthritis, joint contractures, severe calcinosis, and intestinal vasculitis [106]. More than 10 years later, two studies in JDM were published [107, 108]. In the first study of a cohort of Argentine pediatric myositis patients, anti-MJ/NXP-2 antibodies were the most prevalent specificity (25 % of cases), associated with muscle contracture, atrophy, and significant compromise of the functional status [107]. In the other study, based on the JDM National Registry and Repository for UK and Ireland, 23 % prevalence of anti-MJ/NXP-2 in juvenile myositis patients was reported, and they were all JDM with significantly higher prevalence of calcinosis (54 vs. 15 % in anti-MJ-negative patients) [108].

Anti-MJ/NXP-2 antibodies in adult patients with myositis was first reported in a British cohort [109]. Anti-MJ/NXP-2 were found in 3 % (11/393) of PM/DM and 6 % in DM and none in PM. In anti-MJ/NXP-2-positive patients, typical DM skin rash was common and higher prevalence of ILD (64 vs. 28 % in anti-MJ/NXP-2-negative DM) was noted, but only one had calcinosis and none had cancer. This is in contrast to what was reported in another study of anti-MJ/NXP-2 antibodies in adult myositis patients in Japan. The authors estimate 1.6 % (8/507) prevalence of anti-MJ/NXP-2 [110], and they show higher prevalence (four of eight cases) of malignancy associated with this autoantibody [110]. A US study also reported anti-MJ/NXP-2 in 17 % (37/213) of adult DM patients and anti-MJ/NXP-2 was specifically associated with cancer in males (odd ratio, 5.78; 95 % confidence interval, 1.35–24.7) [111]. No similar finding on the association between anti-MJ/NXP-2 antibodies and higher risk of cancer was detected in other cohorts. A French-Canadian study reported

Table 8 Association of anti-TIF1γ/α antibodies with malignancy in adult DM

Author	Country (year (*n*))	Prevalence of anti-TIF1γ/α in cancer-DM vs. non-cancer DM	Prevalence of malignancy in anti-TIF1γ/α (+) DM vs. (−) DM
Targoff [7]	USA (2006 (45))	100 vs. 21 % (P=0.0004)	43 vs. 0 % (P=0.0004)
Kaji [99]	Japan (2007 (52))	50 vs. 4 % (P=0.0017)	71 vs. 11 % (P=0.0017)
Chinoy [27]	UK (2007 (103))	53 vs. 13 % (P=0.0009)	42 vs. 8 % (P=0.0009)
Gunawardena [97]	UK (2008 (20))	100 vs. 18 % (P=0.0175)	50 vs. 0 % (P=0.0175)
Fujikawa [82]	Japan (2009 (30))	100 vs. 0 % (P<0.0001)	100 vs. 0 % (P<0.0001)
Kang [100]	South Korea (2010 (38))	56 vs. 10 % (P=0.0101)	63 vs. 13 % (P=0.0101)
Trallero-Araguas [98]	Spain 2010 (65)	71 vs. 10 % (P<0.0001)	67 vs. 8 % (P<0.0001)
Hamaguchi [74]	Japan (2011 (376))	44 vs. 2 % (P<0.0001)	68 vs. 6 % (P<0.0001)
Ikeda [101]	Japan (2011 (55))	22 vs. 14 % (ns)	44 vs. 30 % (ns)

anti-MJ/NXP-2 in 8 % (2/26) of adult DM cases but none in PM [112]. Another recent study in adult DM patients in the USA reported anti-MJ/NXP-2 in 13 % (16/126) of cases and its association with calcinosis (odds ratio, 15.52; 95 % CI, 2.01–119.90) in this adult cohort [113]. In an Italian study [75], anti-MJ/NXP-2 antibodies were the most prevalent specificity (30 % in DM and 17 % in PM/DM) detected at a prevalence similar to the one observed in JDM Argentinian [107] and UK/Ireland [108] cohorts but much higher than other adult DM studies [109, 110]. Lack of malignancy in anti-MJ/NXP-2-positive patients in the Italian cohort [75] may be related to their young age compared with anti-MJ/NXP-2 (+) patients with malignancy in other cohorts [110]. This may be similar to a strong association of anti-TIF1γ/α antibodies with malignancy in middle to old age DM but not in children or young adults [8, 97].

As shown by these reports, prevalence of anti-MJ/NXP-2 antibodies can be very different in studies performed worldwide, and this could be due to different ethnic background, influence of environmental factors on autoantibody production, or simply for technical reasons. Genetic and/or environmental factors within Caucasians may be important variables, since the prevalence of anti-MJ/NXP-2 within Caucasians seems different.

Anti-SAE

Antibodies to SAE were first identified by Betteridge et al. in 2007 in two DM patients [114]. The target antigens of 40 and 90 kD heterodimer proteins were identified as small ubiquitin-like modifier-activating enzyme A subunit (SAE1) and the SUMO-1 activating enzyme B subunit (SAE2), respectively. These are enzymes involved in the post-translational modification of specific proteins known as SUMOylation. Anti-SAE was found in 10 % (2/20) DM but none in 24 PM patients. In the following study in patients recruited to the Adult Onset Myositis Immunogenetic Collaboration, anti-SAE was found in 4 % (11/266) of PM/DM and 8 % in DM as all anti-SAE positives were DM patients [115]. Among 11 patients with anti-SAE, a high frequency of cutaneous lesions including heliotrope (82 %) and Gottron rash (82 %) were identified. Nine of the 11 patients had systemic features (82 %), and dysphagia was noted in 78 % (seven of nine). A majority (78 %) of them presented with skin disease prior to onset of myositis. There are only a few reports from other countries. A study from Italy also found anti-SAE in 7 % (5/73) of DM patients [116]. Prevalence appears low in Japanese as Fujimoto et al. found 1.5 % (7/456) [117] and Muro et al. reported 1.8 % (2/110) prevalence [118] in Japanese DM patients. Clinical features in seven Japanese anti-SAE-positive patients were similar to those in a UK study except that ILD was common in Japanese patients (71 %) [117] vs. 18 % in the UK study ($P<0.05$) [115].

Immunoprecipitation Detection of Myositis-Specific Autoantibodies

IP analysis of protein components of autoantigens using ^{35}S-methionine-labeled cell extract is a very powerful technique that

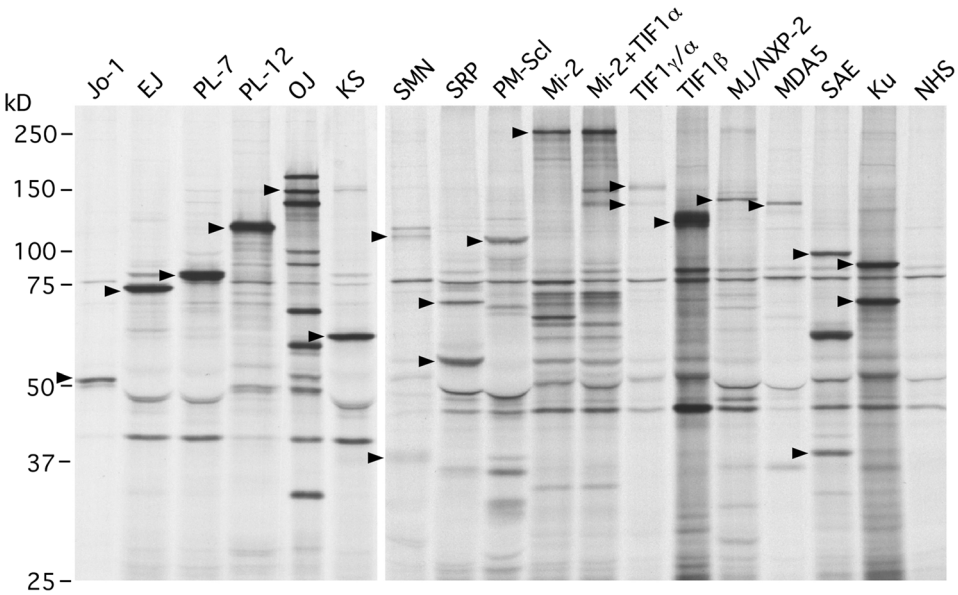

Fig. 2 Immunoprecipitation analysis of protein components of autoantigens recognized by autoantibodies in PM/DM. ^{35}S-methionine-labeled K562 cell extract was immunoprecipitated by sera from patients with PM/DM. Main components of each autoantigen are indicated by *arrowheads*. *Jo-1* histidyl tRNA synthetase, *EJ* glycyl tRNA synthetase, *PL-7* threonyl tRNA synthetase, *PL-12* alanyl tRNA synthetase, *OJ* isoleucyl tRNA synthetase, multienzyme complex, *KS* asparaginyl tRNA synthetase, *SMN* survival of motor neuron, *SRP* signal recognition particle, *PM-Scl* polymyositis-scleroderma, Mi-2, Mi-2+, TIF1α, *TIF1γ/α* transcription intermediary factor 1α, *TIF1β* transcription intermediary factor 1β, *MJ/NXP-2* nuclear matrix protein 2, *MDA5* melanoma differentiation-associated gene 5, *SAE* small ubiquitin-like modifier 1 (SUMO-1) activating enzyme, Ku, *NHS* normal human serum

allows screening for almost all known PM/DM autoantibodies in a single assay. Combining the protein analysis with the analysis of RNA components of the autoantigens by urea-PAGE and silver staining is useful for confirmation of RNA-protein complex. Protein IP of MSA and other autoantibodies seen in PM/DM is shown (Fig. 2). Molecular weights of the target antigens are summarized in Table 2. Many myositis autoantibodies can be interpreted almost conclusively while some may require additional techniques for confirmation [1]. Identifying a multiprotein or multiprotein-nucleic acid complex characterized by a set of proteins, such as anti-OJ, SRP, Mi-2, TIF1γ/α, and SAE is usually not so difficult though IP of unrelated proteins is relatively common in certain molecular weight ranges.

Among MSA listed in Table 3, anti-Jo-1 and Mi-2 were originally defined by DID while all others were defined by IP analysis of proteins, in combination with analysis of RNA components by IP for anti-ARS and anti-SRP. IP is still considered a gold standard for most of them. An apparent weakness of IP is anti-Jo-1, as the Jo-1 antigen is seen as a relatively thin uncharacteristic band in IP, in contrast to other ARS. In addition, it comigrates with IgG heavy chain, making it more difficult to clearly observe. IP analysis of RNA component to confirm the presence of tRNA is helpful; however, it does not tell the tRNA specificity; it only tells immunoprecipitation of "some" tRNA and cannot confirm if it is histidyl tRNA or not. Practically, IP of ~50 kD protein consistent with Jo-1 and the presence of tRNA by RNA analysis is reasonable to strongly suggest it is Jo-1. IP of ~50 kD protein and positive anti-Jo-1 ELISA also is practical.

IP of ~140 kD protein that exactly comigrates with MDA5 or MJ/NXP-2 immunoprecipitated by a reference serum is reasonable and correctly interpreted in most cases. Nevertheless, it will be ideal to confirm the specificity of an uncharacteristic single band such as MDA5 and MJ/NXP-2 with additional test such as ELISA, western blot, or IP-western blot. IP and ELISA using biotinylated in vitro transcription/translation product [11] and IP of ^{35}S-methionine-labeled in vitro transcription/translation product [113] have also been used for these antibody specificities.

SRP72/68, SRP54, SAE1 (40 kD), and SAE2 (90 kD) are confirmed by IP in most cases but there are many proteins of similar size recognized by human sera, and confirmation of exact comigration of the proteins compared with a reference serum may be necessary. For anti-SRP, confirmation by coimmunoprecipitation of small subunits of 19, 14, and 9 kD appears more characteristic than identifying SRP72/68 and SRP54.

Immunofluorescence ANA Staining by Myositis Autoantibodies

Some of the representative immunofluorescence ANA-staining patterns by autoantibodies seen in PM/DM using HEp-2 cells are shown (Fig. 3). Anti-U1RNP antibodies show a coarse speckled pattern (Fig. 3a). Anti-Mi-2 (Fig. 3b), anti-TIF1γ/α (Fig. 3c), and anti-TIF1β (Fig. 3d) all show fine speckled nuclear staining, and the difference in staining pattern is not apparent though anti-Mi-2-positive sera show brighter staining in general. Anti-SAE also shows fine speckled nuclear staining (Fig. 3e). PML body staining is clearly observed by some anti-MJ/NXP-2-positive sera (Fig. 3f) [75], but it may not be always clear and only nuclear fine speckled staining may be observed by some sera (Fig. 3g). Anti-SMN stains a few nuclear dots known as Cajal bodies (Fig. 3h). Anti-PM-Scl antibodies show homogenous nucleolar staining with fine speckled nuclear staining (Fig. 3i, j) though nucleolar staining may be less clear in some cases (Fig. 3j). Anti-U3RNP antibodies show clumpy nucleolar staining (Fig. 3k). Antibodies to Jo-1 (Fig. 3l), PL-7 (Fig. 3m), PL-12 (Fig. 3n), and other ARS stain cytoplasm in a fine speckled pattern but the staining may be weak or absent in some cases. Anti-SRP antibodies show fine speckled cytoplasmic staining (Fig. 3o). MDA5 antigen localizes to the cytoplasm and positive sera may stain the cytoplasm but it is often very weak or negative (Fig. 3p)

Immunofluorescence ANA Pattern and Autoantibody Specificities in PM/DM

IP is a gold standard for detection of the majority of MSA; however, this technique is available only at limited research laboratories. ELISA for anti-Jo-1 and U1RNP are widely available and anti-ARS ELISA that detects anti-Jo-1, EJ, PL-7, PL-12, and KS is available in Japan; mixed antigens are used, and individual specificity is not available [17]. Line immunoassay and other new types of assays have been used for some of the MSA; however, validation of the assay compared with a gold standard is limited and discrepancies in test results have been reported [119]. Thus, being familiar with the localization of the target antigens and immunofluorescence ANA pattern can be useful in two ways. First, specificities of autoantibodies that have higher chance of positivity for a particular patient can be considered when clinicians order specific autoantibody tests selected based on immunofluorescence pattern. Second, when specific autoantibody test result is inconsistent with the ANA pattern, false-positive test result may be considered, e.g. positive anti-Mi-2 with cytoplasmic staining can be considered inconsistent [1].

ANA pattern and corresponding myositis autoantibody specificities are summarized (Fig. 4). Anti-U1RNP, anti-U1/U2RNP, and anti-Ku are considered for a serum with coarse nuclear speckled pattern. For fine nuclear speckled pattern, anti-Mi-2, TIF1γ/α, and anti-SAE may be considered. Staining patterns by anti-Mi-2 and TIF1γ/α are indistinguishable though anti-Mi-2 sera usually show brighter staining. Multiple nuclear dots (PML) pattern is consistent with anti-MJ/NXP-2 staining though this may not be apparent in all cases. Some sera may show nuclear fine speckled pattern without PML body staining [75]. Cajal body staining can be seen by anti-SMN (survival of motor

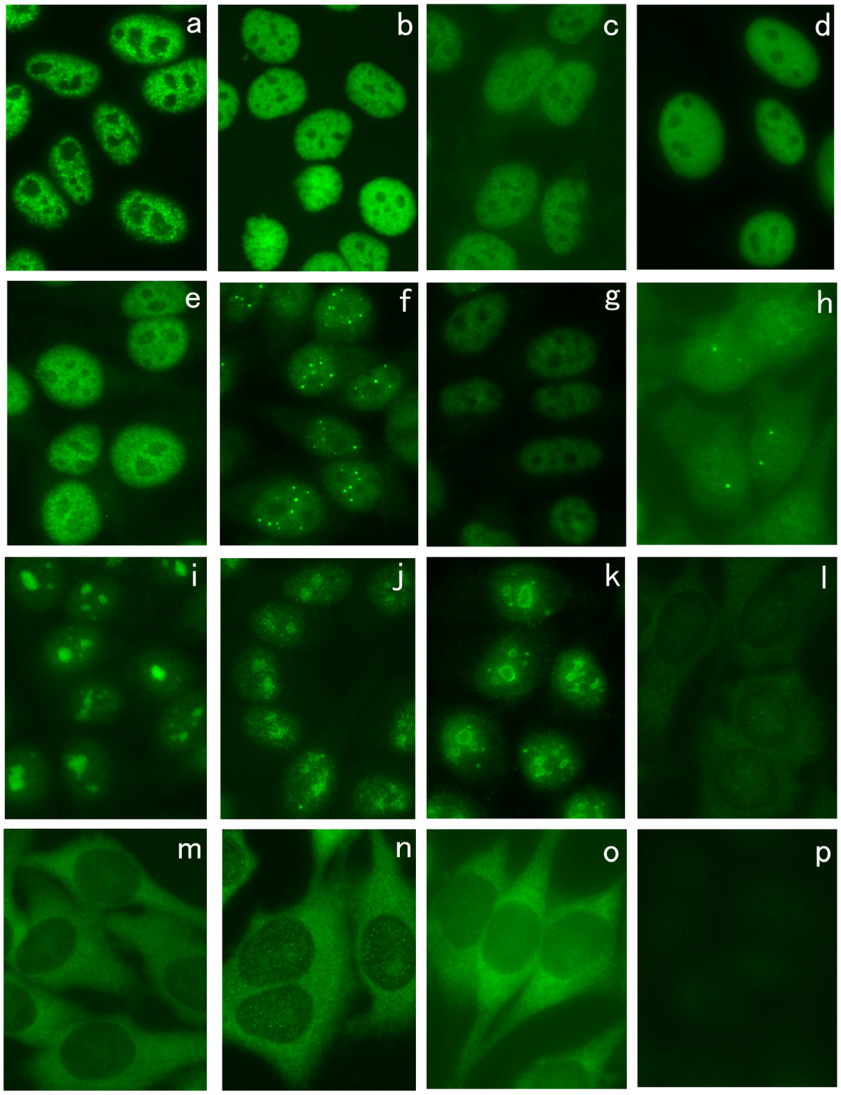

Fig. 3 Immunofluorescence antinuclear antibodies using sera from patients with PM/DM. HEp-2 ANA slides were stained using sera from patients with PM/DM. **a** Anti-U1RNP, **b** anti-Mi-2, **c** anti-TIF1γ/α, **d** anti-TIF1β, **e** anti-SAE, **f**, **g** anti-MJ/NXP-2, **h** anti-SMN, **i**, **j** anti-PM-Scl, **k** anti-U3RNP, **l** anti-Jo-1, **m** anti-PL-7, **n** anti-PL-12, **m** anti-SRP, **p** anti-MDA5

neuron) antibodies found in PM or PM overlap syndrome [120]. Nucleolar stainings associated with myositis are homogeneous staining by anti-PM-Scl and clumpy staining by anti-U3RNP antibodies. Strong cytoplasmic staining is consistent with the presence of anti-ARS or SRP. Although ARS localize in the cytoplasm, staining may be weak or absent. MDA5 antigen localizes to the cytoplasm but cytoplasmic staining is often weak or absent.

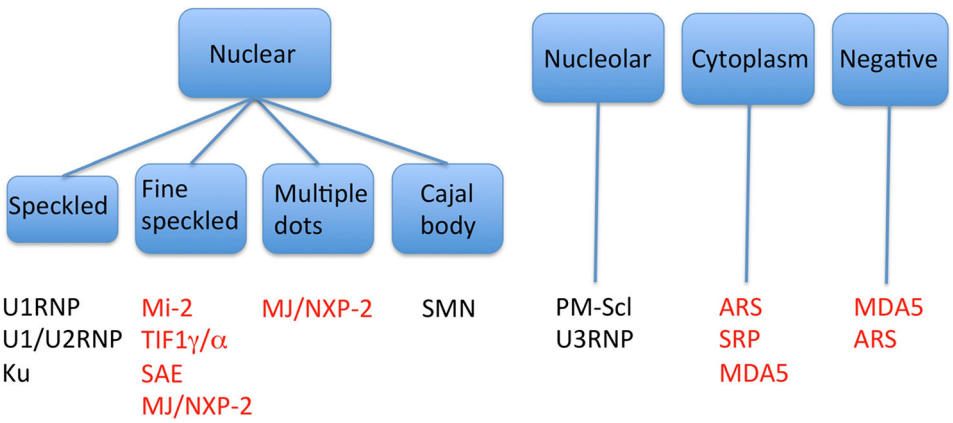

Fig. 4 Summary of HEp-2 cell immunofluorescence patterns corresponding to different autoantibody specificities in PM/DM

Future Direction of MSA Testing

IP is a very powerful and reliable technique that has been used and considered a reliable assay over 30 years, but it has been performed only at a limited number of laboratories and never become a routine assay in clinical practice. Currently, anti-Jo-1 ELISA is the only widely available testing for most clinicians. A new ELISA of anti-ARS to detect antibodies to Jo-1, EJ, PL-7, PL-12, and KS in a single ELISA using a mixture of these antigens has been validated compared with IP and released recently in Japan [17]. Line immunoassay is available in certain countries but has not been used extensively. ELISA and beads assays for several MSAs, in particular the ones with high clinical significance such as anti-TIF1γ and MDA5 are currently under development. They will be widely available in the near future and tests for these autoantibodies will become a part of standard tests in clinical practice of inflammatory myopathy.

While wide availability of new autoantibody immunoassays will be definitely welcomed, performance of new immunoassays without validation is a concern [119]. New immunoassays should be validated against a gold standard such as IP and DID before releasing to the market as performed for some new assays [17] to avoid confusion among clinicians and researchers [119].

Cryptic Epitopes and Mechanisms of MSA Production

Autoantibodies in each PM/DM patient target only a few proteins or protein-RNA complexes. Several autoantibodies are considered MSA; however, presence of more than one MSA in the same patient is uncommon. Patients with antibodies to any ARS could have similar clinical features, known as antisynthetase syndrome; however, detection of antibodies to more than one ARS in each patient is rare for unknown reasons. Thus, it is assumed that there are mechanisms, which select the target antigens of MSA out of thousands of proteins in cells in each patient. We would like to discuss a possibility that certain MSAs, in particular cancer-associated autoantibodies, are triggered by formation of cryptic epitopes resulting from a mutation of the target antigens.

It has been speculated that quantitative (e.g., upregulation or reduced degradation of certain proteins) or qualitative changes (e.g., mutation, aberrant post-translational modification, unusual interaction with other proteins, etc.) or unusual location (e.g., translocation of nuclear proteins to the cell surface) may trigger specific autoimmune responses. A concept of "cryptic epitopes" may be important to understand this idea [121]. Extracellular antigens are typically processed via endosomes and the resulting peptides are presented on MHC class II at the surface of antigen-presenting cells (APCs), whereas intracellular antigens are processed via the proteasomes and the peptides presented on MHC class I. Whether the MHC-peptide complex is recognized as non-self is a critical step toward triggering autoimmune response. Mutation of amino acids can change the pattern of protein digestion and create cryptic epitopes presented on the cell surface MHC. Autoreactive T cells are deleted during T cell development; however, immunological tolerance to epitopes that have little or no expression during this process may be incomplete. When the cryptic epitopes are expressed on APCs in the body in certain conditions, it could trigger autoimmune response. It is not a new concept that changes in the structure or expression levels of certain self-proteins, occurring during tumorigenesis for example, may be associated with triggering autoimmune responses [122].

Perhaps the best-known example is an autoimmune response to a tumor suppressor gene p53 in patients with malignancy. In individuals with a mutation of the tumor suppressor gene p53, loss of normal p53 functions due to amino acid replacement may lead to a development of cancer while the mutated p53 can create cryptic epitopes (Fig. 5), which is recognized as non-self by the immune system and triggers autoantibodies to p53. The immune system is sensitive to detect a single amino acid change and accompanying conformational changes resulting from a point mutation in p53. Autoantibodies to p53 have been reported in patients with

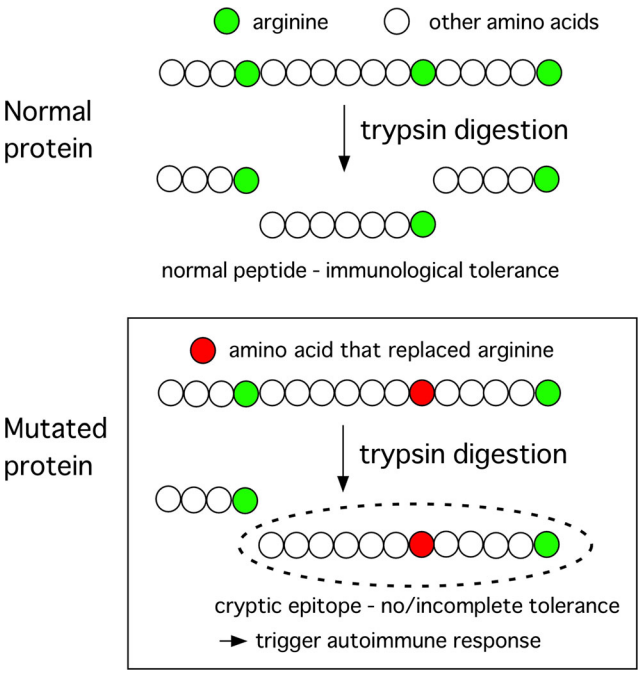

Fig. 5 Formation of cryptic epitopes via a somatic mutation. A somatic mutation that causes amino acid replacement may create cryptic epitopes, which can be recognized as non-self and trigger autoimmune response. *Top*, normal protein digested by trypsin makes normal peptides that are supposed to have immunological tolerance. *Bottom*, if arginine is replaced by other amino acid, trypsin digestion may create cryptic epitopes that have no or incomplete immunological tolerance and trigger autoimmune response

various types of cancer that are associated with certain p53 mutations [123, 124]. Recently, an association of POL3A mutation and production of anti-RNA polymerase III antibodies in SSc patients with malignancy was reported [125]. Six of 8 anti-RNAPIII-positive cancer-SSc patients had somatic mutation or loss of heterozygosity, which suggest the presence of mutation. In contrast, these changes were not observed in SSc patients with cancer in six anti-centromere and six anti-topo I-positive patients. Thus, the mechanism of production of anti-RNAPIII in these patients may be exactly same as that of anti-p53 antibody.

Autoantibodies to TIF-1γ/α have been described recently in strong association with cancer-associated DM [7, 97–99]. Based on the effects of TIF-1γ as a tumor suppressor reported in murine models and humans [96, 126, 127], it seems reasonable to hypothesize that the mechanisms of production of anti-TIF-1γ in patients with cancer-associated DM could be similar to what is speculated in patients with p53-mutated cancer; TIF-1γ mutation could be the primary event that leads to the development of cancer and production of autoantibodies to TIF-1γ (Fig. 6). DM in these patients may be considered as a paraneoplastic syndrome. Roles of TIF-1γ in viral infection and its interactions with viral proteins have been reported [128, 129]. TIF-1α, TIF-1β, and TIF-1γ are identified as adenoviral E1B-55K-binding proteins. TIF-1γ is shown to be a target for degradation by human adenoviruses and it possesses anti-viral activity and limits adenovirus early and late gene product expression during infection [128]. In a mouse model of autoimmune response to p53, it was shown that immunization of viral SVT/self p53 complex induced anti-p53 autoimmune response while immunization of p53 alone did not [130]. One possible explanation was that interaction of viral protein with self p53 modified antigen processing, leading to production of cryptic epitopes. A possible role of viral infection in the pathogenesis of JDM has been discussed for many years without solid and universal evidence. It is tempting to speculate that viral infection, which may create cryptic epitopes of TIF-1γ/α, is the primary event in certain cases of JDM or young adult patients with anti-TIF-1γ/α.

It is also possible that de novo mutations of a gene for other MSA antigens, which occur somewhere in the body, lead to production of autoantibodies. A difference from the p53 model and difficulty when trying to prove or disprove this hypothesis will be the location to test for the mutations in MSA antigens not associated with malignancy. While locating the p53 mutation in the body is relatively easy in a majority of cases, usually at the site of cancer, it will not be easy to locate the site of mutation of genes for MSA antigens in PM/DM without malignancy [131].

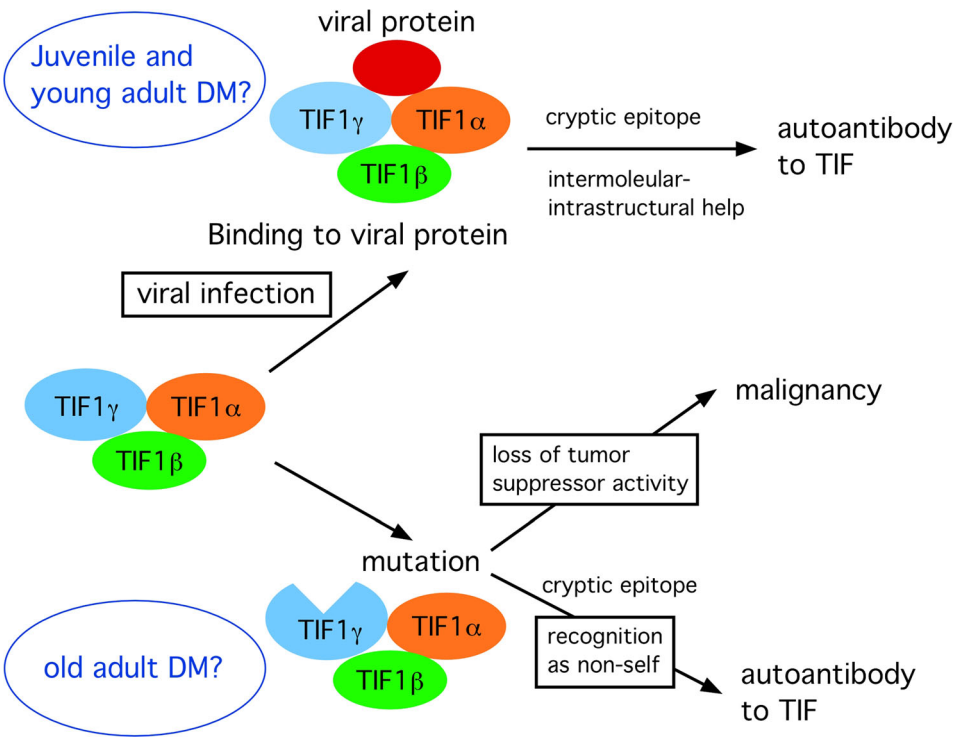

Fig. 6 Hypothesis on the production of anti-TIF1γ/α antibodies based on mutation of TIF1γ or interaction of viral proteins with TIF1. In old adult DM patients with malignancy, TIF1γ mutation may allow development of malignancy while the mutated protein may also trigger autoimmune response to TIF1γ. In JDM or young adult DM patients, interaction of viral proteins with TIF1 proteins may create cryptic epitopes, leading to the autoimmune response

Conclusions

MSA are highly specific for the diagnosis of PM/DM, and many of them are also associated with a unique clinical subset of PM/DM, making them clinically useful biomarkers. There has been significant progress in MSA in the last 10 years and several new MSA with strong clinical significance have been identified. New immunoassays for these new MSA as well as classic MSA will become widely available for clinical practice in the near future. Clinicians are expected to know and understand the significance of MSA and proper use in clinical practice.

ARS, aminoacyl tRNA synthetases;
ALBIA, addressable laser bead assay;
CIE, counter immunoelectrophoresis;
CK, creatine kinase;
CTD, connective tissue disease;
DID, double immunodiffusion;
DM, dermatomyositis;
ELISA, enzyme-linked immunosorbent assay;
ENA, extractable nuclear antigen;
ILD, interstitial lung disease ;
IP, immunoprecipitation;
JDM, juvenile dermatomyositis;
LIA, line immunoassay;
MAA, myositis-associated autoantibodies;
MCTD, mixed connective tissue disease;
MDA5, melanoma differentiation associated gene 5;
MSA, myositis-specific autoantibodies;
NHS, normal human serum;
NXP-2, nuclear matrix protein 2;
PM, polymyositis;RNP, ribonucleoprotein;
PML, promyelocytic leukemia;
RA, rheumatoid arthritis;
RPILD, rapidly progressive interstitial lung disease;
SAE, small ubiquitin-like modifier activating enzyme;
SARD, systemic autoimmune rheumatic diseases;
SRP, signal recognition particle;
SSc, scleroderma;
SUMO, small ubiquitin-like modifier;
TIF1, transcription intermediary factor 1;
UCTD, undifferentiated connective tissue disease;
UsnRNPs, U small nuclear ribonucleoproteins;

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References

1. Satoh M, Chan EKL, Sobel ES, Kimpel DL, Yamasaki Y, Narain S, Mansoor R, Reeves WH (2007) Clinical implication of autoantibodies in patients with systemic rheumatic diseases. Expert Rev Clin Immunol 3:721–738
2. Satoh M, Vazquez-Del Mercado M, Chan EK (2009) Clinical interpretation of antinuclear antibody tests in systemic rheumatic diseases. Mod Rheumatol 19:219–228
3. Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA, Harley JB (2003) Development of autoantibodies before the clinical onset of systemic lupus erythematosus. N Engl J Med 349:1526–1533
4. Nishikai M, Reichlin M (1980) Heterogeneity of precipitating antibodies in polymyositis and dermatomyositis. Characterization of the Jo-1 antibody system. Arthritis Rheum 23:881–888
5. Targoff IN, Reichlin M (1985) The association between Mi-2 antibodies and dermatomyositis. Arthritis Rheum 28:796–803
6. Targoff IN (1992) Autoantibodies in polymyositis. Rheum Dis Clin N Am 18:455–482
7. Targoff IN, Mamyrova G, Trieu EP, Perurena O, Koneru B, O'Hanlon TP, Miller FW, Rider LG (2006) A novel autoantibody to a 155-kD protein is associated with dermatomyositis. Arthritis Rheum 54:3682–3689
8. Fujimoto M, Hamaguchi Y, Kaji K, Matsushita T, Ichimura Y, Kodera M, Ishiguro N, Ueda-Hayakawa I, Asano Y, Ogawa F et al (2012) Myositis-specific anti-155/140 autoantibodies target transcriptional intermediary factor 1 family proteins. Arthritis Rheum 64:513–522
9. Sato S, Hoshino K, Satoh T, Fujita T, Kawakami Y, Kuwana M (2009) RNA helicase encoded by melanoma differentiation-associated gene 5 is a major autoantigen in patients with clinically amyopathic dermatomyositis: association with rapidly progressive interstitial lung disease. Arthritis Rheum 60:2193–2200
10. Sato S, Hirakata M, Kuwana M, Suwa A, Inada S, Mimori T, Nishikawa T, Oddis CV, Ikeda Y (2005) Autoantibodies to a 140-kD polypeptide, CADM-140, in Japanese patients with clinically amyopathic dermatomyositis. Arthritis Rheum 52: 1571–1576
11. Hoshino K, Muro Y, Sugiura K, Tomita Y, Nakashima R, Mimori T (2010) Anti-MDA5 and anti-TIF1-{gamma} antibodies have clinical significance for patients with dermatomyositis. Rheumatology (Oxford) 49:1726–1733
12. Nakashima R, Imura Y, Kobayashi S, Yukawa N, Yoshifuji H, Nojima T, Kawabata D, Ohmura K, Usui T, Fujii T et al (2010) The RIG-I-like receptor IFIH1/MDA5 is a dermatomyositis-specific autoantigen identified by the anti-CADM-140 antibody. Rheumatology (Oxford) 49:433–440
13. Targoff IN (2000) Update on myositis-specific and myositis-associated autoantibodies. Curr Opin Rheumatol 12:475–481
14. Nakashima R, Mimori T (2010) Clinical and pathophysiological significance of myositis-specific and myositis-associated autoantibodies. Int J Clin Rheumatol 5:523–536
15. Targoff IN, Arnett FC (1990) Clinical manifestations in patients with antibody to PL-12 antigen (alanyl-tRNA synthetase). Am J Med 88:241–251
16. Hirakata M, Suwa A, Nagai S, Kron MA, Trieu EP, Mimori T, Akizuki M, Targoff IN (1999) Anti-KS: identification of autoantibodies to asparaginyl-transfer RNA synthetase associated with interstitial lung disease. J Immunol 162:2315–2320
17. Nakashima R, Imura Y, Hosono Y, Seto M, Murakami A, Watanabe K, Handa T, Mishima M, Hirakata M, Takeuchi T et al (2014) The multicenter study of a new assay for simultaneous detection of multiple anti-aminoacyl-tRNA synthetases in myositis and interstitial pneumonia. PLoS ONE 9:e85062
18. Love LA, Leff RL, Fraser DD, Targoff IN, Dalakas M, Plotz PH, Miller FW (1991) A new approach to the classification of idiopathic inflammatory myopathy: myositis-specific autoantibodies

define useful homogeneous patient groups. Medicine (Baltimore) 70:360–374
19. Targoff IN, Miller FW, Medsger TA Jr, Oddis CV (1997) Classification criteria for the idiopathic inflammatory myopathies. Curr Opin Rheumatol 9:527–535
20. Satoh M, Chan EK, Ho LA, Rose KM, Parks CG, Cohn RD, Jusko TA, Walker NJ, Germolec DR, Whitt IZ et al (2012) Prevalence and sociodemographic correlates of antinuclear antibodies in the United States. Arthritis Rheum 64:2319–2327
21. Rider LG, Shah M, Mamyrova G, Huber AM, Rice MM, Targoff IN, Miller FW (2013) The myositis autoantibody phenotypes of the juvenile idiopathic inflammatory myopathies. Medicine (Baltimore) 92:223–243
22. Trallero-Araguas E, Rodrigo-Pendas JA, Selva-O'Callaghan A, Martinez-Gomez X, Bosch X, Labrador-Horrillo M, Grau-Junyent JM, Vilardell-Tarres M (2012) Usefulness of anti-p155 autoantibody for diagnosing cancer-associated dermatomyositis: a systematic review and meta-analysis. Arthritis Rheum 64:523–532
23. Frank MB, McCubbin V, Trieu E, Wu Y, Isenberg DA, Targoff IN (1999) The association of anti-Ro52 autoantibodies with myositis and scleroderma autoantibodies. J Autoimmun 12:137–142
24. Selva-O'Callaghan A, Labrador-Horrillo M, Solans-Laque R, Simeon-Aznar CP, Martinez-Gomez X, Vilardell-Tarres M (2006) Myositis-specific and myositis-associated antibodies in a series of eighty-eight Mediterranean patients with idiopathic inflammatory myopathy. Arthritis Rheum 55:791–798
25. Ghirardello A, Zampieri S, Tarricone E, Iaccarino L, Bendo R, Briani C, Rondinone R, Sarzi-Puttini P, Todesco S, Doria A (2006) Clinical implications of autoantibody screening in patients with autoimmune myositis. Autoimmunity 39:217–221
26. Vancsa A, Gergely L, Ponyi A, Lakos G, Nemeth J, Szodoray P, Danko K (2010) Myositis-specific and myositis-associated antibodies in overlap myositis in comparison to primary dermatopolymyositis: relevance for clinical classification: retrospective study of 169 patients. Joint Bone Spine 77:125–130
27. Chinoy H, Fertig N, Oddis CV, Ollier WE, Cooper RG (2007) The diagnostic utility of myositis autoantibody testing for predicting the risk of cancer-associated myositis. Ann Rheum Dis 66:1345–1349
28. Gunawardena H, Betteridge ZE, McHugh NJ (2009) Myositis-specific autoantibodies: their clinical and pathogenic significance in disease expression. Rheumatology (Oxford) 48:607–612
29. Satoh M, Chan JY, Ceribelli A, Del-Mercado MV, Chan EK (2013) Autoantibodies to argonaute 2 (su antigen). Adv Exp Med Biol 768:45–59
30. Satoh M, Ceribelli A, Hirakata M, Chan EKL (2015) Immunodiagnosis of autoimmune myopathies. In: Detrick B, Hamilton RG, Schmitz JL (eds) Manual of molecular and clinical laboratory immunology, 8th edn. ASM Press, Washington, D. C, **In Press**
31. Arnett FC, Hirsch TJ, Bias WB, Nishikai M, Reichlin M (1981) The Jo-1 antibody system in myositis: relationships to clinical features and HLA. J Rheumatol 8:925–930
32. Yoshida S, Akizuki M, Mimori T, Yamagata H, Inada S, Homma M (1983) The precipitating antibody to an acidic nuclear protein antigen, the Jo-1, in connective tissue diseases. Arthritis Rheum 26:604–611
33. Targoff IN, Arnett FC, Reichlin M (1988) Antibody to threonyl-transfer RNA synthetase in myositis sera. Arthritis Rheum 31: 515–524
34. Arnett FC, Targoff IN, Mimori T, Goldstein R, Warner NB, Reveille JD (1996) Interrelationship of major histocompatibility complex class II alleles and autoantibodies in four ethnic groups with various forms of myositis. Arthritis Rheum 39:1507–1518
35. Bernstein RM, Bunn CC, Hughes GRV, Francoeur AM, Mathews MB (1984) Cellular protein and RNA antigens in autoimmune disease. Mol Biol Med 2:105–120
36. Hirakata M, Mimori T, Akizuki M, Craft J, Hardin JA, Homma M (1992) Autoantibodies to small nuclear and cytoplasmic ribonucleoproteins in Japanese patients with inflammatory muscle disease. Arthritis Rheum 35:449–456
37. Friedman AW, Targoff IN, Arnett FC (1996) Interstitial lung disease with autoantibodies against aminoacyl-tRNA synthetases in the absence of clinically apparent myositis. Semin Arthritis Rheum 26:459–467
38. Yamasaki Y, Yamada H, Nozaki T, Akaogi J, Nichols C, Lyons R, Chin Loy A, Chan EK, Reeves WH, Satoh M (2006) Unusually high frequency of autoantibodies to PL-7 associated with milder muscle disease in Japanese patients with polymyositis/dermatomyositis. Arthritis Rheum 54:2004–2009
39. Sato S, Hirakata M, Kuwana M, Nakamura K, Suwa A, Inada S, Mimori T, Ikeda Y (2005) Clinical characteristics of Japanese patients with anti-PL-7 (anti-threonyl-tRNA synthetase) autoantibodies. Clin Exp Rheumatol 23:609–615
40. Kalluri M, Sahn SA, Oddis CV, Gharib SL, Christopher-Stine L, Danoff SK, Casciola-Rosen L, Hong G, Dellaripa PF, Highland KB (2009) Clinical profile of anti-PL-12 autoantibody. Cohort study and review of the literature. Chest 135:1550–1556
41. Labirua-Iturburu A, Selva-O'Callaghan A, Vincze M, Danko K, Vencovsky J, Fisher B, Charles P, Dastmalchi M, Lundberg IE (2012) Anti-PL-7 (anti-threonyl-tRNA synthetase) antisynthetase syndrome: clinical manifestations in a series of patients from a European multicenter study (EUMYONET) and review of the literature. Medicine (Baltimore) 91:206–211
42. Marie I, Josse S, Decaux O, Dominique S, Diot E, Landron C, Roblot P, Jouneau S, Hatron PY, Tiev KP et al (2012) Comparison of long-term outcome between anti-Jo1- and anti-PL7/PL12 positive patients with antisynthetase syndrome. Autoimmun Rev 11: 739–745
43. Marie I, Josse S, Decaux O, Diot E, Landron C, Roblot P, Jouneau S, Hatron PY, Hachulla E, Vittecoq O et al (2013) Clinical manifestations and outcome of anti-PL7 positive patients with antisynthetase syndrome. Eur J Intern Med 24:474–479
44. Aggarwal R, Cassidy E, Fertig N, Koontz DC, Lucas M, Ascherman DP, Oddis CV (2014) Patients with non-Jo-1 anti-tRNA-synthetase autoantibodies have worse survival than Jo-1 positive patients. Ann Rheum Dis 73:227–232
45. Hamaguchi Y, Fujimoto M, Matsushita T, Kaji K, Komura K, Hasegawa M, Kodera M, Muroi E, Fujikawa K, Seishima M et al (2013) Common and distinct clinical features in adult patients with anti-aminoacyl-tRNA synthetase antibodies: heterogeneity within the syndrome. PLoS ONE 8, e60442
46. Reeves WH, Nigam SK, Blobel G (1986) Human autoantibodies reactive with the signal-recognition particle. Proc Natl Acad Sci U S A 83:9507–9511
47. Nakao Y, Mukai R, Kabashima T, Ohshima Y, Hamaguchi H, Kashiwagi H, Okada N (1982) A novel antibody which precipitates 7.5S RNA is isolated from a patient with autoimmune disease. Biochem Biophys Res Commun 109:1332–1338
48. Okada N, Mimori T, Mukai R, Kashiwagi H, Hardin JA (1987) Characterization of human autoantibodies that selectively precipitate the 7SL RNA component of the signal recognition particle. J Immunol 138:3219–3223
49. Targoff IN, Johnson AE, Miller FW (1990) Antibody to signal recognition particle in polymyositis. Arthritis Rheum 33: 1361–1370
50. Kao AH, Lacomis D, Lucas M, Fertig N, Oddis CV (2004) Anti-signal recognition particle autoantibody in patients with and patients without idiopathic inflammatory myopathy. Arthritis Rheum 50:209–215

51. Hengstman GJ, ter Laak HJ, Vree Egberts WT, Lundberg IE, Moutsopoulos HM, Vencovsky J, Doria A, Mosca M, van Venrooij WJ, van Engelen BG (2006) Anti-signal recognition particle autoantibodies: marker of a necrotising myopathy. Ann Rheum Dis 65:1635–1638
52. Miller T, Al-Lozi MT, Lopate G, Pestronk A (2002) Myopathy with antibodies to the signal recognition particle: clinical and pathological features. J Neurol Neurosurg Psychiatry 73:420–428
53. Takada T, Hirakata M, Suwa A, Kaneko Y, Kuwana M, Ishihara T, Ikeda Y (2009) Clinical and histopathological features of myopathies in Japanese patients with anti-SRP autoantibodies. Mod Rheuamtol 19:156–164
54. Benveniste O, Drouot L, Jouen F, Charuel JL, Bloch-Queyrat C, Behin A, Amoura Z, Marie I, Guiguet M, Eymard B et al (2011) Correlation of anti-signal recognition particle autoantibody levels with creatine kinase activity in patients with necrotizing myopathy. Arthritis Rheum 63:1961–1971
55. Aggarwal R, Oddis CV, Goudeau D, Fertig N, Metes I, Stephens C, Qi Z, Koontz D, Levesque MC (2015) Anti-signal recognition particle autoantibody ELISA validation and clinical associations. Rheumatology (Oxford) 54:1194–1199
56. Suzuki S, Nishikawa A, Kuwana M, Nishimura H, Watanabe Y, Nakahara J, Hayashi YK, Suzuki N, Nishino I (2015) Inflammatory myopathy with anti-signal recognition particle antibodies: case series of 100 patients. Orphan J Rare Dis 10:61
57. Vincze M, Molnar PA, Tumpek J, Szollosi L, Gyetvai A, Kapitany A, Danko K (2010) An unusual association: anti-Jo1 and anti-SRP antibodies in the serum of a patient with polymyositis. Clin Rheumatol 29:811–814
58. Sugie K, Tonomura Y, Ueno S (2012) Characterization of dermatomyositis with coexistence of anti-Jo-1 and anti-SRP antibodies. Intern Med 51:799–802
59. Malkan A, Cappelen-Smith C, Beran R, Griffith N, Toong C, Wang MX, Cordato D (2015) Anti-synthetase syndrome associated with anti PL-12 and anti-signal recognition particle antibodies and a necrotizing auto-immune myositis. J Clin Neurosci 22:396–398
60. Satoh T, Okano T, Matsui T, Watabe H, Ogasawara T, Kubo K, Kuwana M, Fertig N, Oddis CV, Kondo H et al (2005) Novel autoantibodies against 7SL RNA in patients with polymyositis/dermatomyositis. J Rheumatol 32:1727–1733
61. Ellis E, Ann Tan J, Lester S, Tucker G, Blumbergs P, Roberts-Thomson P, Limaye V (2012) Necrotizing myopathy: clinicoserologic associations. Muscle Nerve 45:189–194
62. Wang L, Liu L, Hao H, Gao F, Liu X, Wang Z, Zhang W, Lv H, Yuan Y (2014) Myopathy with anti-signal recognition particle antibodies: clinical and histopathological features in Chinese patients. Neuromuscul Disord 24:335–341
63. Suzuki S, Yonekawa T, Kuwana M, Hayashi YK, Okazaki Y, Kawaguchi Y, Suzuki N, Nishino I (2014) Clinical and histological findings associated with autoantibodies detected by RNA immunoprecipitation in inflammatory myopathies. J Neuroimmunol 274:202–208
64. Ellis AE, De Sousa M (1974) Phylogeny of the lymphoid system. I. A study of the fate of circulating lymphocytes in plaice. Eur J Immunol 4:338–343
65. Nilasena DS, Trieu EP, Targoff IN (1995) Analysis of the Mi-2 autoantigen of dermatomyositis. Arthritis Rheum 38:123–128
66. Zhang Y, LeRoy G, Seelig HP, Lane WS, Reinberg D (1998) The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. Cell 95:279–289
67. Roux S, Seelig HP, Meyer O (1998) Significance of Mi-2 autoantibodies in polymyositis and dermatomyositis. J Rheumatol 25: 395–396
68. Brouwer R, Hengstman GJ, Vree Egberts W, Ehrfeld H, Bozic B, Ghirardello A, Grondal G, Hietarinta M, Isenberg D, Kalden JR et al (2001) Autoantibody profiles in the sera of European patients with myositis. Ann Rheum Dis 60:116–123
69. Hausmanowa-Petrusewicz I, Kowalska-Oledzka E, Miller FW, Jarzabek-Chorzelska M, Targoff IN, Blaszczyk-Kostanecka M, Jablonska S (1997) Clinical, serologic, and immunogenetic features in Polish patients with idiopathic inflammatory myopathies. Arthritis Rheum 40:1257–1266
70. Komura K, Fujimoto M, Matsushita T, Kaji K, Kondo M, Hirano T, Orito H, Horikawa M, Hamaguchi Y, Hasegawa M et al (2005) Prevalence and clinical characteristics of anti-Mi-2 antibodies in Japanese patients with dermatomyositis. J Dermatol Sci 40:215–217
71. Ghirardello A, Zampieri S, Iaccarino L, Tarricone E, Bendo R, Gambari PF, Doria A (2005) Anti-Mi-2 antibodies. Autoimmunity 38:79–83
72. Ronnelid J, Barbasso Helmers S, Storfors H, Grip K, Ronnblom L, Franck-Larsson K, Nordmark G, Lundberg IE (2009) Use of a commercial line blot assay as a screening test for autoantibodies in inflammatory myopathies. Autoimmun Rev 9:58–61
73. Ghirardello A, Rampudda M, Ekholm L, Bassi N, Tarricone E, Zampieri S, Zen M, Vattemi GA, Lundberg IE, Doria A (2010) Diagnostic performance and validation of autoantibody testing in myositis by a commercial line blot assay. Rheumatology (Oxford) 49:2370–2374
74. Hamaguchi Y, Kuwana M, Hoshino K, Hasegawa M, Kaji K, Matsushita T, Komura K, Nakamura M, Kodera M, Suga N et al (2011) Clinical correlations with dermatomyositis-specific autoantibodies in adult Japanese patients with dermatomyositis: a multicenter cross-sectional study. Arch Dermatol 147:391–398
75. Ceribelli A, Fredi M, Taraborelli M, Cavazzana I, Franceschini F, Quinzanini M, Tincani A, Ross SJ, Chan JY, Pauley BA et al (2012) Anti-MJ/NXP-2 autoantibody specificity in a cohort of adult Italian patients with polymyositis/dermatomyositis. Arthritis Res Ther 14:R97
76. Muro Y, Sugiura K, Akiyama M (2013) A new ELISA for dermatomyositis autoantibodies: rapid introduction of autoantigen cDNA to recombinant assays for autoantibody measurement. Clin Dev Immunol 2013:856815
77. Petri MH, Satoh M, Martin-Marquez BT, Vargas-Ramirez R, Jara LJ, Saavedra MA, Cruz-Gonzalez C, Andrade-Ortega L, Vera-Lastra OL, Salazar-Paramo M et al (2013) Implications in the difference of anti-Mi-2 and -p155/140 autoantibody prevalence in two dermatomyositis cohorts from Mexico City and Guadalajara. Arthritis Res Ther 15:R48
78. Okada S, Weatherhead E, Targoff IN, Wesley R, Miller FW (2003) Global surface ultraviolet radiation intensity may modulate the clinical and immunologic expression of autoimmune muscle disease. Arthritis Rheum 48:2285–2293
79. Love LA, Weinberg CR, McConnaughey DR, Oddis CV, Medsger TA Jr, Reveille JD, Arnett FC, Targoff IN, Miller FW (2009) Ultraviolet radiation intensity predicts the relative distribution of dermatomyositis and anti-Mi-2 autoantibodies in women. Arthritis Rheum 60:2499–2504
80. Euwer RL, Sontheimer RD (1991) Amyopathic dermatomyositis (dermatomyositis sine myositis). Presentation of six new cases and review of the literature. J Am Acad Dermatol 24:959–966
81. Gerami P, Schope JM, McDonald L, Walling HW, Sontheimer RD (2006) A systematic review of adult-onset clinically amyopathic dermatomyositis (dermatomyositis sine myositis): a missing link within the spectrum of the idiopathic inflammatory myopathies. J Am Acad Dermatol 54:597–613
82. Fujikawa K, Kawakami A, Kaji K, Fujimoto M, Kawashiri S, Iwamoto N, Aramaki T, Ichinose K, Tamai M, Kamachi M et al (2009) Association of distinct clinical subsets with myositis-

specific autoantibodies towards anti-155/140-kDa polypeptides, anti-140-kDa polypeptides, and anti-aminoacyl tRNA synthetases in Japanese patients with dermatomyositis: a single-centre, cross-sectional study. Scand J Rheumatol 38:263–267
83. Gono T, Kawaguchi Y, Satoh T, Kuwana M, Katsumata Y, Takagi K, Masuda I, Tochimoto A, Baba S, Okamoto Y et al (2010) Clinical manifestation and prognostic factor in anti-melanoma differentiation-associated gene 5 antibody-associated interstitial lung disease as a complication of dermatomyositis. Rheumatology (Oxford) 49:1713–1719
84. Gono T, Kawaguchi Y, Ozeki E, Ota Y, Satoh T, Kuwana M, Hara M, Yamanaka H (2011) Serum ferritin correlates with activity of anti-MDA5 antibody-associated acute interstitial lung disease as a complication of dermatomyositis. Mod Rheumatol 21:223–227
85. Kobayashi I, Okura Y, Yamada M, Kawamura N, Kuwana M, Ariga T (2011) Anti-melanoma differentiation-associated gene 5 antibody is a diagnostic and predictive marker for interstitial lung diseases associated with juvenile dermatomyositis. J Pediatr 158: 675–677
86. Chen F, Wang D, Shu X, Nakashima R, Wang G (2012) Anti-MDA5 antibody is associated with A/SIP and decreased T cells in peripheral blood and predicts poor prognosis of ILD in Chinese patients with dermatomyositis. Rheumatol Int 32:3909–3915
87. Muro Y, Sugiura K, Hoshino K, Akiyama M (2011) Disappearance of anti-MDA-5 autoantibodies in clinically amyopathic DM/interstitial lung disease during disease remission. Rheumatology (Oxford) 51:800–804
88. Cao H, Pan M, Kang Y, Xia Q, Li X, Zhao X, Shi R, Lou J, Zhou M, Kuwana M et al (2012) Clinical manifestations of dermatomyositis and clinically amyopathic dermatomyositis patients with positive expression of anti-melanoma differentiation-associated gene 5 antibody. Arthritis Care Res (Hoboken) 64:1602–1610
89. Fiorentino D, Chung L, Zwerner J, Rosen A, Casciola-Rosen L (2011) The mucocutaneous and systemic phenotype of dermatomyositis patients with antibodies to MDA5 (CADM-140): a retrospective study. J Am Acad Dermatol 65:25–34
90. Hall JC, Casciola-Rosen L, Samedy LA, Werner J, Owoyemi K, Danoff SK, Christopher-Stine L (2013) Anti-MDA5-associated dermatomyositis: expanding the clinical spectrum. Arthritis Care Res (Hoboken) 65:1307–1315
91. Ceribelli A, Fredi M, Taraborelli M, Cavazzana I, Tincani A, Selmi C, Chan JY, Chan EK, Satoh M, Franceschini F (2014) Prevalence and clinical significance of anti-MDA5 antibodies in European patients with polymyositis/dermatomyositis. Clin Exp Rheumatol 32:891–897
92. Labrador-Horrillo M, Martinez MA, Selva-O'Callaghan A, Trallero-Araguas E, Balada E, Vilardell-Tarres M, Juarez C (2014) Anti-MDA5 antibodies in a large Mediterranean population of adults with dermatomyositis. J Immunol Res 2014:290797
93. Sato S, Kuwana M, Fujita T, Suzuki Y (2013) Anti-CADM-140/MDA5 autoantibody titer correlates with disease activity and predicts disease outcome in patients with dermatomyositis and rapidly progressive interstitial lung disease. Mod Rheumatol 23:496–502
94. Targoff IN, Trieu EP, Levy-Neto M, Prasertsuntarasai T, Miller FW (2006) Autoantibodies to transcriptional intermediary factor 1-gamma (TIF1-γ) in dermatomyositis. Arthritis Rheum 54(Suppl):S518
95. Satoh M, Chan JY, Ross SJ, Li Y, Yamasaki Y, Yamada H, Mercado MV, Petri MH, Jara LJ, Saavedra MA et al (2012) Autoantibodies to transcription intermediary factor (TIF)1beta associated with dermatomyositis. Arthritis Res Ther 14:R79
96. Herquel B, Ouararhni K, Khetchoumian K, Ignat M, Teletin M, Mark M, Bechade G, Van Dorsselaer A, Sanglier-Cianferani S, Hamiche A et al (2011) Transcription cofactors TRIM24, TRIM28, and TRIM33 associate to form regulatory complexes that suppress murine hepatocellular carcinoma. Proc Natl Acad Sci U S A 108:8212–8217
97. Gunawardena H, Wedderburn LR, North J, Betteridge Z, Dunphy J, Chinoy H, Davidson JE, Cooper RG, McHugh NJ (2008) Clinical associations of autoantibodies to a p155/140 kDa doublet protein in juvenile dermatomyositis. Rheumatology (Oxford) 47: 324–328
98. Trallero-Araguas E, Labrador-Horrillo M, Selva-O'Callaghan A, Martinez MA, Martinez-Gomez X, Palou E, Rodriguez-Sanchez JL, Vilardell-Tarres M (2010) Cancer-associated myositis and anti-p155 autoantibody in a series of 85 patients with idiopathic inflammatory myopathy. Medicine (Baltimore) 89:47–52
99. Kaji K, Fujimoto M, Hasegawa M, Kondo M, Saito Y, Komura K, Matsushita T, Orito H, Hamaguchi Y, Yanaba K et al (2007) Identification of a novel autoantibody reactive with 155 and 140 kDa nuclear proteins in patients with dermatomyositis: an association with malignancy. Rheumatology (Oxford) 46:25–28
100. Kang EH, Nakashima R, Mimori T, Kim J, Lee YJ, Lee EB, Song YW (2010) Myositis autoantibodies in Korean patients with inflammatory myositis: anti-140-kDa polypeptide antibody is primarily associated with rapidly progressive interstitial lung disease independent of clinically amyopathic dermatomyositis. BMC Musculoskelet Disord 11:223
101. Ikeda N, Takahashi K, Yamaguchi Y, Inasaka M, Kuwana M, Ikezawa Z (2011) Analysis of dermatomyositis-specific autoantibodies and clinical characteristics in Japanese patients. J Dermatol 38:973–979
102. Targoff IN, Trieu EP, Levy-Neto M, Fertig N, Oddis CV (2007) Sera with autoantibodies to the MJ antigen react with NXP2. Arthritis Rheum 56:S787
103. Mimura Y, Takahashi K, Kawata K, Akazawa T, Inoue N (2010) Two-step colocalization of MORC3 with PML nuclear bodies. J Cell Sci 123:2014–2024
104. Kimura Y, Sakai F, Nakano O, Kisaki O, Sugimoto H, Sawamura T, Sadano H, Osumi T (2002) The newly identified human nuclear protein NXP-2 possesses three distinct domains, the nuclear matrix-binding, RNA-binding, and coiled-coil domains. J Biol Chem 277:20611–20617
105. Takahashi K, Yoshida N, Murakami N, Kawata K, Ishizaki H, Tanaka-Okamoto M, Miyoshi J, Zinn AR, Shime H, Inoue N (2007) Dynamic regulation of p53 subnuclear localization and senescence by MORC3. Mol Biol Cell 18:1701–1709
106. Oddis CV, Fertig N, Goel A, Espada G, Confalone Gregorian M, Maldonado Cocco JA, Londino AV (1997) Clinical and serological characterization of the anti-MJ antibody in childhood myositis. Arthritis Rheum 40:S139
107. Espada G, Maldonado Cocco JA, Fertig N, Oddis CV (2009) Clinical and serologic characterization of an Argentine pediatric myositis cohort: identification of a novel autoantibody (anti-MJ) to a 142-kDa protein. J Rheumatol 36:2547–2551
108. Gunawardena H, Wedderburn LR, Chinoy H, Betteridge ZE, North J, Ollier WE, Cooper RG, Oddis CV, Ramanan AV, Davidson JE et al (2009) Autoantibodies to a 140-kD protein in juvenile dermatomyositis are associated with calcinosis. Arthritis Rheum 60:1807–1814
109. Betteridge ZE, Gunawardena H, Chinoy H, Venkovsky J, Allard SA, Gordon PA, Cooper RG, McHugh NJ (2009) Autoantibodies to the p140 autoantigen NXP-2 in adult dermatomyositis. Arthritis Rheum 60:S304
110. Ichimura Y, Matsushita T, Hamaguchi Y, Kaji K, Hasegawa M, Tanino Y, Inokoshi Y, Kawai K, Kanekura T, Habuchi M et al (2012) Anti-NXP2 autoantibodies in adult patients with idiopathic inflammatory myopathies: possible association with malignancy. Ann Rheum Dis 71:710–713
111. Fiorentino DF, Chung LS, Christopher-Stine L, Zaba L, Li S, Mammen AL, Rosen A, Casciola-Rosen L (2013) Most patients

with cancer-associated dermatomyositis have antibodies to nuclear matrix protein NXP-2 or transcription intermediary factor 1gamma. Arthritis Rheum 65:2954–2962
112. Troyanov Y, Targoff IN, Payette MP, Raynauld JP, Chartier S, Goulet JR, Bourre-Tessier J, Rich E, Grodzicky T, Fritzler MJ et al (2014) Redefining dermatomyositis: a description of new diagnostic criteria that differentiate pure dermatomyositis from overlap myositis with dermatomyositis features. Medicine (Baltimore) 93:318–332
113. Valenzuela A, Chung L, Casciola-Rosen L, Fiorentino D (2014) Identification of clinical features and autoantibodies associated with calcinosis in dermatomyositis. JAMA Dermatol 150:724–729
114. Betteridge Z, Gunawardena H, North J, Slinn J, McHugh N (2007) Identification of a novel autoantibody directed against small ubiquitin-like modifier activating enzyme in dermatomyositis. Arthritis Rheum 56:3132–3137
115. Betteridge ZE, Gunawardena H, Chinoy H, North J, Ollier WE, Cooper RG, McHugh NJ (2009) Clinical and human leucocyte antigen class II haplotype associations of autoantibodies to small ubiquitin-like modifier enzyme, a dermatomyositis-specific autoantigen target, in UK Caucasian adult-onset myositis. Ann Rheum Dis 68:1621–1625
116. Tarricone E, Ghirardello A, Rampudda M, Bassi N, Punzi L, Doria A (2012) Anti-SAE antibodies in autoimmune myositis: identification by unlabelled protein immunoprecipitation in an Italian patient cohort. J Immunol Methods 384:128–134
117. Fujimoto M, Matsushita T, Hamaguchi Y, Kaji K, Asano Y, Ogawa F, Yamaoka T, Fujikawa K, Tsukada T, Sato K et al (2013) Autoantibodies to small ubiquitin-like modifier activating enzymes in Japanese patients with dermatomyositis: comparison with a UK Caucasian cohort. Ann Rheum Dis 72:151–153
118. Muro Y, Sugiura K, Akiyama M (2013) Low prevalence of anti-small ubiquitin-like modifier activating enzyme antibodies in dermatomyositis patients. Autoimmunity 46:279–284
119. Satoh M, Tanaka S, Chan EK (2015) The uses and misuses of multiplex autoantibody assays in systemic autoimmune rheumatic diseases. Front Immunol 6:181
120. Satoh M, Chan JY, Ross SJ, Ceribelli A, Cavazzana I, Franceschini F, Li Y, Reeves WH, Sobel ES, Chan EK (2011) Autoantibodies to survival of motor neuron (SMN) complex in patients with polymyositis—immunoprecipitation of D-E-F-G without other components of small nuclear ribonucleoproteins. Arthritis Rheum 63:1972–1978
121. Lanzavecchia A (1995) How can cryptic epitopes trigger autoimmunity? J Exp Med 181:1945–1948
122. Tan EM (2001) Autoantibodies as reporters identifying aberrant cellular mechanisms in tumorigenesis. J Clin Invest 108: 1411–1415
123. Abu-Shakra M, Buskila D, Ehrenfeld M, Conrad K, Shoenfeld Y (2001) Cancer and autoimmunity: autoimmune and rheumatic features in patients with malignancies. Ann Rheum Dis 60:433–441
124. Reuschenbach M, von Knebel DM, Wentzensen N (2009) A systematic review of humoral immune responses against tumor antigens. Cancer Immunol Immunother 58:1535–1544
125. Joseph CG, Darrah E, Shah AA, Skora AD, Casciola-Rosen LA, Wigley FM, Boin F, Fava A, Thoburn C, Kinde I et al (2014) Association of the autoimmune disease scleroderma with an immunologic response to cancer. Science 343:152–157
126. Vincent DF, Yan KP, Treilleux I, Gay F, Arfi V, Kaniewski B, Marie JC, Lepinasse F, Martel S, Goddard-Leon S et al (2009) Inactivation of TIF1gamma cooperates with Kras to induce cystic tumors of the pancreas. PLoS Genet 5, e1000575
127. Aucagne R, Droin N, Paggetti J, Lagrange B, Largeot A, Hammann A, Bataille A, Martin L, Yan KP, Fenaux P et al (2011) Transcription intermediary factor 1gamma is a tumor suppressor in mouse and human chronic myelomonocytic leukemia. J Clin Invest 121:2361–2370
128. Forrester NA, Patel RN, Speiseder T, Groitl P, Sedgwick GG, Shimwell NJ, Seed RI, Catnaigh PO, McCabe CJ, Stewart GS et al (2012) Adenovirus E4orf3 targets transcriptional intermediary factor 1gamma for proteasome-dependent degradation during infection. J Virol 86:3167–3179
129. Herquel B, Ouararhni K, Martianov I, Le Gras S, Ye T, Keime C, Lerouge T, Jost B, Cammas F, Losson R et al (2013) Trim24-repressed VL30 retrotransposons regulate gene expression by producing noncoding RNA. Nat Struct Mol Biol 20:339–346
130. Dong X, Hamilton KJ, Satoh M, Wang J, Reeves WH (1994) Initiation of autoimmunity to the p53 tumor suppressor protein by complexes of p53 and SV40 large T antigen. J Exp Med 179: 1243–1252
131. Satoh M, Ceribelli A, Chan EK (2012) Common pathways of autoimmune inflammatory myopathies and genetic neuromuscular disorders. Clin Rev Allergy Immunol 42:16–25

INTERNAL MEDICINE

□ PICTURES IN CLINICAL MEDICINE □

Spontaneous Improvement of Interstitial Pneumonia with Autoimmune Features

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Key words: interstitial pneumonia with autoimmune features, anti-EJ antibody

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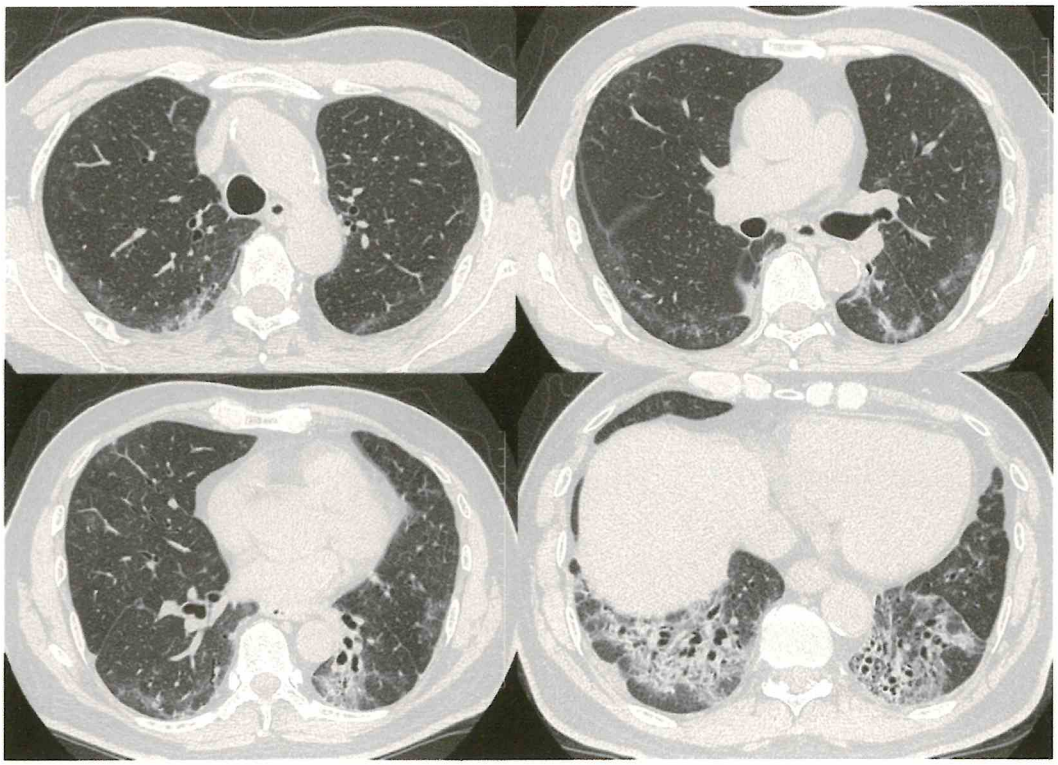

Picture 1.

A 66-year-old man, diagnosed with interstitial pneumonia based on high-resolution computed tomography (HRCT) findings (Picture 1) presented to our hospital due to a persistent dry cough with desaturation. Pulmonary function tests showed a reduced forced vital capacity of 1.90 L (51% predicted) and a decreased diffusing capacity for carbon monoxide (65% predicted). A surgical lung biopsy revealed nonspecific interstitial pneumonia (Picture 2A). Anti-EJ (glycyl tRNA synthetase) antibodies were positive on immunoprecipitation using ^{35}S-methionine-labeled K562 cell extract (Picture 2B). He did not meet the criteria for polymyositis and dermatomyositis and was diagnosed with interstitial pneumonia with autoimmune features (IPAF) (1). He declined corticosteroids and/or immunosuppressant therapy. A year later, spontaneous improvement of the features, including the desaturation, HRCT findings (Picture 3) and pulmonary function tests was noted. IPAF can show various clinical courses (2); we herein report the first known case of

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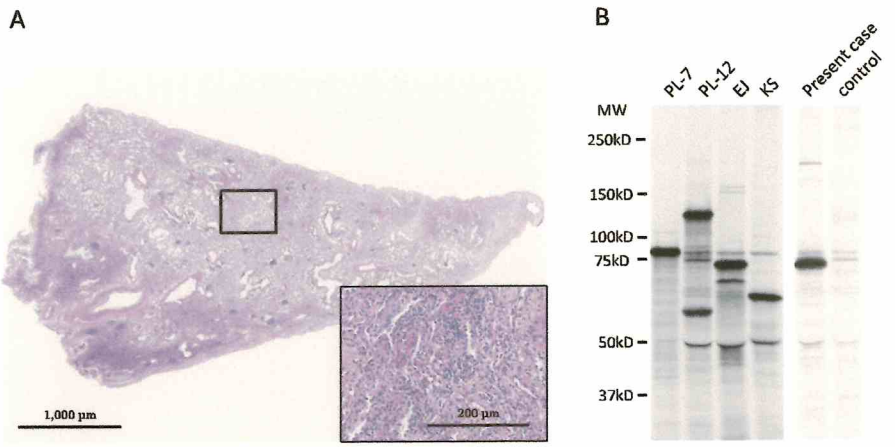
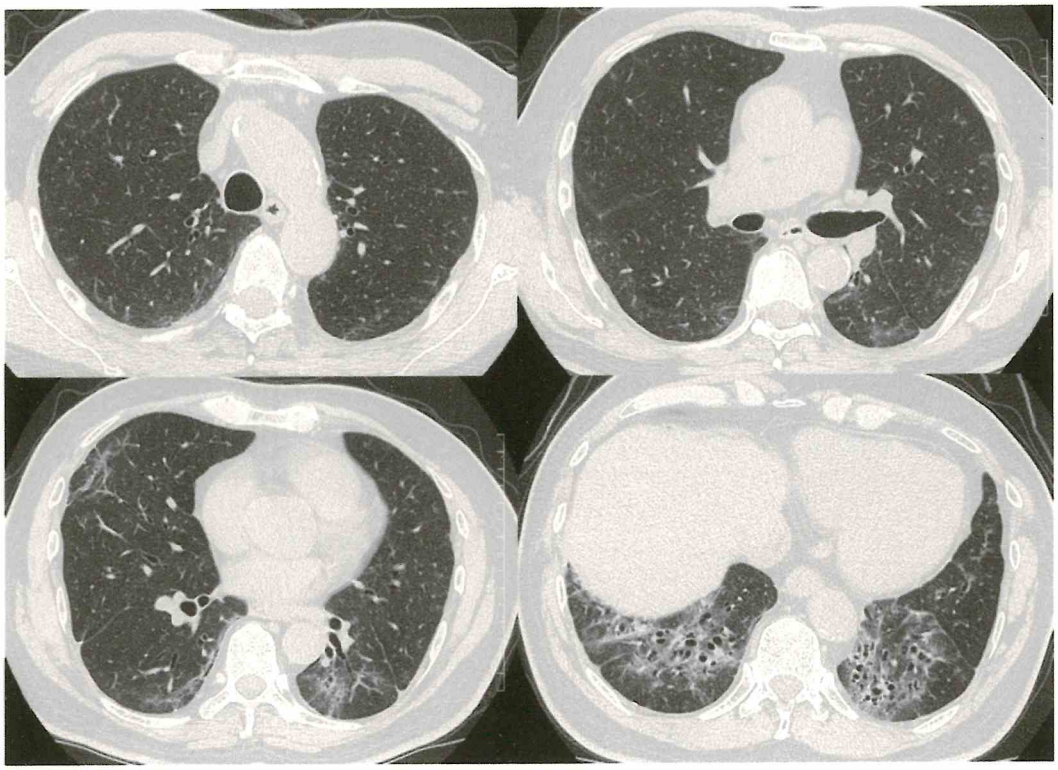


Picture 2.



Picture 3.

IPAF that spontaneously improved without any treatment.

The authors state that they have no Conflict of Interest (COI).

References

1. Fischer A, Antoniou KM, Brown KK, et al. An official European Respiratory Society/American Thoracic Society research statement: interstitial pneumonia with autoimmune features. Eur Respir J **46**: 976-987, 2015.
2. Takato H, Waseda Y, Watanabe S, et al. Pulmonary manifestations of anti-ARS antibody positive interstitial pneumonia: with or without PM/DM. Respir Med **107**: 128-133, 2013.

産業ストレス研究(Job Stress Res.), 24, 187-188(2017)

特集によせて

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産業ストレス研究はここ30年で飛躍的な発展を遂げた。それだけ働く人々にとって職場のストレスは重要な悩みごととなり，生活に脅威を与えていると言える。職場のストレスは実に多様な疾患の原因となっていることもここ数十年の研究から明らかにされているが，その生物学的メカニズムに関する研究は意外にも多くない。これまでに分かったことは，ストレスを正確に検出する単独の生物学的指標はまだ見つかっていないこと，人種や文化の違いによって結果が異なること，個体差が大きいことなど，実に未解明な部分が多いことである。

そこで本特集では，職場の心理社会的因子と内分泌免疫系の関連を明らかにする研究に活発に取り組んできた先生方に，自験例を交えて最新の知見について解説していただいた。

江口先生には，職業性心理社会的因子と心血管疾患の発症を予測する因子として近年富に注目されている慢性微弱炎症（low-grade systemic inflammation）について，これまで出版された11本の論文を解説していただいた。特にC反応蛋白（CRP）とインターロイキン6（IL-6）の動態に焦点を絞っているために，分かりやすく読みやすい。これらの研究から分かったことは，ストレスに対する認知や結果指標に人種が大きく影響することであり，それ故，解釈にあたっては十分注意する必要があることである。

太田先生には，視床下部—下垂体—副腎皮質系ホルモンとして近年注目を集めているデヒドロエピアンドロステロン（DHEA）とその硫酸抱合体（DHEAS）と職業性心理社会的要因，特に仕事の要求度—コントロール—社会的支援モデルならびに努力報酬不均衡モデルとの関係について解説いただいた。過去の研究を整理すると，職業性心理社会的要因とこれらのホルモンの関連について必ずしも一貫しておらず，その理由として曝露の持続性，測定のタイミング，バイアスなどが原因となっている可能性を指摘されている。しかし，逆に生理的指標の側からより良いストレスモデルの構築の可能性や予防・治療に生かすという新しい視点からのご提言もあり，今後の太田先生の研究成果に大いに期待したい。

井澤先生には，毛髪や爪のコルチゾール値を測定することにより職業性ストレスを評価する新しい試みについて最新の知見を整理していただいた。一般に，血液や唾液のコルチゾールは急性ストレスを反映し，逆に毛髪や爪のコルチゾールは慢性的・蓄積的なストレスを反映するものと考えられるため，慢性的な職業性ストレスを客観的にとらえる上でより正確な指標となるかもしれない。なんと言っても，サンプル採取にあたって侵襲性が低く，被験者自らが採取できるという利点と簡便性は職業性ストレス研究において魅力がある。まだ研究数が少ないことや血液等との比較検証が十分に行われていないなどの課題はあるものの，将来的に有望な評価法についていち早くご紹介いただけたことは筆者自身，大変勉強になった。

坪井先生には，精神神経内分泌免疫学における新しい潮流である，末梢から中枢へのシグナルについて慢性微弱炎症の視点から解説していただいた。慢性微弱炎症を引き起こす原因として，不適切な生活習慣の持続が挙げられ，生活習慣を整えることが逆に慢性微弱炎症を抑制し，メンタルヘルスの改善にもつながる可能性について論じていただいた。

最後に，筆者自身は職業性心理社会的因子と細胞性免疫，特にNK細胞系と炎症系マーカーの関連について紹介させていただいた。これまでに筆者が共同研究者と共に行った研究では，一貫してNK細胞数やNK細胞活性が職業性ストレスによって低下することから，職場ストレスのNK細胞に対する影響は間違いないことが分かったが，

採血量が多いことや費用がかかることが大きな課題であった。一方，炎症マーカーについての最先端の情報は江口先生に分かりやすく解説いただいたので，筆者はデータの解析等について役に立ちそうな情報を追加した。

すべての論文に目を通していただくとお気付きになると思われるが，この領域の研究はまだ端緒についたばかりである。研究成果はまだ少なく，結果は必ずしも一貫しておらず，それ故解釈も多様である。逆に言えば，それだけやりがいのある新しい分野であることも確かだ。

この特集号を読み，特に産業ストレスのバイオロジーに興味を持っていただいた若手の研究者には是非積極的に研究に挑戦していただき，産業ストレス研究の奥深さ，幅の広さを味わっていただけたらと思う。

産業ストレス研究(Job Stress Res.), 24, 197-204(2017)

【特集　産業ストレスのバイオロジー：生物学的指標の現在と展望】

職業性ストレスの免疫学的指標－細胞性免疫とサイトカインを中心に

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抄録：本総説は職業性ストレスの免疫指標として，細胞性免疫と炎症マーカーに焦点を絞って現在までの知見を整理したものである。これまでに蓄積された研究を概観した結果，職業上の心理社会的ストレスへの曝露（高い要求度，低い裁量権，高い仕事のストレイン，低い職務満足感，高い努力報酬不均衡，オーバーコミットメント，失業，長時間残業）はNK細胞活性やNK細胞数の低下ならびに炎症マーカーの亢進に測定しうる程度の影響をもたらすことが判明した。その結果，職業上の心理社会的ストレスは細胞性免疫と炎症マーカーに負の影響をもたらすことが示されたが，因果関係をより明確化する研究や職業性ストレスに対してより鋭敏で利用しやすい指標の開発が必要であることが示された。

Key words: Job stress（職業性ストレス），Inflammatory marker（炎症マーカー），cellular immunity（細胞性免疫），lymphocyte（リンパ球），Cytokine（サイトカイン），Psychosocial factor（心理社会的因子），Workers（労働者）

1．はじめに

過去30年の職業性ストレスの研究により，仕事のストレスが健康にどのような影響を及ぼすか，多くのエビデンスが蓄積されてきた。最近になって，これらのエビデンスに基づきメタ分析が報告されるようになり，職業性ストレスが様々な疾患の発症にどの程度の影響を及ぼすかも明らかになりつつある。例えば，仕事の要求度—コントロールモデルに基づく“仕事のストレイン”に注目した場合，仕事のストレインが高い者は低い者に比べ1.24倍脳卒中の発症リスク（相対危険度）が高く[1]，心疾患の発症リスクは1.26倍[2]であることが明らにされている。同じく，２型糖尿病の発症リスクは1.29倍[3]，高血圧の発症リスクは1.24倍[4]と報告されている。さらに，呼吸器系では慢性閉塞性肺疾患（COPD）の増悪は1.32倍[5]，うつ病の発症リスクは1.77倍（未出版データのみでもリスクは1.27倍で有意）である[6]。一方，仕事のストレインはがん（前立腺，乳，大腸）の発症[7]，炎症性腸疾患[8]や気管支喘息の増悪[9]を有意には増加させないことも報告されている。これらのメタ分析に含まれている多くの研究は，欧米で行われたものを中心としているため我が国の労働者において必ずしも当てはまるとは限らないが，仕事のストレインという一つの指標だけをとっても多様な疾患の発症にかかわっており，疾患によっては明白な影響を及ぼしていることが考えられる。

仕事のストレインはこれらの疾患の媒介因子となる喫煙[10]，運動[11]，飲酒[12]等の生活因子や肥満／低体重[13]とも関連することがメタ分析によって示されている。例えば，喫煙者は非喫煙者よりも有意に仕事のストレインが高く，また，仕事のストレインが高い喫煙者は低い喫煙者に比べると１週間当たりの喫煙本数が３本多いことが報告されており，仕事のストレインは喫煙者の喫煙行動を促進する方向に働く[10]。一方，非飲酒者や重度の飲酒者は，中等度の飲酒者よりも仕事のストレインが高いことが横断研究の分析によって判

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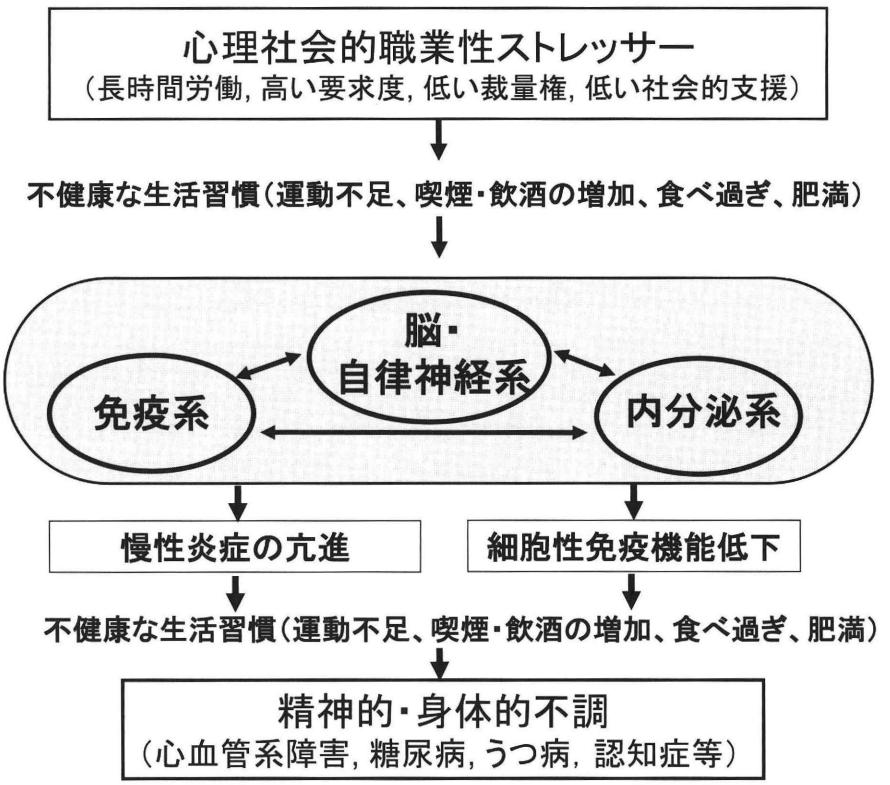


図1　心理社会的職業性ストレッサーの神経・内分泌・免疫系を介しての生体への影響

注：心理社会的職業性ストレッサーは不健康な生活習慣を招き，神経・内分泌・免疫系に影響し，慢性炎症の亢進ならびに細胞性免疫の機能の低下を導く。さらに，ネガティブ・フィードバックにより不健康な生活習慣が強化され，精神的・身体的不調が引き起こされると考えられる。

明しているが，縦断研究の結果からは仕事のストレインと飲酒の関連は明確には認められていない[12]。また，運動に関しては，仕事のストレインが高い者は運動不足の者が多く，定期的な運動習慣があった者でもストレインが高くなるに従って運動不足になることが示されている[11]。肥満や低体重に関しては，横断的には標準体重の者よりも低体重や肥満の者で仕事のストレインが高く，縦断的には標準体重の者の仕事ストレインが増加した場合肥満が増加し，逆に肥満であった者は仕事ストレインの増加によって体重が低下するという現象も認められている。これらのことから，職業性ストレスは働く人々の健康行動を悪化させることによって長期的に悪影響を及ぼすものと考えられる（図1）。

さらに，最近では仕事のストレインが後年の認知症の発症とも関連する証拠も提示されており[14,15]，仕事のストレインが仕事から離れた後も長期間にわたって影響することも明らかにされている。

仕事のストレインによって発症しうるこれらの様々な疾患に共通するメカニズムとして，近年，慢性炎症が注目されている。一般に炎症反応はその経過によって急性と慢性に分類される。急性炎症は感染や火傷，重金属や有機溶剤への曝露などによって引き起こされ，経過がすみやかで早期に終息するものをいう。一方，慢性炎症は組織障害が長期にわたる場合や，原因となる病原がなかなか処理されない場合に起こる。加齢や肥満さらに喫煙は慢性炎症を促進し，逆に運動は抑制することが示唆されていたが，職業性ストレスのような心理社会的因子によっても慢性炎症反応が促進されることが報告されるようになった[16]。

2．職業性ストレスと慢性炎症

職業性ストレスと慢性炎症に関する研究は，主に欧米において研究が活発に行われてきた。その理由として，主に白人や黒人において心理社会的ストレスとC

反応性蛋白（C-reactive protein；CRP），インターロイキン（Interleukin；IL）-6，TNF（腫瘍壊死因子（Tumor Necrosis Factor；TNF）- α，白血球数，単球数などの炎症マーカーが中等度の相関を示し[17]，これらの上昇が循環器系疾患を予測する因子として有用と考えられたからである[18]。しかし，黄色人種の炎症マーカー（CRP，IL-6）の値は白人や黒人に比べるとかなり低い[19]ことから，欧米での知見が必ずしもアジア圏の人種に当てはまるとは限らないと考えられた。しかし，近年炎症マーカーの一つであるCRPの値が白人や黒人に比べ低い人種（日本，中国，香港，韓国，マカオ，モンゴル，台湾等）においても脳卒中などを予測する上で有用であることが示されており[20]，予測マーカーとしては信頼性があると考えられている。

さて，職業性ストレスと炎症マーカーの関連については，詳しくは本特集号の筆者の一人である江口尚先生に譲るとして，測定結果の解釈や測定上の注意点，数値のとらえ方についてこれまでの筆者の経験から気付いた点をいくつか挙げておく。まず，これらの多くの研究では，炎症マーカーが1種類しか測定されておらず，他のマーカーも並行して測定していないため結果の解釈には十分注意が必要である。また，外れ値（高値）があった場合に感染などの急性炎症による増加なのか，あるいは測定上の問題（抗体のロットの違いによる系統誤差等）なのか十分に考慮し，また，場合によっては再測定するためにサンプルを保存しておく必要がある。逆に極端な低値は抗生物質等の薬物によって低下している可能性もあり，検査対象者の薬物使用の有無等についても記録し，交絡因子として考慮する必要がある。このような対象者がいれば，解析に含めた場合と含めない場合で結果がどのように異なるかも検討する必要があろう。疫学研究では大量のサンプルを同時測定するため，検査時にこの種の見落としがあると取り返しがつかなくなることもある。

筆者は可能な限り複数の炎症マーカーを測定し，測定値のみの信頼性についても検討するようにしている。男性健常者191名を対象に炎症マーカーを同時測定したデータでは，高感度CRP（hs-CRP），IL-6とTNF-αは正の相関が認められた。スペアマンの順位相関係数（rs）を計算したところ，hs-CRPとIL-6はrs=.412（$p<0.001$），hs-CRPとTNF-αはrs=.378（$p<0.001$），IL-6とTNF-αはrs=.456（$p<0.001$）であった。異なる測定系で得られた値も得るようにすれば（この場合，hs-CRPとサイトカイン），測定値自身の信頼性も得られると考えられる。

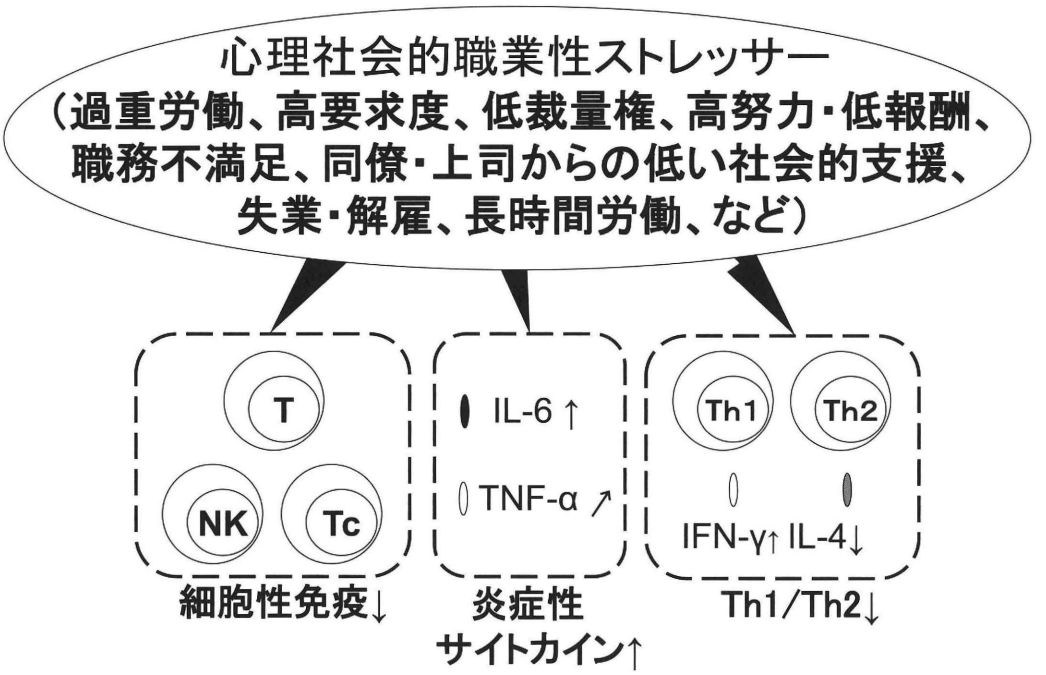
その他，検査の値に関して正規分布を前提とした統計解析を行う場合，対数変換等の変換処理を事前に行う必要がある。対数変換によっても正規化しない場合は，Box-Cox変換，平方根変換やBlom's normal score変換などによって正規分布に近づけるなどの工夫も必要である。

3．職業性ストレスと細胞性免疫

職業性ストレス研究において，細胞性免疫機能の測定は比較的早くから行われていた。ナチュラル・キラー（Natural killer；NK）細胞活性やリンパ球幼若化反応はリンパ球の機能を調べる上で有用であり，これらの測定値が低ければ免疫機能の低下が疑われた。

欧米では1980年代に失業と細胞性免疫能の関連が活発に研究され，失業直後よりも失業12ヶ月後でリンパ球幼若化反応が低下する現象が観察された[21]。このことは，長引くストレスの影響が間隔を置いて免疫機能に反映されることを示唆している。失業とNK細胞活性に着目した研究では，失業状態から再就職した（つまりストレッサーが取り抜かれた）場合，免疫機能がどのように回復するかという研究もなされている。Cohenら[22]は失業者100名と年齢と性別がマッチする雇用が安定した就労者100名のNK細胞活性を月1回の割合で4ヶ月にわたって測定した。その結果，失業者全体のNK細胞活性は4ヶ月間一貫して就労者よりも有意に低かった。経過観察の途中で就職が決まった25名の翌月のNK細胞活性は前月に比べ44－72％の増加が認められた。この回復は生活習慣やNK細胞率の変化では説明されなかったことから，失業というストレスから解放されるとNK細胞の機能は比較的速やかに回復する可能性があることが判明した。

次にNK細胞の数と機能の両者と職業要因の関連を検討した筆者らの一連の報告について言及する。我々の研究では，男性労働者を対象に仕事の要求度－コントロール－社会的支援モデルとリンパ球分画数の関連を検討し，仕事の要求度とNK細胞数との間に負の相関，仕事のストレインとNK細胞数の間に負の相関を認めている[23]。一方，社会的支援と細胞障害性T細胞との間には正の相関を認めた。これらのことから職業



T=T細胞、Tc=Cytotoxic T細胞、Th1=T helper 1細胞、Th2=T helper 2細胞、IFN=Interferon

図2　心理社会的職業性ストレッサーの細胞性免疫と炎症マーカーへの影響

性ストレスの標的細胞はNK細胞と推定し，努力報酬不均衡，職務満足感ならびに長時間残業とNK細胞の関連を男女別に検討した。その結果，男性では報酬の低下はNK細胞数とNK活性の両者の低下と関連し，努力はNK細胞数の低下と関連した[24]。女性では，努力，報酬ならびに努力報酬不均衡はいずれも関連が認められなかった。なお，オーバーコミットメントは男性において弱い負の相関を示した。また，職務満足感は女性のNK細胞数とNK細胞活性の上昇と関連し，男性はNK細胞活性のみ上昇するという結果を得た[25]。さらに，長時間の残業はNK細胞数と負の相関を示すことも示された[26]。

これら一連の結果から，様々なストレスモデルに基づく職業性ストレスの変数や労働時間はNK細胞と関連することが判明し，NK細胞は職業性ストレスの免疫指標として有用である可能性が示された。しかし，これらの指標を健診などに取り入れるには実際上の問題もある。例えば，NK細胞活性の測定には1検体につき少なくとも5－7ml程度の全血が必要であり，リンパ球幼若化反応も同時に測定するとなると同じ程度の量の血液量が必要になる。そうなると検査対象者の負担は多く，健診などでは実用的ではない。その点を考慮すると，細胞性免疫指標としては1ml未満の血液量で測定が行えるNK細胞数がより妥当と思われる。

健診の測定項目として取り入れることを考える場合，血清50μl程度でサイトカインを一括して測定できるマルチプレックスサスペンションアレーというシステムも確立されていることから，複数の炎症性サイトカインや抗炎症性サイトカインを同時測定する方が実用上有用かもしれない。

なお，これまでに述べた知見を図として整理したものを示す（図2）。

まとめ

本稿では，職業性ストレスと細胞性免疫，炎症マーカーとの関連について最新の知見を整理した。その結果，過剰な仕事のストレスはNK細胞性の機能を抑制し，炎症マーカーの増加を促すことが示唆された。しかし，産業保健の現場でこの知見を応用・利用するにはいくつかの課題が残されている。例えば，これまでの研究から，集団レベルにおいて過剰なストレスは免疫機能を抑制することが判明しているが，個人単位で

みた場合，免疫系の抑制が仕事要因によるのか，それとも仕事外要因によるのか，あるいは個人の遺伝的・素質的要因なのかの判別が難しい。また，ストレスの影響が免疫系に対して同時期に反映されていれば因果関係を理解しやすいが，急性ストレスでは免疫機能は増強され，慢性ストレスによって抑制されることから，免疫系に反映されるまでに時間差があることなどから，結果の解釈が難しい面もある。さらに，免疫指標の測定はコストが高いこと，様々な測定条件を整えなければならないという課題もある。そのため，ストレスを鋭敏に定量化でき，現在よりも安価でリアルタイム（point-of-care）な測定が可能なマーカーの探索や測定機器の開発が望まれる。これらの課題を踏まえて，今後新たな知見が集積されることが期待される。

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参考文献

1）Fransson, E. I., Nyberg, S. T., Heikkila, K., Alfredsson, L., Bjorner, J. B., Borritz, M., Burr, H., Dragano, N., Geuskens, G. A., Goldberg, M., Hamer, M., Hooftman, W. E., Houtman, I. L., Joensuu, M., Jokela, M., Knutsson, A., Koskenvuo, M., Koskinen, A., Kumari, M., Leineweber, C., Lunau, T., Madsen, I. E., Hanson, L. L., Nielsen, M. L., Nordin, M., Oksanen, T., Pentti, J., Pejtersen, J. H., Rugulies, R., Salo, P., Shipley, M. J., Steptoe, A., Suominen, S. B., Theorell, T., Toppinen-Tanner, S., Vahtera, J., Virtanen, M., Vaananen, A., Westerholm, P. J., Westerlund, H., Zins, M., Britton, A., Brunner, E. J., Singh-Manoux, A., Batty, G. D. and Kivimaki, M.: Job strain and the risk of stroke: an individual-participant data meta-analysis. Stroke, **46**, 557-559 (2015)

2）Xu, S., Huang, Y., Xiao, J., Zhu, W., Wang, L., Tang, H., Hu, Y. and Liu, T.: The association between job strain and coronary heart disease: a meta-analysis of prospective cohort studies. Ann Med, **47**, 512-518 (2015)

3）Nyberg, S. T., Fransson, E. I., Heikkila, K., Alfredsson, L., Casini, A., Clays, E., De Bacquer, D., Dragano, N., Erbel, R., Ferrie, J. E., Hamer, M., Jockel, K. H., Kittel, F., Knutsson, A., Ladwig, K. H., Lunau, T., Marmot, M. G., Nordin, M., Rugulies, R., Siegrist, J., Steptoe, A., Westerholm, P. J., Westerlund, H., Theorell, T., Brunner, E. J., Singh-Manoux, A., Batty, G. D., Kivimaki, M. and Consortium, I. P.-W.: Job strain and cardiovascular disease risk factors: meta-analysis of individual-participant data from 47,000 men and women. PLoS One, **8**, e67323 (2013)

4）Babu, G. R., Jotheeswaran, A. T., Mahapatra, T., Mahapatra, S., Kumar, A., Sr., Detels, R. and Pearce, N.: Is hypertension associated with job strain? A meta-analysis of observational studies. Occup Environ Med, **71**, 220-227 (2014)

5）Heikkila, K., Madsen, I. E., Nyberg, S. T., Fransson, E. I., Ahola, K., Alfredsson, L., Bjorner, J. B., Borritz, M., Burr, H., Knutsson, A., Koskenvuo, M., Koskinen, A., Nielsen, M. L., Nordin, M., Pahkin, K., Pentti, J., Rugulies, R., Salo, P., Shipley, M. J., Suominen, S. B., Theorell, T., Vaananen, A., Vahtera, J., Virtanen, M., Westerholm, P. J., Batty, G. D., Singh-Manoux, A., Kivimaki, M. and Consortium, I. P.-W.: Job strain and COPD exacerbations: an individual-participant meta-analysis. Eur Respir J, **44**, 247-251 (2014)

6）Madsen, I. E., Nyberg, S. T., Magnusson Hanson, L. L., Ferrie, J. E., Ahola, K., Alfredsson, L., Batty, G. D., Bjorner, J. B., Borritz, M., Burr, H., Chastang, J. F., de Graaf, R., Dragano, N., Hamer, M., Jokela, M., Knutsson, A., Koskenvuo, M., Koskinen, A., Leineweber, C., Niedhammer, I., Nielsen, M. L., Nordin, M., Oksanen, T., Pejtersen, J. H., Pentti, J., Plaisier, I., Salo, P., Singh-Manoux, A., Suominen, S., Ten Have, M., Theorell, T., Toppinen-Tanner, S., Vahtera, J., Vaananen, A., Westerholm, P. J., Westerlund, H., Fransson, E. I., Heikkila, K., Virtanen, M., Rugulies, R., Kivimaki, M. and Consortium, I. P.-W.: Job strain as a risk factor for clinical depression: systematic review and meta-analysis with additional individual participant data. Psychol Med, 1-15 (2017)

7）Heikkila, K., Nyberg, S. T., Theorell, T., Fransson, E. I., Alfredsson, L., Bjorner, J. B., Bonenfant, S., Borritz, M., Bouillon, K., Burr, H., Dragano, N., Geuskens, G. A., Goldberg, M., Hamer, M., Hooftman, W. E., Houtman, I. L., Joensuu, M., Knutsson, A., Koskenvuo, M., Koskinen, A., Kouvonen, A., Madsen, I. E., Magnusson Hanson, L. L., Marmot, M. G., Nielsen, M. L., Nordin, M., Oksanen, T., Pentti, J., Salo, P., Rugulies, R., Steptoe, A., Suominen, S., Vahtera, J., Virtanen, M., Vaananen, A., Westerholm, P., Westerlund, H., Zins, M., Ferrie, J. E., Singh-Manoux, A., Batty, G. D., Kivimaki, M. and Consortium, I. P.-W.: Work stress and risk of cancer: meta-analysis of 5700 incident cancer events in 116,000 European men and women. BMJ, **346**, f165 (2013)

8）Heikkila, K., Madsen, I. E., Nyberg, S. T., Fransson, E. I., Ahola, K., Alfredsson, L., Bjorner, J. B., Borritz, M., Burr, H., Dragano, N., Ferrie, J. E., Knutsson, A., Koskenvuo, M.,

Koskinen, A., Nielsen, M. L., Nordin, M., Pejtersen, J. H., Pentti, J., Rugulies, R., Oksanen, T., Shipley, M. J., Suominen, S. B., Theorell, T., Vaananen, A., Vahtera, J., Virtanen, M., Westerlund, H., Westerholm, P. J., Batty, G. D., Singh-Manoux, A., Kivimaki, M. and Consortium, I. P.-W.: Job strain and the risk of inflammatory bowel diseases: individual-participant meta-analysis of 95,000 men and women. PLoS One, **9**, e88711 (2014)

9) Heikkila, K., Madsen, I. E., Nyberg, S. T., Fransson, E. I., Westerlund, H., Westerholm, P. J., Virtanen, M., Vahtera, J., Vaananen, A., Theorell, T., Suominen, S. B., Shipley, M. J., Salo, P., Rugulies, R., Pentti, J., Pejtersen, J. H., Oksanen, T., Nordin, M., Nielsen, M. L., Kouvonen, A., Koskinen, A., Koskenvuo, M., Knutsson, A., Ferrie, J. E., Dragano, N., Burr, H., Borritz, M., Bjorner, J. B., Alfredsson, L., Batty, G. D., Singh-Manoux, A., Kivimaki, M. and Consortium, I. P.-W.: Job strain and the risk of severe asthma exacerbations: a meta-analysis of individual-participant data from 100 000 European men and women. Allergy, **69**, 775-783 (2014)

10) Heikkila, K., Nyberg, S. T., Fransson, E. I., Alfredsson, L., De Bacquer, D., Bjorner, J. B., Bonenfant, S., Borritz, M., Burr, H., Clays, E., Casini, A., Dragano, N., Erbel, R., Geuskens, G. A., Goldberg, M., Hooftman, W. E., Houtman, I. L., Joensuu, M., Jockel, K. H., Kittel, F., Knutsson, A., Koskenvuo, M., Koskinen, A., Kouvonen, A., Leineweber, C., Lunau, T., Madsen, I. E., Magnusson Hanson, L. L., Marmot, M. G., Nielsen, M. L., Nordin, M., Pentti, J., Salo, P., Rugulies, R., Steptoe, A., Siegrist, J., Suominen, S., Vahtera, J., Virtanen, M., Vaananen, A., Westerholm, P., Westerlund, H., Zins, M., Theorell, T., Hamer, M., Ferrie, J. E., Singh-Manoux, A., Batty, G. D., Kivimaki, M. and Consortium, I. P.-W.: Job strain and tobacco smoking: an individual-participant data meta-analysis of 166,130 adults in 15 European studies. PLoS One, **7**, e35463 (2012)

11) Fransson, E. I., Heikkila, K., Nyberg, S. T., Zins, M., Westerlund, H., Westerholm, P., Vaananen, A., Virtanen, M., Vahtera, J., Theorell, T., Suominen, S., Singh-Manoux, A., Siegrist, J., Sabia, S., Rugulies, R., Pentti, J., Oksanen, T., Nordin, M., Nielsen, M. L., Marmot, M. G., Magnusson Hanson, L. L., Madsen, I. E., Lunau, T., Leineweber, C., Kumari, M., Kouvonen, A., Koskinen, A., Koskenvuo, M., Knutsson, A., Kittel, F., Jockel, K. H., Joensuu, M., Houtman, I. L., Hooftman, W. E., Goldberg, M., Geuskens, G. A., Ferrie, J. E., Erbel, R., Dragano, N., De Bacquer, D., Clays, E., Casini, A., Burr, H., Borritz, M., Bonenfant, S., Bjorner, J. B., Alfredsson, L., Hamer, M., Batty, G. D. and Kivimaki, M.: Job strain as a risk factor for leisure-time physical inactivity: an individual-participant meta-analysis of up to 170,000 men and women: the IPD-Work Consortium. Am J Epidemiol, **176**, 1078-1089 (2012)

12) Heikkila, K., Nyberg, S. T., Fransson, E. I., Alfredsson, L., De Bacquer, D., Bjorner, J. B., Bonenfant, S., Borritz, M., Burr, H., Clays, E., Casini, A., Dragano, N., Erbel, R., Geuskens, G. A., Goldberg, M., Hooftman, W. E., Houtman, I. L., Joensuu, M., Jockel, K. H., Kittel, F., Knutsson, A., Koskenvuo, M., Koskinen, A., Kouvonen, A., Leineweber, C., Lunau, T., Madsen, I. E., Magnusson Hanson, L. L., Marmot, M. G., Nielsen, M. L., Nordin, M., Pentti, J., Salo, P., Rugulies, R., Steptoe, A., Siegrist, J., Suominen, S., Vahtera, J., Virtanen, M., Vaananen, A., Westerholm, P., Westerlund, H., Zins, M., Theorell, T., Hamer, M., Ferrie, J. E., Singh-Manoux, A., Batty, G. D., Kivimaki, M. and Consortium, I. P.-W.: Job strain and alcohol intake: a collaborative meta-analysis of individual-participant data from 140,000 men and women. PLoS One, **7**, e40101 (2012)

13) Nyberg, S. T., Heikkila, K., Fransson, E. I., Alfredsson, L., De Bacquer, D., Bjorner, J. B., Bonenfant, S., Borritz, M., Burr, H., Casini, A., Clays, E., Dragano, N., Erbel, R., Geuskens, G. A., Goldberg, M., Hooftman, W. E., Houtman, I. L., Jockel, K. H., Kittel, F., Knutsson, A., Koskenvuo, M., Leineweber, C., Lunau, T., Madsen, I. E., Hanson, L. L., Marmot, M. G., Nielsen, M. L., Nordin, M., Oksanen, T., Pentti, J., Rugulies, R., Siegrist, J., Suominen, S., Vahtera, J., Virtanen, M., Westerholm, P., Westerlund, H., Zins, M., Ferrie, J. E., Theorell, T., Steptoe, A., Hamer, M., Singh-Manoux, A., Batty, G. D., Kivimaki, M. and Consortium, I. P.-W.: Job strain in relation to body mass index: pooled analysis of 160 000 adults from 13 cohort studies. J Intern Med, **272**, 65-73 (2012)

14) Wang, H. X., Wahlberg, M., Karp, A., Winblad, B. and Fratiglioni, L.: Psychosocial stress at work is associated with increased dementia risk in late life. Alzheimers Dement, **8**, 114-120 (2012)

15) Johansson, L., Guo, X., Waern, M., Ostling, S., Gustafson, D., Bengtsson, C. and Skoog, I.: Midlife psychological stress and risk of dementia: a 35-year longitudinal population study. Brain, **133**, 2217-2224 (2010)

16) Nakata, A.: Psychosocial job stress and immunity: a systematic review. Methods Mol Biol, **934**, 39-75 (2012)

17) Hansel, A., Hong, S., Camara, R. J. and von Kanel, R.: Inflammation as a psychophysiological biomarker in chronic psychosocial stress. Neurosci Biobehav Rev, **35**, 115-121 (2010)

18) Kaptoge, S., Seshasai, S. R., Gao, P., Freitag, D. F., Butterworth, A. S., Borglykke, A., Di Angelantonio, E., Gudnason, V., Rumley, A., Lowe, G. D., Jorgensen, T. and Danesh, J.: Inflammatory cytokines and risk of coronary heart disease: new prospective study and updated meta-analysis. Eur Heart J, **35**, 578-589 (2014)

19) Coe, C. L., Love, G. D., Karasawa, M., Kawakami, N., Kitayama, S., Markus, H. R., Tracy, R. P. and Ryff, C. D.: Population differences in proinflammatory biology: Japanese have healthier profiles than Americans. Brain Behav Immun, **25**, 494-502 (2011)

20) Saito, I., Maruyama, K. and Eguchi, E.: C-reactive protein and cardiovascular disease in East asians: a systematic review. Clin Med Insights Cardiol, **8**, 35-42 (2014)

21) Arnetz, B. B., Brenner, S. O., Levi, L., Hjelm, R., Petterson, I. L., Wasserman, J., Petrini, B., Eneroth, P., Kallner, A., Kvetnansky, R. and Vigas M.: Neuroendocrine and immunologic effects of unemployment and job insecurity. Psychother Psychosom, **55**, 76-80 (1991)

22) Cohen, F., Kemeny, M. E., Zegans, L. S., Johnson, P., Kearney, K. A. and Stites, D. P.: Immune function declines with unemployment and recovers after stressor termination. Psychosom Med, **69**, 225-234 (2007)

23) Nakata, A., Araki, S., Tanigawa, T., Miki, A., Sakurai, S., Kawakami, N., Yokoyama, K. and Yokoyama, M.: Decrease of suppressor-inducer (CD4+ CD45RA) T lymphocytes and increase of serum immunoglobulin G due to perceived job stress in Japanese nuclear electric power plant workers. J Occup Environ Med, **42**, 143-150 (2000)

24) Nakata, A., Takahashi, M. and Irie, M.: Effort-reward imbalance, overcommitment, and cellular immune measures among white-collar employees. Biol Psychol, **88**, 270-279 (2011)

25) Nakata, A., Takahashi, M., Irie, M. and Swanson, N. G.: Job satisfaction is associated with elevated natural killer cell immunity among healthy white-collar employees. Brain Behav Immun, **24**, 1268-1275 (2010)

26) Nakata, A., Takahashi, M. and Irie, M.: Association of overtime work with cellular immune markers among healthy daytime white-collar employees. Scand J Work Environ Health, **38**, 56-64 (2012)

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Immune markers for job stress research: A focus on inflammatory and cellular immune markers

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Abstract The purpose of this review was to provide current knowledge about the possible association between psychosocial job stress and immunity focusing on inflammatory and cellular immune markers. In general, exposure to psychosocial job stressors (high job demands, low job control, high job strain, low job satisfaction, high effort-reward imbalance, over-commitment, unemployment, and excessive overtime) had a measurable impact on immune parameters including reduced NK cell activity and NK cell counts, and increased inflammatory responses. The evidence supports that psychosocial job stresses are related to disrupted immune responses but further research is needed to demonstrate cause-effect relationships and develop more sensitive and usable markers.

Job Stress Res., 24 (2), 197-204 (2017)

商品開発・評価のための
生理計測とデータ解析ノウハウ

～生理指標の特徴、測り方、実験計画、データの解釈・評価方法～

日本人間工学会PIE研究部会編
監修 三宅 晋司

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第4章　免疫指標

産業医科大学　中田　光紀

1　はじめに

近年，労働の現場では仕事のストレス，蓄積疲労，過重労働，長時間労働，燃え尽き，睡眠不足，うつ病等の問題がますます加速化している。ニュースや報道では，電通の女性新人社員が過重労働・長時間残業で自らの命を絶つという残念な事件が記憶に新しい(平成27年12月25日)。過労死等予防対策や自殺予防対策などの施策が施行されるなか，このような犠牲者が次々と出てくるのはなぜなのだろうか。筆者は長年，ストレスや過重労働の生物学的機序についての研究に取り組んできたが，こうした犠牲者を未然に検出することができない理由には，①職場においては多様なストレス・環境要因があり(図1)，既存の評価尺度だけでは十分に予測ができないこと，②上記に掲げたストレス，過重労働，疲労等の曝露評価が主観に依存し

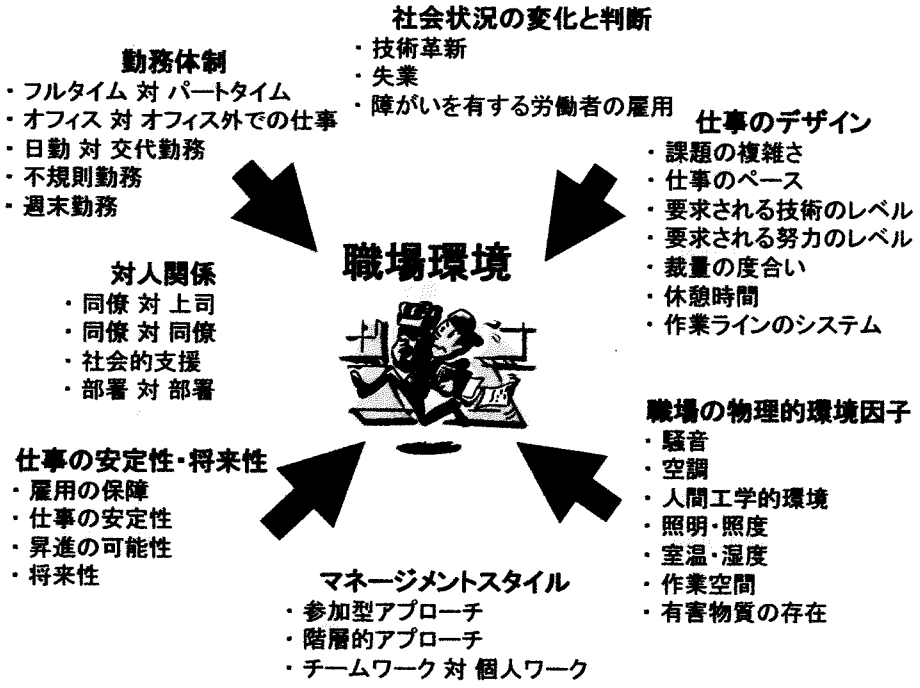

図1　職場で発生しうる様々なストレス要因

ていること，③個人によって物事の考え方や反応の仕方に差があり，同程度のストレスでも異なる反応を示すこと（個体の脆弱性の差），ならびに④曝露影響を客観的に評価し，客観的な基準を設けにくいことである。さらに，組織のモラルや体質の問題等，数多くの乗り越えるべき課題があると感じている。

そこで，筆者は曝露影響を客観的に評価する物差しの１つとして，生体防御に中心的な役割を果たす“免疫指標”を追究してきた。本稿では仕事のストレスの評価，結果指標としての免疫指標についてさまざまな研究を紹介するとともに，実際にどのような指標がストレスの検知に役立ちうるのか，各種指標の利点・欠点，測定上の注意点等について解説する。

2　日本人労働者の現状

「はじめに」の項でも紹介したように，日本の職場における仕事のストレス，蓄積疲労，過重労働，長時間労働，睡眠不足，うつ病等の現状について，国の統計情報に基づいて簡潔に紹介する。

厚生労働省が2012年に実施した労働者健康状況調査によれば，「仕事や職業生活に関する強い不安，悩み，ストレス」を有する日本人労働者の割合は全体の60.9%（男性61.9%，女性60.1%）に及ぶことが示された[1]。この傾向は2007年に少し減少したものの（全体58.0%），再び上昇傾向に転じている。同調査による睡眠時間については，１日６時間未満の労働者の割合は46.5%（５時間未満8.4%，５時間以上６時間未満38.1%）であった。また，（独）労働政策研究・研修機構が2009年に実施した「日本人の就業実態に関する総合調査」では，就業者にふだんの仕事で感じる疲れ・ストレスを調査した結果，「身体の疲れ」をとても感じている者が22.9%，やや感じている者が51.4%で，合計すると疲労を感じている就業者の割合は74.4%であった[2]。さらに，厚生労働省が2015年12月から2016年１月に実施した，企業約１万社（回答は1,743社）を対象とした調査結果である過労死防止対策白書（2016）によれば，１カ月の残業が最も長かった正社員の残業時間が「過労死ライン」の80時間を超えた企業は22.7%であった。なかでも情報通信業が44.4%，学術研究，専門・技術サービス業が40.6%で４割を超えていた[3]。さらに，残業時間が100時間を超えた企業の割合は11.9%であった。

このように高い仕事のストレス，睡眠の不足，疲労や過度な残業という条件の下で働く日本人労働者は過半数をはるかに超えると考えられ，安倍首相が唱える「1億総活躍社会」の前に「1億総疲弊社会」を迎えてしまう可能性すらある。

こうした事情を背景に，ストレス，睡眠不足，疲労，過度な残業などの影響を客観的にとらえようとする研究が医学，心理学，生物学，社会学等の領域で行われるようになり，それらが融合し，精神神経免疫学（Psychoneuroimmunology）あるいは精神神経内分泌免疫学（Psychoneuroendocrinoimmunology）という新しい分野として開拓され，1980年代半ばより欧米を中心に発展した。筆者は主に職場のストレスと免疫系についての研究を行ってきたことから，本稿では主にその点に焦点を当てることとするが，動物を用いた研究も進めてきたことから，次の項では，まず心理的ストレスと免疫系についての古典的な動物実験系を紹介し，その後に人を対象として行った研究と近年の知見について紹介する。

3 ストレスの認知的評価と免疫指標
（動物実験におけるストレスと免疫系）

少し話は古くなるが，米国には1980年代にストレスを認知の観点から評価することを提唱した，心理学者のリチャード・ラザルスという人物がいる。ラザルスはストレスとストレッサーを分けることが重要であると考え，人間と環境の相互作用を考慮することを提案した（図2）[4]。つまり，人は環境とかかわる中で，何が，どの程度のストレスになるのかを決定すると考えたのである。こうした決定を，「認知的評価」と呼び，このような決定を行いながら，周囲のさまざまな要求や，それに随伴する感情を処理する過程をコーピング（対処）と呼ぶようになった。言い換えると，ストレッサーが存在しても，そのストレッサーに上手に対処・処理することが可能であれば生体にとってあまり大きなストレスとはならないが，その対処に失敗すると過大なストレスとなる，ということである。

ラットを対象とした動物実験において，この考え方を取り入れた画期的な実験系が存在する。その方法をトリアディック・デザイン（3つ組法）と呼ぶ（図3）[5][6]。この実験デザインはストレッサーである電気刺激に対して，対処できるか否かの条件を与え，能動的・積極的にストレスに対して挑戦・克服出来るかという対処可能群と受動的・消極的にストレス刺激に対して受け身の状態である対処不可能群を設定し，その差を観察するという系である。図3の一番左のラットに注目していただこう。このラットは対処可能ラットで，電撃を与えられることにより目の前にぶら下がるディスクにかみついたり，手をかけて引いたりと逡巡を繰り返す内に，偶然ディスクを引き電撃が

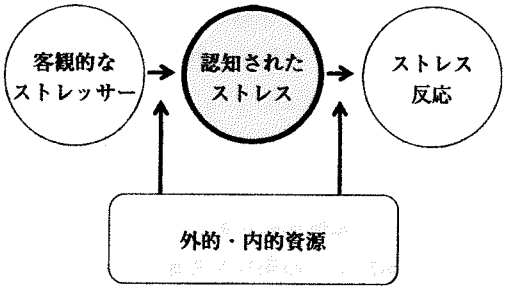

図2　ストレス認知モデル[4]

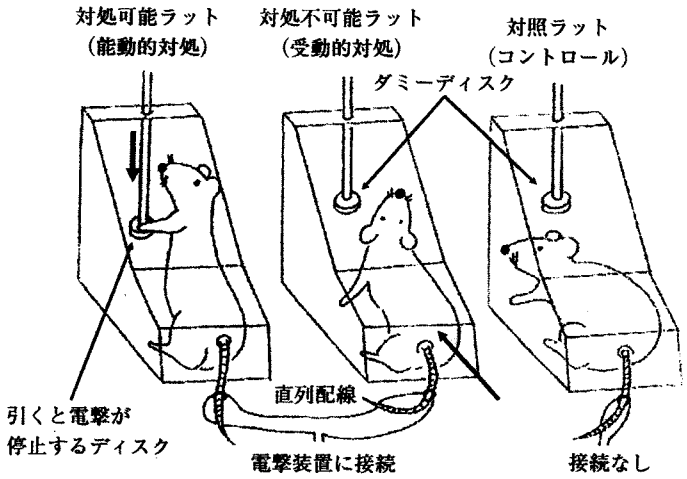

図3　トリアディック・デザイン[5][6]

停止することを学習する。私の経験では，100回の試行でほとんどのラットが電撃を停止できるようになる。一方，真ん中の対処不可能ラットは，目の前にぶら下がるディスクを何回引いても電撃を停止することはできない。すなわち，目の前のディスクはダミーなのである。ただし，対処可能ラットが電撃を停止すれば，自動的に停止する。このことから，対処可能と対処不可能ラットの電撃を受ける総量は同一であるが，対処可能性の可否によって生体が被る影響が異なる。

これまでの研究では，対処不可能ラットで体重や食欲が低下し，胃潰瘍や脳内アドレナリン量が増加する一方，対処可能ラットにおいてはそのような大きな変化は観察されないことが報告されている[7]。同様の実験を“免疫系”に関連するアウトカムを用いた研究では，移植癌に対する拒絶率が対処不可能ラットでは対処可能ラットに比べ有意に拒絶率が低くなること[8]，脾臓のナチュラル・キラー(NK)細胞の機能を測定するNK細胞活性[9]ならびにT細胞の機能を測定するためのリンパ球幼若化反応[10]も同様の傾向を示すことが報告されている。なかでも，Con Aというリンパ球刺激物質に対しての反応は，対処可能ラットでは対照ラットのそれよりも反応が増強することが判明し，克服可能なストレッサーへの曝露は，生体にとっては必ずしも悪い刺激とはならない可能性が示唆された。

さて，筆者らはこの実験系を用いて，Tリンパ球分画への影響を検討した。各々の群に16匹のラットを用いて100回の電撃を与え，24時間後に電撃の影響を想起させるための少量の電撃を再び与え，末梢血，脾臓，胸腺のリンパ球全体に対するCD4+，CD8+，CD25+Tリンパ球の割合を測定した[6]。その結果，対処可能ラットでは胸腺のCD4+Tリンパ球率の低下と脾臓のCD4+/CD8+比が低下したのみであったが，対処不可能ラットでは末梢血，脾臓，胸腺において多様な変化を示した(図4)。以上から，生体が曝露するストレッサーの条件の違いによって免疫反応が異なることが示され，ストレスの免疫影響を調べる際は，このように対象可能性などの条件を考慮する必要があることが判明した。しかし，課題が複雑(5回ディスクを引かないと停止しない)になると対処可能群の方がかえってストレス度が高くなること

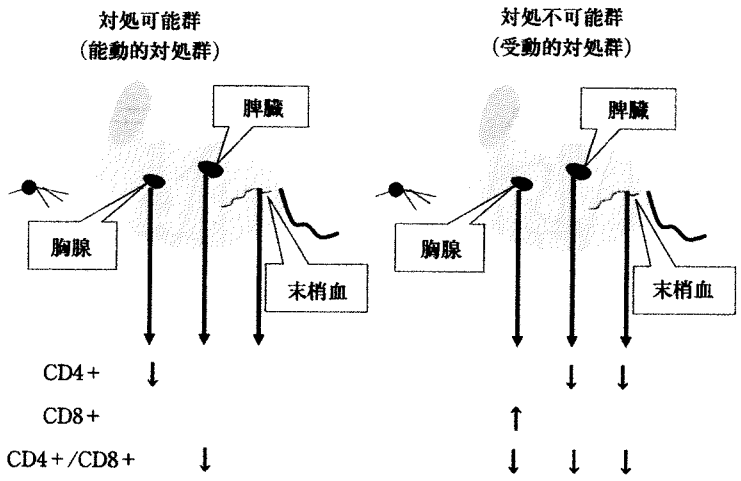

図4　対処可能性とリンパ球分画(対照群との比較)[6]

等，条件によって生体への影響が異なることも報告されている[11]。

4　ストレス研究における免疫指標測定の種類と測定法

ここでこれらの実験で使用された免疫指標にはどのような種類があるのか，各指標についてポイントを解説する(表1，2)。上記の動物実験では，①細胞の機能を測定する指標として，NK細胞活性やTリンパ球幼若化反応が測定されている。細胞の機能を測定する場合は，1検体につき少なくとも3～7mL程度の全血が必要になり，その後の処理も迅速に行わなければならない(通常24時間以内)。そのように処理しなければならない理由として，採血後は細胞自体が徐々に死滅するためである。

NK細胞活性とリンパ球幼若化反応の測定法について各々その原理と方法を解説する。まず，NK細胞活性は一定数のがん細胞と一定数の健常者のリンパ球を混ぜ，がん細胞が消滅する割合を計算する。がん細胞は放射性物質クロム-51(^{51}Cr)で標識されており，NK細胞によって死滅するがん細胞が多いほど^{51}Crが遊離される。逆にいえば，^{51}Crが遊離される割合が低いほど，NK細胞の機能不全が起きている可能性がある。なお，NK細胞活性とがん罹患に関する成人男女3600名に関する埼玉がんセンターの大規模コホート研究では，被験者をベースラインでNK細胞活性の高い順に3群に分け，11年後の全がん罹患率を比較したところ，最も

表1　免疫指標の種類と測定に必要な採血量

①機能的マーカー（全血5～7mL）	1 NK細胞活性
	2 リンパ球幼若化反応(PHA，Con A，PWM等)
②数・量的マーカー	1 リンパ球サブセット(NK，T，B，CD4＋細胞数)(全血2mL)
	2 非顕性ウイルスの再活性化(EBV等の抗体価)(全血5mL)
	3 各種炎症マーカー(IL-6，TNF-α，CRP)(血清1～2mL)
③バランスを測定するマーカー	1 Th1/Th2(IFN-γ/IL-4)比(血清1mL)
	2 CD4＋/CD8＋比(全血1mL)

表2　免疫指標と機能・役割

マーカー	機能・役割
ヘルパー(CD4＋)T細胞	抗体産生細胞(B細胞)やキラー細胞の活性化
NK細胞	初期のがん細胞やウイルス感染細胞を殺傷
B細胞	抗体産生
免疫グロブリン	異物の抗原性を不活性化させる等(IgG，IgM，IgA等)
CRP，IL-6，TNF，単球	生体内の炎症反応と関連
Th1/Th2	サイトカイン産生による免疫系のバランス
特異的ウイルスの抗体価	非顕性ウイルスの再活性化(HHV，EBVに対する抗体価等)

NK細胞活性が高い群に比べ最も低い群で1.59倍全がん罹患率のリスクが高まることを報告している(図5)[12]。

一方，リンパ球幼若化反応(リンパ球芽球化反応とも呼ぶ)はリンパ球を単位体積当たり一定数の濃度にし，DNA前駆物質である放射性物質トリチウムチミジン(^{3}H)とともにリンパ球を刺激する薬剤(PHA, Con A, PWM等)を添加し，一定時間培養する(5% CO_2インキュベーター，37℃)。リンパ球はリンパ球刺激剤に曝露すると，刺激を受け大型化し，分裂・増殖する。その際にDNA合成時に取り込まれた^{3}H量を測定し(取り込まれ量をcount per minute(cpm)で表す)，リンパ球の分裂・増殖能力を測る。分裂・増殖能力が低いとリンパ球の機能の低下の可能性が示唆される。測定の単位は刺激指数(添加時の^{3}H取り込みを非添加時の^{3}H取り込みで除した値)として計算される。なお，リンパ球刺激剤の種類によっても刺激するリンパ球が異なることがあるため，解釈には注意を要する。例えば，PHAはCD8+T細胞よりもCD4+T細胞を強く活性化するが，Con AはCD8+T細胞を強く活性化する。

次に，②数・量的マーカーについて解説する。まず1番目のリンパ球サブセットは，NK細胞，T細胞，B細胞，ヘルパー(CD4+)T細胞，細胞傷害性T細胞がリンパ球全体の中でどの程度の割合を占めるかを測定する手法である。これらはフローサイトメーターという細胞測定装置によって測定可能である。単位体積当たりの数を割り出すためには，リンパ球数を全自動血球計数器で測定し，個々のリンパ球サブセットの割合にリンパ球数を乗じて絶対数を算出する。

このようにNK細胞数やヘルパーTリンパ球数を算出するには次のような意義がある。例えば，NK細胞活性が高くてもその数が極端に少なければ，がん細胞を消滅させる効率が悪い可能性がある。同じようにヘルパーT細胞数が極端に少なければ，免疫系を制御するリンパ球が少ないことになり，機能不全につながる。後天性免疫不全症候群(AIDS)はヘルパーT細胞が標的細胞であり，ヘルパーT細胞数はAIDSウイルスの感染によって激減する。それによって日和見感染，カポジ肉腫やカンジタ症など，免疫機能が通常通り機能していれば発症しない感染症が増加する。逆にヘルパーT細胞数が多すぎると，炎症の亢進が起きている可能性が考えられる。

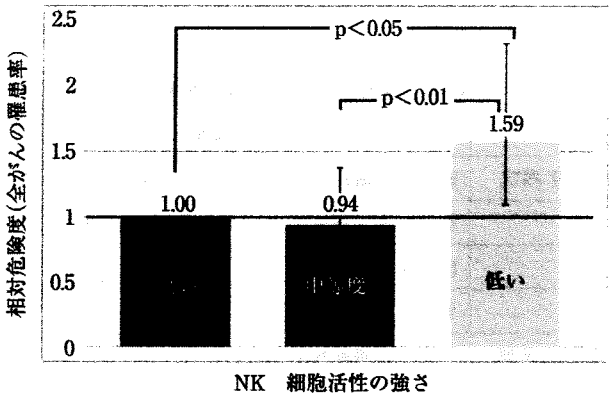

図5　NK細胞活性の強さによる全がん発生率の11年間の疫学調査[12]
(文献12より作成)

一方，2番目の非顕性ウイルスの再活性化は個体の免疫機能が低下した際によく見受けられる現象である。例えば，サイトメガトウイルス，EBウイルスやヘルペスウイルス(HHV)は通常，幼小児期に不顕性感染の形で感染し，生涯その宿主に潜伏感染する。免疫機能が抑制された状態で再活性化し，種々の病態を引き起こす。これらのウイルスは血中の特異的IgMやIgGを蛍光抗体法や酵素抗体法によって測定する。通常，これらの値が高いほど，ウイルスの再活性化が起こっていると想定され，免疫不全と関連すると考えられている。なお，最近では，抗ヘルペスウイルス抗体価は唾液によっても測定できるようになった[13]。

3番目の各種炎症マーカーであるが，炎症マーカーとして良く用いられているのは健康診断でも度々測定されるC反応蛋白(C reactive protein，CRP)である。CRPは体内で炎症や組織細胞の破壊などが起こると肝臓で生産されて血液中に放出され，増加する。通常，何らかの疾患(急性心筋梗塞，悪性腫瘍，自己免疫疾患，肺炎等)や外傷や熱傷(けが，火傷)による急性炎症がない限りは，血液の中にはほんの微量しか含まれない蛋白質である。そのほか，CRPを誘導する炎症性サイトカインであるInterleukin(IL)-6や腫瘍壊死因子(Tumor Necrosis Factor)-αなどがCRPに次いで良く測定されるが1検体あたりの価格は比較的高額である。ストレスとの関連では，これらの炎症マーカーの高値が持続する慢性炎症が問題となり，心疾患，糖尿病，脳卒中などの早期マーカーとして注目されている。なお，最近は血清50 μL程度でサイトカインを一括して測定するマルチプレックスサスペンションアレーというシステムが開発され，炎症性サイトカイン数種類を含む，10種類以上のサイトカインが同時測定可能である。

CRPとIL-6は健常者においては正の相関がある。健常者191名を対象に高感度CRP(hs-CRP)，IL-6ならびにTNF-αを同時測定し，スペアマンの順位相関係数(rs)を計算したところ，hsCRPとIL-6は$rs=.412$($p<0.001$)，hsCRPとTNF-αは$rs=.378$($p<0.001$)，IL-6とTNF-αは$rs=.456$($p<0.001$)であり，中等度の関連の強さであった。なお，これらの値は正規分布を示さないことが多いので，パラメトリック検定にて統計処理を行う場合は対数変換等の変換処理を事前に行う必要がある。対数変換によっても正規化しない場合は，Box-Cox変換，平方根変換やBlom's normal score変換などによって正規分布に近くなるような処理をする必要がある。

最後に③バランスを測定するマーカーについて解説する。バランスを測定するマーカーで最も重要と考えられるのは，ヘルパーT細胞が産生するサイトカインのバランスである。**図6**にヘルパーT細胞の分化の選択的方向性と種類を示す。ヘルパーT細胞は，特定のサイトカイン産生パターンを有しないナイーブヘルパーT細胞(ナイーブTh細胞：Th0細胞)が大部分を占めるが，APC(抗原提示細胞)が産生するサイトカインにより細胞性免疫に関与する1型ヘルパーT(Th1)細胞に分化するか，プロスタグランジンE_2(PGE_2)や細胞傷害性T細胞，NK細胞等が産生するサイトカインによって液性免疫に関与する2型ヘルパーT(Th2)細胞へと分化するかが決定される。Th1細胞は細胞内病原体，ウイルス，バクテリアに対する免疫作用を有する一方(細胞性免疫)，Th2細胞は寄生生物に対する免疫作用を有する(液性免疫)。Th1細胞が産生するサイトカインであるIFN-γ，IL-2，TNF-βはTh2細胞の反応を抑制し，Th2細胞が産生するサイトカインであるIL-4，IL-5，IL-6，IL-10，IL-13はTh1細胞の

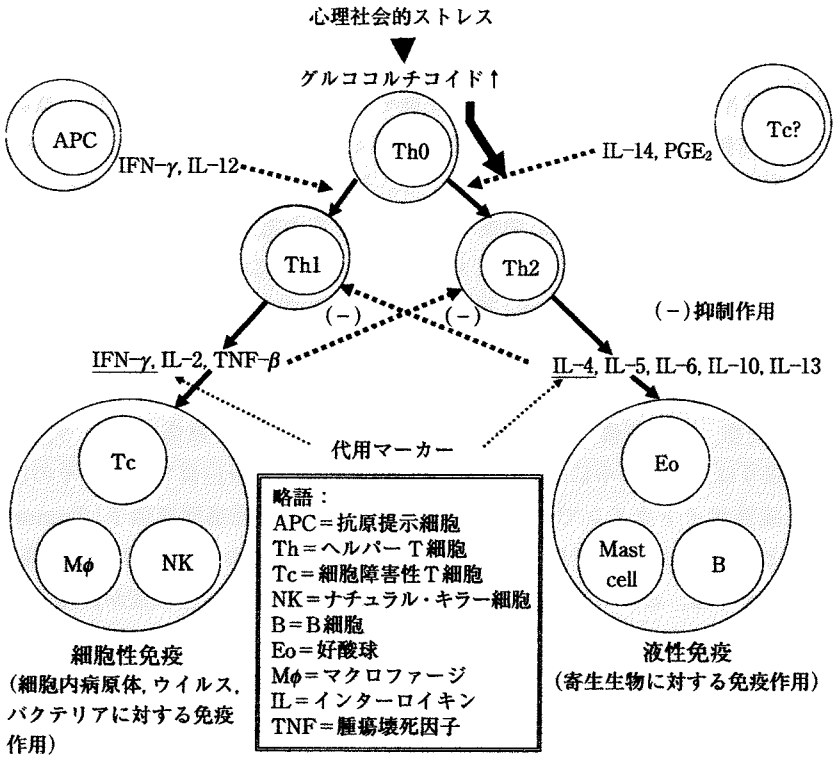

図6　心理社会的ストレスによるTh1ならびにTh2細胞への影響と細胞性・液性免疫の調節

反応を抑制し，相互抑制的に働きバランスをコントロールする。

ストレスが負荷されるとグルココルチコイドが視床下部-下垂体-副腎(HPA)系を介して産生される。グルココルチコイドはTh0細胞からTh2細胞へと分化を促す。Th1細胞にはストレスホルモン受容体があるため，その刺激でTh1サイトカインの分泌が低下し，細胞内病原体やウイルス感染を抑制する細胞性免疫機能が抑制され，Th2細胞の活性が上昇して液性免疫反応が亢進される。その結果，IgEなどの免疫グロブリンの産生が亢進される。ストレスの負荷により，アレルギーや喘息の症状が悪化するのはそのためである。ストレス研究では，Th1が産生するIFN-γとTh2が産生するIL-4の比を計算することで間接的に細胞性免疫と液性免疫のバランスを測定する方法がある(代用マーカー)。

そのほかに，バランスを測定するマーカーとして用いられている指標として，CD4+T細胞率とCD8+T細胞率の比であるCD4+/CD8+比がある。通常，CD4+/CD8+比はAIDSや伝染性単核症などへの感染や心筋梗塞，骨髄移植後に低下するが，ストレスによっても低下することが報告されている。しかし，CD4+/CD8+比はストレスによって上昇することも報告されていることから，感染等の免疫不全がない場合，ストレスによる免疫影響を示す単独の指標として十分ではないと考えられる。以上，3種の異なる指標について解説した。ストレスの免疫影響を考える際，これらを組み合わせて総合的に考慮する必要がある。

5　ヒトを対象とした仕事のストレスと免疫系の研究

仕事のストレスといってもその原因となる要素は多種多様である。単純に仕事の量が多いことや働く時間が長いことをはじめとして，やりがいを感じない仕事，仕事に満足できない，裁量権が低い，通勤時間が長い，上司や同僚とのコミュニケーション不足による感情的対立が強い，リストラされるかもしれないという不安等の個人的要因から，部署間の折り合いが悪い，倒産，企業合併，経済不況等のグループ，企業，国・世界単位のストレスもある。その他，職場における騒音，換気や照明の悪さ等の物理的に不衛生な環境等に加え，有期，パート，派遣労働などの身分不安定によるストレスなど枚挙に暇がない。さらに，ストレスによる蓄積疲労や長時間労働による慢性睡眠不足等によって引き起こされる抑うつ等がある。これらの全てが単一の原因によって労働者の不健康が引き起こされるわけはなく，多くの労働者は長い労働生活の中でさまざまなストレスを経験し，ストレスの蓄積によって身体的・精神的疾患が発症する。

では，仕事のストレスは免疫系にどの様な影響を与えるであろうか。ここでは，これまでに開発された職業性ストレスモデルに基づいた研究結果について解説する。

5.1　仕事の要求度-コントロールモデルと免疫指標

職業性ストレスモデルの中で最も有名かつ多用されたモデルは「仕事の要求度-コントロールモデル」である（図7）[14]。このモデルは，仕事の量的および心理的負担を示す要求度と，職務に対する権限や技術の活用度を示すコントロールの2つの要素から構成され，仕事の要求度が高くコントロールが低い組み合わせのストレイン状態に曝露すると，各種の疾患に対する罹患の可能性が高くなると仮定している。実際，高ストレインの持続は，冠動脈性心疾患[15]，脳卒中[16]，糖尿病[17]，筋骨格系障害[18]ならびに抑うつ[19]などの増加を予測することが確認されている。仕事の要求度-コントロールモデルと免疫指標との関連は，これまでに10本以上の論文が報告されている[20]。

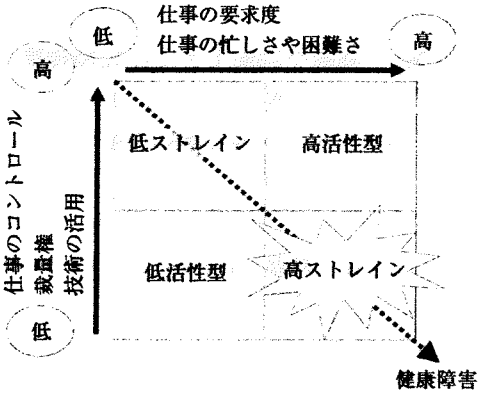

図7　仕事の要求度-コントロールモデル[14]

初期の研究では，空港管理官，内科医，ウエイター，音楽家など多業種のスウェーデン人勤労者の仕事のストレインと免疫グロブリンとの関連，仕事のストレインが上昇するとともにIgG値が上昇することが報告されている[21]。また，仕事のストレインが高い者ほど社会的支援の緩衝効果が働き，IgG値の上昇が抑えられることが判明した。しかし，その後この研究グループによりサービス業の労働者を対象に同様の効果を検討したところ追認できなかったが[22]，我が国の研究では仕事のストレインに伴うIgG値の上昇が確認された[23]。

仕事の要求度-コントロールモデルと細胞性免疫，なかでもリンパ球分画数との関係は我が

国[23]やオランダ[24]から報告されている。これらで共通して認められたのは，仕事のストレインがCD4＋T細胞数やNK細胞数が低下することである。また，若年者では仕事の要求度が高いこと，管理職の多い中高年者では仕事のコントロールが低いこと[25]がCD4＋T細胞数を低下させる要因であることから，年齢や職位によってその関連が異なる可能性が認められた。

仕事の要求度-コントロールモデルは冠動脈性心疾患を予測する[26]。このことから，ストレスが原因で血管内の慢性炎症反応が亢進する可能性が考えられる。CRP，IL-6，TNF-α，総白血球数や単球数など炎症関連因子の血中での増加が心疾患や無症候性アテローム性動脈硬化のマーカーとなることから[27]，要求度-コントロールモデルとこれら炎症関連因子との関係が報告されるようになった[28-32]。

Shiromら[33]は仕事の要求度，コントロール，社会的支援のレベルが18ヶ月後の白血球数とCRPを予測するか否かを調べたが，有意な関連は見出されなかった。一方，逆の仮説，すなわち，炎症関連物質の上昇が職業性ストレスの上昇を予測するかということも検討したが，支持されなかった。このことから，仕事のストレインによる冠動脈性心疾患の増加は別の作用機序で説明される可能性があると彼らは結論づけた。同様の結果は，Claysら[30]，Hemingwayら[28]，Sunら[32]の横断研究でも明らかにされているが，Schnorpfeilら[29]が要求度とCRPの正の相関，コントロールと負の相関を報告していることから，必ずしも関連がないとは言いきれない。今後，より優れた研究デザインで検討される必要がある。

5.2　努力-報酬不均衡モデルと免疫指標

「努力-報酬不均衡モデル」は，仕事の要求度-コントロールモデルに次いで頻用されたストレスモデルであり(**図8**)，ドイツの社会学者シーグリストにより開発された[34]。このモデルでは，経済(金銭)的，心理(尊重)的あるいはキャリア(仕事の安定や昇進)的報酬要素を重視し，仕事において費やす努力と，そこから得られるべき，もしくは得られることが期待される報酬が得られない高努力/低報酬状態をストレスフルとする。また，高努力/低報酬状態はオーバーコミットメント(内在的努力)，すなわち状況面からのみならず，仕事に過度に傾注する個人の態度や行動パターンによってさらに増強されるとする。

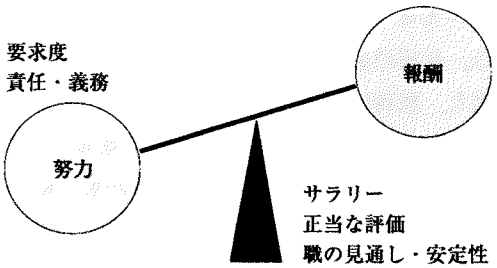

図8　努力・報酬不均衡モデル[34]

このモデルによる健康障害の予測性は優れており，近年数多くの研究が発表されている[35]。最近になって努力-報酬不均衡モデルと仕事の要求度-コントロールモデルを組み合わせて健康障害を予測する研究が報告されるようになり，高ストレインかつ高努力-低報酬状態は高い鋭敏性をもって，幅広い健康障害と関連することが判明した[36]。努力-報酬不均衡モデルがさまざまな生理指標と関連することも近年になって明らかにされ，高努力/低報酬状態が続くと心拍数[37]や血圧の上昇[38]やカテコールアミン[39]やコルチゾール[40]が増加することが報告されており，高努力/低報酬で心疾患の増加の生理的背景が明らかになりつつある。

努力-報酬不均衡モデルと免疫指標との関連は今日までにあまり多くの報告がなされていないが，ドイツ，オーストラリア，ヨルダンならびに日本からの報告がある[41)-44)]。Boschら[41)]はドイツ人工場労働者において高努力/低報酬状態が免疫系の老化と関連するかを検討した。高努力/低報酬状態はCD4+/CD8+比とCD8+CD27-CD28-T細胞数が正の相関を示すことを報告し，免疫機能の老化の進行を早めている可能性を見出した。一方，Bellingrathら[42)]は，高努力 低報酬の状態が続くとT細胞やNK細胞数は低下するが，さらに新たなストレスが加わると，それらの細胞が適切に反応しにくいことを報告した。高努力/低報酬状態は粘膜免疫能の低下を示す，唾液中のIgAの分泌の低下65)やCRPの上昇[43)]と関連することも示されている。

日本人ホワイトカラー従業員の場合では，高努力 低報酬状態はNK細胞数の低下と関連し，報酬はNK細胞活性とNK細胞数の両者の上昇と関連した[45)]。報酬の3つの要素の中でも，尊重と仕事の安定・昇進はNK細胞活性とNK細胞数を増加させる上で重要な役割を果たしていることが明らかにされている。しかしこの関係は，男性においてのみ認められた。なお，オーバーコミットメントとNK細胞の間では有意な関連は見出されなかった。

最近，努力-報酬不均衡モデルと免疫指標に関するシステマティックレビューが報告され，7本の論文についてまとめられた[46)]。その結果，ストレス状態が高い高努力/低報酬であると各種の免疫機能が低下状態に陥るとされている。具体的には，NK細胞活性などの細胞性免疫の低下，唾液IgAの低下，炎症マーカー(CRP，IL-6，TNF)の亢進が認められた。以上から，努力-報酬不均衡モデルは，職業性ストレスに対する生理学的変化を捉える鋭敏な指標となりえる可能性を秘めており，今後この領域におけるさらなる研究が望まれる。

5.3　NIOSH職業性ストレスモデルと免疫指標

NIOSHの職業性モデルには多様な職業要因が考慮されており，図9に示すように，仕事のストレッサーのみではなく，仕事外の要因(家族の要求，介護・育児の負担，等)，緩衝要因(社会的支援)，個人要因(年齢，性別，職種，パーソナリティー，自尊感情，等)を総合的に考慮する。ここでは，その中でもほかのストレスモデルでは扱われていない，対人関係と職務満足感と免疫指標について概説する。

NIOSHの職業性ストレスモデルの中には，対人関係に注目した尺度が2つある。それぞれ，グループ内対人葛藤とグループ間対人葛藤と呼ばれ，前者は自分が所属するグループ，部署，

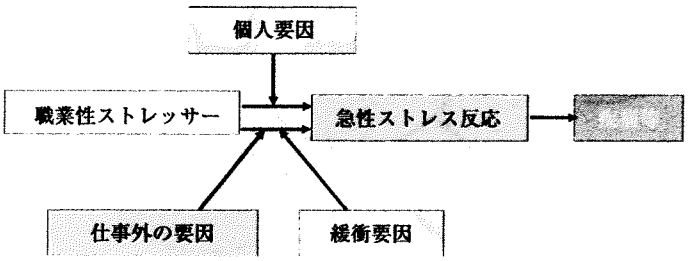

図9　NIOSHの職業性ストレスモデル

チーム内などの比較的身近な人間関係における心理的葛藤であり，後者はグループ対グループ，部署対部署などの一定の距離がある人間関係における心理的葛藤である。韓国人の女性看護師41名を対象とした研究では[47]，対人葛藤が高いとIL-1βならびに唾液IgAの値が高くなると報告した。しかし，対人葛藤の種類については明示されていない。筆者らは173名の健康なホワイトカラー労働者を対象に対人葛藤（グループ内，グループ間）とNK細胞活性，リンパ球サブセット数（NK，T，B，CD4），血清免疫グロブリン（IgG，IgA，IgM）の関連を横断研究により検討した。その結果，グループ内対人葛藤の得点が高いほどNK細胞数の低下とIgA値の上昇が認められた（交絡因子調整後）[48]。一方，グループ間対人葛藤と免疫指標の関連は認められなかった。日本人では，職場において対人関係によるストレスの発生率が高いこと，特に身近な人間関係の問題が多いと推察されることから，対人関係における心理的葛藤を緩和するプログラムが望まれる。

次に，職務満足感に関する研究であるが，職務満足感は組織心理学領域において最も研究が発展したと言える。職務満足感の健康影響に関する485の論文に基づいたメタ分析によると，職務満足感が低いと不安（ρ=.420），燃え尽き（ρ=.478），抑うつ（ρ=.428），低い自己評価（ρ=.429），自己申告による身体的不健康（ρ=.287）および心疾患（ρ=.121）と強い関連を示すことが明らかにされている[49]。職務満足感は職業上の諸要因すなわち，賃金，仕事の安定性や将来性，昇進の機会，独立性，仕事のストレス，職場の公平性など総合的判断に基づき規定されると考えられている（図10）。近年の不況により，世界的に職務満足感が低下傾向にあることも報告されており[50]，職務満足感を高めることが労働者の健康を守る上で重要な要素であると考えられる。

職務満足感と免疫系の関連では，スウェーデン人女性労働者の職務満足感がIL-6の低下と関連するが，男性では関連がないことが報告されている[51]。イタリア人看護師を対象とした調査では，職務満足感が低いと1年後のIL-1β，IL-6，CD8+CD57+T細胞数が上昇することが報告されている[52]。日本のデータでは，職務満足感がNK細胞活性とNK細胞数の両方と正の相関を示すことが報告されている（図11）[53]。なお，職務満足感が低い者ほど風邪の罹患

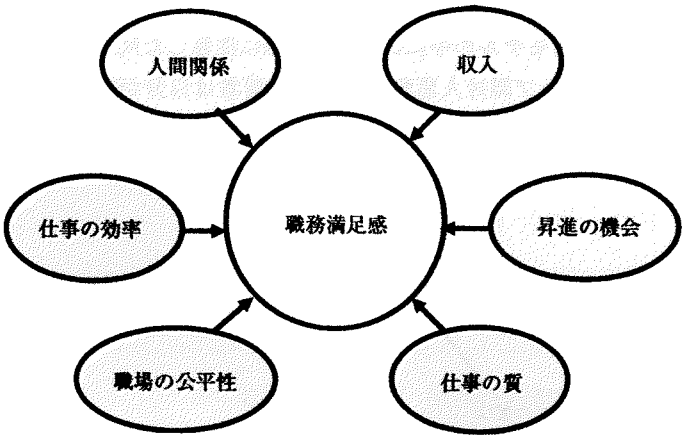

図10　職務満足感を規定しうる職業因子

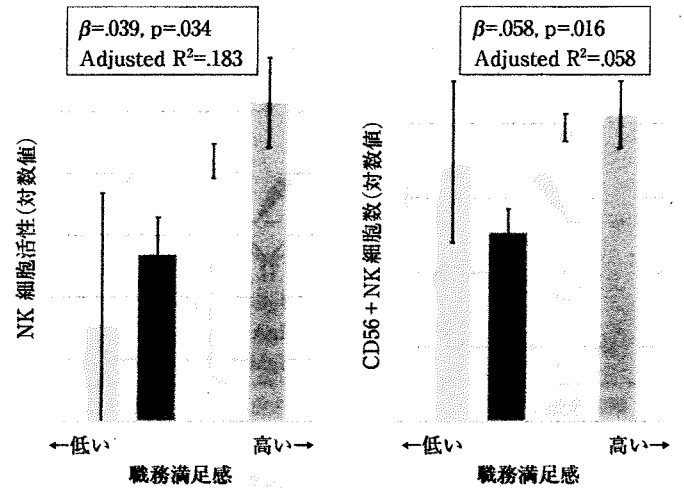

図11　職務満足感とNK細胞活性(左)とNK細胞数(右)の関連[53]

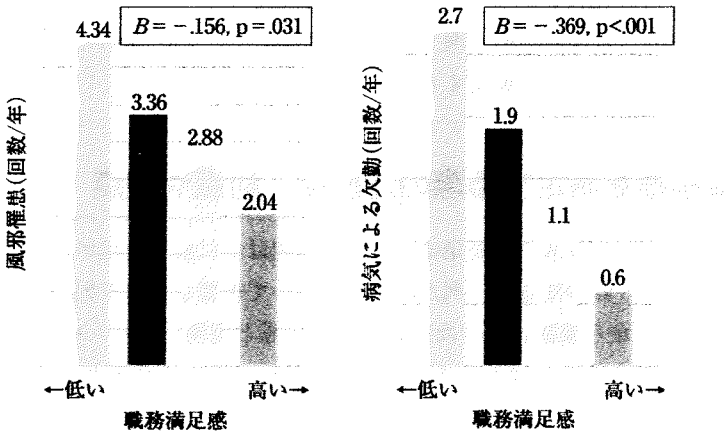

図12　職務満足感と過去1年間の風邪罹患回数ならびに病欠回数の関連[54]

回数や病欠回数が強いことも報告されている(**図12**)[54]。

一方，職務満足感と免疫系の関係は，ノルウェー人女性看護師の職務満足感と血清IgA値と補体C3が負の相関を示し，IgGやIgM値とは関連がないことが報告されている[55]。イスラエル人労働者を対象とした研究では，男性労働者において個別的満足感とCRPと負の相関，女性では統計的有意性のない負の相関が認められている[31]。これらの結果から，職務満足感と健康の関連を裏づける，免疫学的メカニズムが存在することが考えられた。

以上の職業性ストレスの免疫指標への影響をまとめると**図13**のようになると考えられる。すなわち，図6に示したように職業性の心理社会的ストレスによって①細胞性免疫系の抑制，②炎症性物質の増加，③免疫グロブリン特にIgGやIgMの増加，そして④Th1/Th2のサイトカインのアンバランスが引き起こされる。これらの現象が同時に起こるのか否かはストレッ

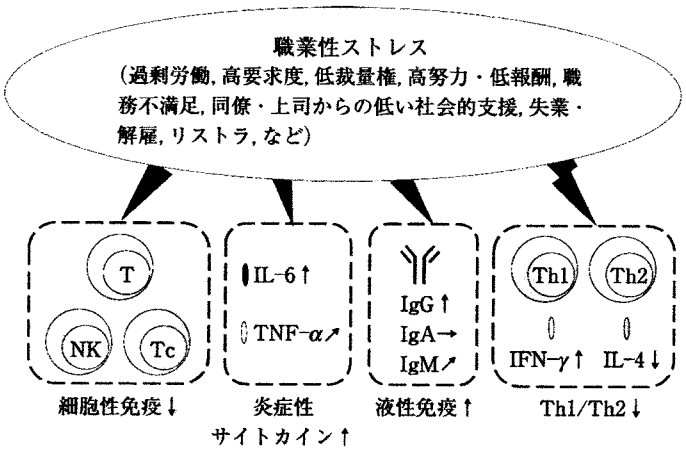

図13　職業性ストレスの各種免疫指標への影響のまとめ

サーへの曝露のタイミングや持続時間(急性，慢性)，ストレスの種類や強度によって異なると考えられる。

次の項ではストレスと免疫指標に関する研究において，上記を含めて測定上の注意点，指標の利点・欠点，解釈等について概説する。

6　免疫指標を測定する際の注意点，利点や欠点

ここで取り上げたストレスの免疫指標に関する研究は主に血液を用いたものである。そこで，血液の免疫指標を扱う際の注意点，利点・欠点について重要な項目を列挙する。まず，採血は侵襲性を有することから被験者への研究参加への意思と説明(インフォームドコンセント)をしっかり行う必要があることは言うまでもない(**表3**)。研究への参加率に関していえば，職域では産業保健スタッフと十分なコミュニケーションを取れば8割程度は望める。男女の参加率に関しては，筆者の経験では，男性の被験者の参加率は女性のそれよりも低い傾向であった。一方，血液を扱う実験者も感染のリスクがあること，採血後は速やかに分析を行える準備を整えておく必要がある。また，蛍光抗体等の消耗品は比較的高額であること，実験の条件統制(どの時間帯に採血するか，被験者の実験当日の体調等)を十分に考慮する必要がある。筆者が職域を対象に行った調査では，バイオリズムを統制するために日勤労働者の午前9時から11時の間に採血を行い，すべて24時間以内に測定を終了した。そのほか，信頼出来る免疫データを得るためには予備実験を繰り返し，十分な準備を行うこと，免疫指標に影響する多様な交絡因子を考慮することなどがある。交絡因子として重要なのは，まず性別，年齢や人種に加え，教育歴，婚姻歴，生活習慣(喫煙，睡眠，運動習慣，食生活，飲酒習慣)，薬物(降圧剤，

表3　免疫指標を測定する際の注意点

1	侵襲性を有する
2	それ故，被験者を説得しにくい
3	実験者も感染のリスクがある
4	消耗費等の研究費が高い
5	多様な交絡因子を考慮
6	条件統制が困難なこともある
7	実験設備・人手・経験が必要

ストロイド等)使用の有無，体格指数(BMI)，メンタルヘルス，仕事外の要因(介護・育児の有無)，経済状況，職務内容や職位等が挙げられる。

一方，利点としてはストレスと疾患の関連の免疫学的メカニズムの解明にアプローチできることや結果指標として客観的マーカーであること，生活習慣との関連が密接であること等がある(表4)。

表4　免疫指標を測定する際の利点

1	客観性が高い
2	メカニズムの解明に迫れる
3	様々な疾患(感染症，アレルギー，がんなど)の媒介因子となりえる
4	生活習慣と関連が深い

7　ストレスへの曝露後のどの時点で免疫指標を測定すべきか

ストレスへの曝露後の一般的な免疫反応の動態について，模式図を示した(図14)。急性ストレスへの曝露では，免疫反応は一時的に亢進する。この反応はストレスの強度や種類にもよるが，スピーチ課題と暗算課題のような軽度のストレスの組み合わせ(これをトリーア社会的ストレステスト，Trier Social Stress Test(TSST)と呼ぶ)では，ストレス曝露直後から90分以内に各種の免疫指標の上昇が起こる(図の左側)。例えば，次のような実験がある。被験者を，仕事のストレスが高い群と低い群にあらかじめ2群に分け，これらの被験者に新たな急性ストレス(暗算課題とスピーチ課題)を負荷する[56]。ストレス負荷45分前と課題終了1，10，20，30，90分後にリンパ球分画数(ヘルパーT細胞とNK細胞)ならびにサイトカイン(TNF-α，IL-6ならびにIL-10)の変化を調べたところ，ストレス負荷直後1分でリンパ球分画数とTNF-αは両群とも10%～150%増加した。しかし，群別に見ると，低ストレス群では急性ストレスの曝露によりIL-6ならびにIL-10の濃度は上昇するが，高ストレス群では逆に抑制された。この研究結果から，ストレス負荷直後よりリンパ球分画数とサイトカイン値は比較的鋭敏に反応するが，生体のストレス負荷の状態(ストレスの高低)によって免疫指標への影響は異なることである。

一方，ストレスへの曝露が慢性化し，強度が強くなるにつれて，免疫反応の低下状態が持続する(図の右側)。例えば，失業のような慢性ストレスが負荷された状態では，NK細胞活性は10～20%低下した状態を維持するが，ストレスから解放されると(新しい仕事を見つけた場合)，その1ヶ月後には失業前のレベルあるいは雇用されている対照群と同レベルまで回復する[57]。

以上から，ストレス負荷後の免疫反応は時間経過とともに変化することから，測定は可能な限り継時的(特に急性期)に行うことをお勧めする。

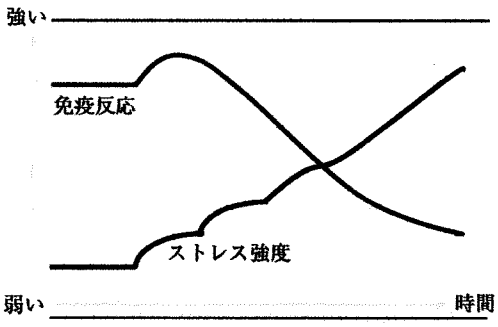

図14　ストレスの強さと免疫機能
弱い急性のストレスだと免疫機能の増強が起こるが，強い慢性のストレスだと免疫機能の低下が起こる

8 おわりに

ストレスと免疫指標の関連について，これまでに国内外で行われた研究を中心に概説した。また，免疫指標を測定する上での問題点や注意点，測定のタイミング等を筆者の経験に基づき列挙した。今後は，ストレス評価にセンサー，加速度計やソシオメーターなどの客観的なマーカーを用いて評価することが期待される。その他，仕事にはやり甲斐や楽しみ，生きる喜び等のポジティブな側面もあるため，それらの点についても考慮し，ストレスの免疫指標の精度を高める必要がある。

文　献

1) 厚生労働省：労働者健康状況調査(2012).
2) 日本人の就業実態に関する総合調査：労働政策研究・研修機構(2009).
3) 厚生労働省：平成28年度版過労死等防止対策白書(2016).
4) Lazarus, R.S., Folkman, S.: Stress, appraisal, and coping. Springer Publishing Company, New York. (1984).
5) Weiss, J. M.: Effects of coping behavior in different warning signal conditions on stress pathology in rats. *J Comp Physiol Psychol*, **77**, 1-13(1971).
6) Nakata, A., Araki, S., Tanigawa, T., Sakurai, S. and Yokoyama, M.: Effects of uncontrollable and controllable electric shocks on T lymphocyte subpopulations in the peripheral blood, spleen, and thymus of rats. *Neuroimmunomodulat*, **3**, 336-341(1996).
7) 谷川武, 荒記俊一, 中田光紀：制御可能性が免疫系に及ぼす影響．行動医学研究, **3**, 16-19(1996).
8) Lewis, J. W., Shavit, Y., Terman, G. W., et al. : Stress and morphine affect survival of rats challenged with a mammary ascites tumor(MAT 13762B). *Nat Immun Cell Growth Regul*, **3**, 43-50(1983).
9) Shavit, Y., Ryan, S. M., Lewis, J. W., Laudenslager, M. L., Terman, G. W., Gale, R. P. and Liebeskind, J. C. : Inescapable but not escapable stress alters immune function. *Physiologist*, **26**, A64(1983).
10) Laudenslager, M. L., Ryan, S. M., Drugan, R. C., Hyson, R. L. and Maier, S. F.: Coping and immunosuppression: Inescapable but not escapable shock suppresses lymphocyte proliferation. *Science*, **221**, 568-570(1983).
11) 田中正敏：ストレスそのとき脳は？講談社(1987).
12) Imai, K., Matsuyama, S., Miyake, S., Suga, K. and Nakachi, K.: Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet*, **356**, 1795-1799(2000).
13) 近藤一博：ヘルペスウイルス感染と疲労．ウイルス, 55, 9-18(2005).
14) Karasek, R.A. : Job demands, job decision latitude, and mental strain: Implication for job design. *Admin Sci Q*, **24**, 285-307(1979).
15) Kivimaki, M., Virtanen, M., Elovainio, M., et al.: Work stress in the etiology of coronary heart disease--a meta-analysis. *Scand J Work Environ Health*, **32**, 431-442(2006).
16) Tsutsumi, A., Kayaba, K. and Ishikawa, S. : Impact of occupational stress on stroke across occupational classes and genders. *Soc Sci Med*, **72**, 1652-1658(2011).
17) Heraclides, A., Chandola, T., Witte, D. R. and Brunner, E. J. : Psychosocial stress at work doubles the risk of type 2 diabetes in middle-aged women: evidence from the Whitehall II study. *Diabetes Care*, **32**, 2230-2235(2009).
18) Rugulies, R. and Krause, N. : Job strain, iso-strain, and the incidence of low back and neck injuries. A 7.5-year prospective study of San Francisco transit operators. *Soc Sci Med*, **61**, 27-39(2005).
19) Bonde, J. P.: Psychosocial factors at work and risk of depression: a systematic review of the epidemiological evidence. *Occup Environ Med*, **65**, 438-445(2008).

20) 中田光紀，池田智子：職業性ストレスの免疫学的評価—系統的展望．産業医学レビュー，**24**, 253-269 (2012).

21) Theorell, T., Orth-Gomer, K. and Eneroth, P. : Slow-reacting immunoglobulin in relation to social support and changes in job strain: a preliminary note. *Psychosom Med*, **52**, 511-516 (1990).

22) Ohlson, C. G., Soderfeldt, M., Soderfeldt, B., Jones, I. and Theorell, T.: Stress markers in relation to job strain in human service organizations. *Psychother Psychosom*, **70**, 268-275 (2001).

23) Nakata, A., Araki, S., Tanigawa, T., et al.: Decrease of suppressor-inducer (CD4 + CD45RA +) T lymphocytes and increase of serum immunoglobulin G due to perceived job stress in Japanese nuclear electric power plant workers. *J Occup Environ Med*, **42**, 143-150 (2000).

24) Meijman, T. F., van Dormolen, M., Herber, R. F. M., Rogen, H. and Kuiper, S.: Job strain, neuroendocrine activation, and immune status. In S. L. Sauter and L. R. Murphy (eds), Organizational risk factors for job stress. Washington, DC: American Psychological Association, 113-126 (1995).

25) Nakata, A., Tanigawa, T., Fujioka, Y., et al.: Association of low job control with a decrease in memory (CD4 + CD45RO +) T lymphocytes in Japanese middle-aged male workers in an electric power plant. *Ind Health*, **40**, 142-148 (2002).

26) Kivimaki, M., Virtanen, M., Elovainio, M., et al.: Work stress in the etiology of coronary heart disease--a meta-analysis. *Scand J Work Environ Health*, **32**, 431-442 (200).

27) Danesh, J., Wheeler, J. G., Hirschfield, G. M., et al. : C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. *N Engl J Med*, **350**, 1387-1397 (2004).

28) Hemingway, H., Shipley, M., Mullen, M. J., et al.: Social and psychosocial influences on inflammatory markers and vascular function in civil servants (the Whitehall II study). *Am J Cardiol*, **92**, 984-987 (2003).

29) Schnorpfeil, P., Noll, A., Schulze, R., et al.: Allostatic load and work conditions. *Soc Sci Med*, **57**, 647-656 (2003).

30) Clays, E., De Bacquer, D., Delanghe, J., et al.: Associations between dimensions of job stress and biomarkers of inflammation and infection. *J Occup Environ Med*, **47**, 878-883 (2005).

31) Shirom, A., Toker, S., Berliner, S., Shapir, I. and Melamed, S.: Work-related vigor and job satisfaction relationships with inflammation biomarkers among employed adults. In A. Fave (ed), Dimensions of well-being: Research and intervention. Milano: Franco Angeli, 254-274 (2006).

32) Sun, J., Wang, S., Zhang, J. Q. and Li, W.: Assessing the cumulative effects of stress: The association between job stress and allostatic load in a large sample of Chinese employees. *Work & Stress*, **21**, 333-347 (2007).

33) Shirom, A., Toker, S. and Berliner, S. and Shapira, I. : The job demand-control-support model and stress-related low-grade inflammatory responses among healthy employees: A longitudinal study. *Work & Stress*, **22**, 138-152 (2008).

34) Siegrist, J.: Adverse health effects of high-effort/low-reward conditions. *J Occup Health Psychol*, **1**, 27-41 (1996).

35) Tsutsumi, A. and Kawakami, N.: A review of empirical studies on the model of effort-reward imbalance at work: reducing occupational stress by implementing a new theory. *Soc Sci Med*, **59**, 2335-2359 (2004).

36) Dai, J. M., Collins, S., Yu, H. Z. and Fu, H.: Combining job stress models in predicting burnout by hierarchical multiple regressions: a cross-sectional investigation in Shanghai. *J Occup Environ Med*, **50**, 785-790 (2008).

37) Hintsanen, M., Elovainio, M., Puttonen, S., et al. :Effort-reward imbalance, heart rate, and heart rate variability: the Cardiovascular Risk in Young Finns Study. *Int J Behav Med*, **14**, 202-212 (2007).

38) Kjeldsen, S. E., Knudsen, K., Ekrem, G., et al. : Is there an association between severe job strain, transient rise in blood pressure and increased mortality? *Blood Press*, **15**, 93-100 (2006).

39) Lee, K. M., Kang, D., Yoon, K., et al. : A pilot study on the association between job stress and repeated measures of immunological biomarkers in female nurses. *Int Arch Occup Environ Health*, **83**, 779-789 (2010).

40) Chandola, T., Britton, A., Brunner, E., et al. :Work stress and coronary heart disease: what are the mechanisms? *Eur Heart J*, **29**, 640-648(2008).

41) Bosch, J. A., Fischer, J. E. and Fischer, J. C. : Psychologically adverse work conditions are associated with CD8 + T cell differentiation indicative of immunesenescence. *Brain Behav Immun*, **23**, 527-534(2009).

42) Bellingrath, S., Rohleder, N. and Kudielka, B. M.: Healthy working school teachers with high effort-reward-imbalance and overcommitment show increased pro-inflammatory immune activity and a dampened innate immune defense. *Brain Behav Immun*, **24**, 1332-1339(2010).

43) Wright, B. J: Effort-reward imbalance is associated with salivary immunoglobulin a and cortisol secretion in disability workers. *J Occup Environ Med*, **53**, 308-312(2011).

44) Almadi, T., Cathersm, I., Hamdan Mansour, A. M. and Chow, C. M. : The association between work stress and inflammatory biomarkers in Jordanian male workers. *Psychophysiology*, **50**, 821-830(2013).

45) Nakata, A., Takahashi, M. and Irie, M.: Effort-reward imbalance, overcommitment, and cellular immune measures among white-collar employees. *Biol Psychol*, **88**, 270-279(2011).

46) Eddy, P., Heckenberg, R., Wertheim, E. H., Kent, S. and Wright, B. J.: A systematic review and meta-analysis of the effort-reward imbalance model of workplace stress with indicators of immune function. J Psychosom Res(in press).

47) Yoon, H. S., Lee, K. M. and Kang, D.: Intercorrelation between immunological biomarkers and job stress indicators among female nurses: a 9-month longitudinal study. *Front Public Health*, **2**, 157(2014).

48) Sonoda, N., Nakata, A., Irie, M. and Takahashi, M.: The association of interpersonal conflict at the workplace immunity: A cross-sectional study among white-collar employees at a trading company. *Int J Psychophysiol*, **94**, 232-233(2014).

49) Faragher, E. B., Cass, M. and Cooper, C. L. : The relationship between job satisfaction and health: a meta-analysis. *Occup Environ Med*, **62**, 105-112(2005).

50) Sousa-Poza, A., Sousa-Poza, A., A. : Well-being at work: a cross-sectional analysis of the levels and determinants of job satisfaction. *J Socio-Econ*, **29**, 517-538(2000).

51) Theorell, T., Hasselhorn, H. M., Vingard, E. and Andersson, B., MUSIC-Norrtalje-Study-Group. : Interleukin 6 and cortisol in acute musculoskeletal disorders: results from a case-referent study in Sweden. *Stress Med*, **16**, 27-35(2000).

52) Amati, M., Tomasetti, M., Ciuccarelli, M., et al. : Relationship of job satisfaction, psychological distress and stress-related biological parameters among healthy nurses: a longitudinal study. *J Occup Health*, **52**, 31-38(2010).

53) Nakata, A., Takahashi, M., Irie, M. and Swanson, N. G.: Job satisfaction is associated with elevated natural killer cell immunity among healthy white-collar employees. *Brain Behav Immun*, **24**, 1268-1275(2010).

54) Nakata, A., Takahashi, M., Irie, M., Ray, T. and Swanson, N. G. : Job satisfaction, common cold, and sickness absence among white-collar employees: a cross-sectional survey. *Ind Health*, **49**, 116-121(2010).

55) Endresen, I. M., Vaernes, R., Ursin, H. and Tonder, O. : Psychological stress-factors and concentration of immunoglobulins and complement components in Norwegian nurses. *Work & Stress*, **1**, 365-375(1987).

56) Bellingrath, S., Rohleder, N. and Kudielka, B. M. : Healthy working school teachers with high effort-reward-imbalance and overcommitment show increased pro-inflammatory immune activity and a dampened innate immune defence. *Brain Behav Immun*, **24**, 1332-1339(2010).

57) Cohen, F., Kemeny, M. E., Zegans, L. S., Johnson, P., Kearney, K. A. and Stites, D. P.: Immune function declines with unemployment and recovers after stressor termination. *Psychosom Med*, **69**, 225-234(2007).

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産業ストレス研究(Job Stress Res.), 24, 213-218(2017)

【特集　産業ストレスのバイオロジー：生物学的指標の現在と展望】

毛髪・爪試料を利用した慢性的・蓄積的なストレスホルモン分泌の評価：産業ストレス研究における展望

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抄録：ストレスホルモンとして知られているコルチゾールは心理社会的なストレスと冠動脈疾患やうつなどの疾患を結びつける生物学的なメカニズムとして考えられている。本総説では毛髪や爪の試料からのコルチゾールの評価について紹介した。毛髪や爪のコルチゾールは過去に遡って数か月，あるいは数週間にわたるホルモン産生を反映していると考えられている。職場環境における心理社会的ストレスと毛髪・爪コルチゾールの関連を調べた研究はわずかな数であった。毛髪や爪の試料は職業性ストレスと健康の生物学的なメカニズムを探索する上で大いに役に立つと考えられ，さらなる研究が必要である。

Key words: cortisol（コルチゾール），hair（毛髪），fingernail（爪），job stress（職業性ストレス）

1．コルチゾールと職業性ストレス

職場環境における心理社会的ストレスが健康を阻害することが欧米では多く報告されている。その傾向は日本においても同様であり，最近では，いくつかのコホート研究によって，職業性ストレスは脳血管疾患の発症やうつなどのメンタルヘルスの悪化に関与していることが報告されている[1-3)]。

それとあわせて，職業性ストレスと健康を結びつける生物学的メカニズムについても多くの研究がおこなわれている。そのうち，有力なものの一つにコルチゾールをあげることができる。コルチゾールは副腎皮質から放出されるステロイドホルモンであり，急性のストレスは血中や唾液中のコルチゾール濃度を増加させることが一般的に知られている[4)]。また，それと同時にコルチゾールは，免疫系，血管系，中枢神経系に対して様々な生理的作用を有すことも知られており[5)]，また，精神疾患や心臓血管疾患との関わりも報告されている[6)]。そのようなことから，コルチゾールはストレスと病気を結びつけるホルモンとして注目されてきている。

職業性ストレスとコルチゾールの関連は過去に多く調べられている。例えば，井澤ら[7)]は，仕事の要求度―コントロールモデルと努力―報酬不均衡モデルにより評価された職業性ストレスとコルチゾールの関連について系統的なレビューを行っている。それによると，仕事の要求度―コントロールモデルと唾液中コルチゾールの増加の関連は比較的一貫した傾向としては観察されたものの，全体的には，関連を認めない結果や逆に負の関連性を認める研究成果も報告されており，両者の間に一貫した関連性を認めているとは言い難い状況である。

このような状況の原因としていくつかの点をあげることができる。一つの大きな問題は日内変動であり，コルチゾールは起床後に高く，夜にかけて徐々に低下することがわかっている。したがって，コルチゾール

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の測定値は測定時刻や起床時刻による影響が大きく，この誤差が結果を大きく左右している可能性が考えられる。また，急性ストレスの問題もあり，例えば，試料の採取の前に一過性のストレスとなるような出来事があった場合，このストレスによる影響が職業性ストレスの影響に混在してしまう可能性が考えられる。別の問題としては，コルチゾールの評価の回数をあげることができる。職業性ストレスのような慢性的なストレスと対応させるのであれば，コルチゾールを長期間にわたって複数回評価するのが望ましいが，多くの研究（特に血液サンプルを扱った研究）では，単回，あるいは同一日に複数回コルチゾールを評価するにとどまっている。

2．毛髪のコルチゾール

血液や唾液のサンプルに関する前述のような点を考慮して，近年では毛髪に含まれるコルチゾールに注目が集まっている。もとはスポーツ選手のステロイドなどの薬物使用の判別のために注目されていた方法であるが，これをステロイドホルモンの一種であるコルチゾールに利用した形である。毛髪は，形成される際に毛細血管からコルチゾールを含むステロイドが拡散すると考えられている（図1）。毛髪は1か月で約1cm伸びるため，例えば，根元から3cmの部分の毛髪に含まれるコルチゾールは，3か月間に蓄積されたコルチゾールを表すといわれている。毛髪は過去のコルチゾール分泌の“記録媒体”として考えられている。

初期のパイロット研究では，例えば，失業[10]や介護[11]などの慢性的なストレスを経験している人においては，毛髪のコルチゾール濃度が高いことが報告されている。また，大うつ病[12]や急性心筋梗塞[13]との関連も報告されている。

職業性ストレスとの関連については，研究数は多くないものの，いくつかの結果が報告されている。Manenschijnら[14]は33名の交代勤務労働者と89名の日勤労働者を比較し，交代勤務労働者では毛髪コルチゾールが高かったことを報告している。Steinischら[15]は衣料品工場の175名の労働者を対象に調査を行い，仕事の要求度に関連する項目（今後の販売促進の見込み）と毛髪コルチゾールの間に正の相関を報告している。Qiら[16]は39名の幼稚園の先生を対象に調査を行い，努力—報酬不均衡の得点と毛髪のコルチゾールの間に正の相関を報告している。Janssensら[17]は102名の製造会社で働く労働者を対象に調査を行い，自記式のストレスの質問紙と毛髪コルチゾールの間に相関は認めなかったが，抑うつ症状が高い者では毛髪コルチゾールが高かったことを報告している。Gidlowら[18]は132名のパブリック・セクターの労働者を対象に調査

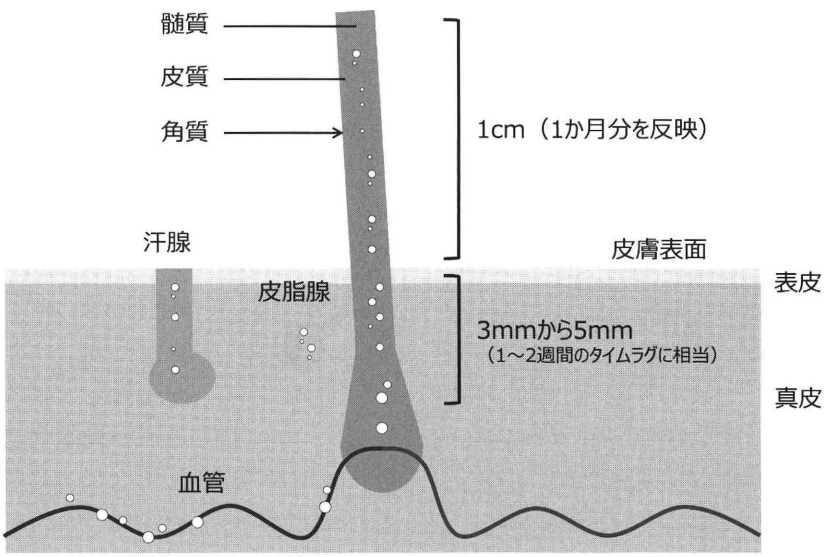

図1　毛髪に含まれるコルチゾール

血中のコルチゾール（白い○で表現）は毛髪が形成される際に毛幹の中心部（髄質）に受動的に拡散すると考えられている。汗腺や皮脂腺に含まれるコルチゾールの影響については明確なことはわかっていない（先行研究8，9）を参考に著者が図を作成）。

を行い，勤務中の自覚ストレスや努力—報酬不均衡モデルによる職業性ストレスと毛髪コルチゾールの間に有意な相関は認められなかったことを報告している。

毛髪試料の特徴的な点としては，把握できる期間の長さである。個人の毛髪の長さにも依存するが，過去6か月程度までのコルチゾールを評価することが可能であると考えられている。なお，毛髪の先端にいくほど，コルチゾール濃度は低くなることが観察されている[19]。一方で，毛髪からのコルチゾールの評価にあたっては何点かの留意すべき点がある。例えば，コルチゾールの測定には数十本の毛髪が必要であり，またハサミを利用して後頭部から採取するため，自身で採取することは難しい。また太陽光やヘアダイ・染髪によってコルチゾール値が低下することも指摘されており[20-22]，保存が長期間（2年以上）に及ぶ毛髪ではコルチゾール濃度が低かったことも報告されている[20]。しかしながら，このような点を考慮しても，長期的なコルチゾールの動態を把握できるという点において毛髪は非常に有用な試料であり，多くの研究で利用され始めている。

3．爪のコルチゾール

爪に関しては非常に研究数が少ない状況であるが，毛髪と類似した原理であり，ステロイドなどのホルモンが爪母基（nail matrix）に受動拡散によって輸送され，ケラチンが形成される際に取り込まれると考えられている（図2）[23,24]。手指の爪は10日間で約1mm伸びるため[25]，例えば，1mmの爪であれば，過去の10日間に蓄積されたコルチゾールを表すと考えられている。ただし，手指の爪は根元から先端までに伸びるのに数か月を要するため，先端の爪のコルチゾールは数か月前のものを反映していると考えられている。この点について検討した最近の研究[26]では，爪のコルチゾールは4か月前に評価した唾液中コルチゾールと中程度の相関があることが報告されている。

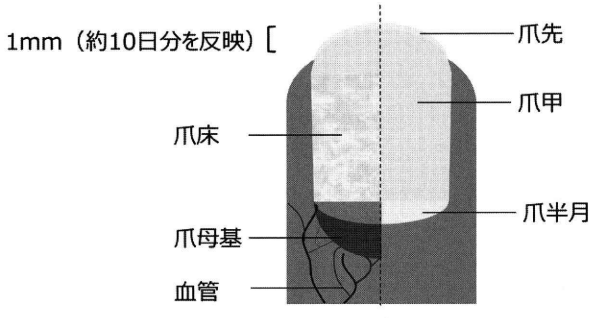

図2　爪に含まれるコルチゾール
血中のステロイドなどのホルモンは爪母基に受動拡散によって輸送され，ケラチンが形成される際に取り込まれると考えられている（イラストの左側は皮膚や爪が透けて，その中の構造を示している）。先端の1mmの爪は約10日分の長さに相当する。ただし，爪の根元部分が先端部分に移動するまでに数か月を要する。

ストレスとの関連について検討した研究としては，例えば，Warnockら[24]は学生を対象に試験や論文締め切りによるストレスと爪のコルチゾールやデヒドロエピアンドロステロン（DHEA）の関連を検討している。爪が根元から先端に伸びる時間差を考慮して，爪試料は，学生のストレスが低いと考えられる時期の3か月後（ベースライン期）と，試験や論文提出の時期の3か月後（ストレス期）にそれぞれ採取された。その結果，爪のコルチゾールは，ベースライン期とストレス期の間では差は認められなかったが，コルチゾール／DHEAの値はストレス期で有意に高いという結果が得られた（コルチゾールとDHEAの比は一つのストレスの指標として考えられている[27]）。しかしながら，この研究では対照群は設けられておらず，ホルモン値や爪の成長速度の季節による変化が結果に影響を与えている可能性を否定できない。また，最近の著者らの研究[28]では，ライフイベントや職業性ストレスと爪コルチゾールの関連を検討し，ライフイベントを経験している人やコントロール度に対する要求度の比が高い人は，コルチゾールが高かったことが報告されている。これらの結果はいずれも予備的なものであり，今後の研究が待たれる。

爪試料の特徴的な点としては，まず，採取が簡便である点があげられる。採取量も少なく済み，自身で採取することが可能である。爪が短い場合でも1～2週間の期間を設ければ，必要量を採取することは可能である。また，室温による保存が可能であり，郵送で回収することも可能である。このような側面は疫学研究などでの利用に適している。また，毛髪と比較して爪は構造上の劣化が少ないことが予想され，また，コルチゾールはケラチンとの結合性が高いため，日常生活における外的な要因の影響を受けにくいことが示唆されている[29]。一方で，他試料との相関について検討した研究はまだ一つであり[26]，今後，妥当性に関する

データの蓄積は必要である。まだ交絡要因について不明な点も多く，例えば，マニキュアなどの影響や爪の成長速度の影響などについてもあわせて検討が必要である。

4．まとめ

本総説では，コルチゾールの評価の方法として，近年，注目されている毛髪や爪の試料の研究を紹介した。各試料の特徴を表1にまとめた。従来までの血液や唾液の試料は“その時点”のホルモン値を強く反映するのに対して，毛髪や爪は，数週間から数か月にわたるホルモンの長期的な動態を反映する点が特徴的である。職場の慢性的なストレスやそれに伴う慢性的なコルチゾールの上昇が健康に悪影響を及ぼすことを考えると，このような特徴は研究などでストレスの現象を検討する上では非常に有利な点である。研究数はまだ少なく，まだ不明な点も多いが，毛髪や爪の試料を積極的に研究に取り入れることによって，職業性ストレスと健康の関連の解明がさらに進むことが期待される。

表1 コルチゾールの評価における各試料の特徴

	血液・唾液	毛髪	爪
慢性ストレスの評価	急性ストレスや日内変動による影響が大きい	数か月単位の状態を把握	数週間単位の状態を把握（ただし数か月前）
検体採取の容易さ	侵襲的（血液） 安静状態の確保が必要 冷凍保存が必要	自己採取難しい 検体量必要（毛髪が短い人やない人からは採取できない） 冷凍手続き不要	自己採取可能 少ない検体量 冷凍手続き不要
検体の状態による測定結果への影響	検体が冷凍保存されていれば問題ない	ヘアダイ・パーマ，太陽光による試料の劣化の可能性	（未検討）
妥当性の検証（他試料との相関）	血液と強い相関（唾液）	唾液との相関を認める研究もあるが，研究数は少ない（特に爪試料）	

文 献

1）Inoue, A., Kawakami, N., Haratani, T., Kobayashi, F., Ishizaki, M., Hayashi, T., Fujita, O., Aizawa, Y., Miyazaki, S., Hiro, H., Masumoto, T., Hashimoto, S. and Araki, S.: Job stressors and long-term sick leave due to depressive disorders among Japanese male employees: findings from the Japan Work Stress and Health Cohort study. J. Epidemiol. Community Health, **64**, 229-235 (2010)

2）Tsutsumi, A., Kayaba, K., Ojima, T., Ishikawa, S., Kawakami, N. and Jichi Medical School Cohort Study Group.: Low control at work and the risk of suicide in Japanese men: a prospective cohort study. Psychother. Psychosom, **76**, 177-185 (2007)

3）Tsutsumi, A., Kayaba, K., Kario, K. and Ishikawa, S.: Prospective study on occupational stress and risk of stroke. Arch. Intern. Med, **169**, 56-61 (2009)

4）Dickerson, S.S. and Kemeny, M.E.: Acute stressors and cortisol responses: a theoretical integration and synthesis of laboratory research. Psychol. Bull, **130**, 355-391 (2004)

5）Sapolsky, R.M., Romero, L.M. and Munck, A.U.: How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev, **21**, 55-89 (2000)

6）McEwen, B.S.: Allostasis and allostatic load: implications for neuropsychopharmacology. Neuropsychopharmacology, **22**, 108-124 (2000)

7）井澤修平，松浦桂，原谷隆史：職場環境における心理社会的ストレスとコルチゾールの関連—系統的レビューによる検討—．産業ストレス研究，**18**，161-172（2011）

8）Gow, R., Thomson, S., Rieder, M., Van Uum, S. and Koren, G.: An assessment of cortisol analysis in hair and its clinical

applications. Forensic Sci Int, **196**, 32-37 (2010)

9) Russell, E., Koren, G., Rieder, M. and Van Uum, S.: Hair cortisol as a biological marker of chronic stress: current status, future directions and unanswered questions. Psychoneuroendocrinology, **37**, 589-601 (2012)

10) Dettenborn, L., Tietze, A., Bruckner, F. and Kirschbaum, C.: Higher cortisol content in hair among long-term unemployed individuals compared to controls. Psychoneuroendocrinology, **35**, 1404-1409 (2010)

11) Stalder, T., Tietze, A., Steudte, S., Alexander, N., Dettenborn, L. and Kirschbaum, C.: Elevated hair cortisol levels in chronically stressed dementia caregivers. Psychoneuroendocrinology, **47**, 26-30 (2014)

12) Dettenborn, L., Muhtz, C., Skoluda, N., Stalder, T., Steudte, S., Hinkelmann, K., Kirschbaum, C. and Otte, C.: Introducing a novel method to assess cumulative steroid concentrations: increased hair cortisol concentrations over 6 months in medicated patients with depression. Stress, **15**, 348-353 (2012)

13) Pereg, D., Gow, R., Mosseri, M., Lishner, M., Rieder, M., Van Uum, S. and Koren, G.: Hair cortisol and the risk for acute myocardial infarction in adult men. Stress, **14**, 73-81 (2011)

14) Manenschijn, L., van Kruysbergen, R.G., De Jong, F.H., Koper, J.W. and Van Rossum, E.F.: Shift work at young age is associated with elevated long-term cortisol levels and body mass index. J. Clin. Endocrinol. Metab, **96**, E1862-1865 (2011)

15) Steinisch, M., Yusuf, R., Li, J., Stalder, T., Bosch, J.A., Rahman, O., Strümpell, C., Ashraf, H., Fischer, J.E. and Loerbroks, A.: Work stress and hair cortisol levels among workers in a Bangladeshi ready-made garment factory - Results from a cross-sectional study. Psychoneuroendocrinology, **50**, 20-27 (2014)

16) Qi, X., Zhang, J., Liu, Y., Ji, S., Chen, Z., Sluiter, J.K. and Deng, H.: Relationship between effort-reward imbalance and hair cortisol concentration in female kindergarten teachers. J. Psychosom. Res, **76**, 329-332 (2014).

17) Janssens, H., Clays, E., Fiers, T., Verstraete, A.G., de Bacquer, D. and Braeckman, L.: Hair cortisol in relation to job stress and depressive symptoms. Occup. Med. (Lond) [Epub ahead of print]

18) Gidlow, C.J., Randall, J., Gillman, J., Silk, S. and Jones, M.V.: Hair cortisol and self-reported stress in healthy, working adults. Psychoneuroendocrinology, **63**, 163-169 (2016)

19) Kirschbaum, C., Tietze, A., Skoluda, N. and Dettenborn, L.: Hair as a retrospective calendar of cortisol production-Increased cortisol incorporation into hair in the third trimester of pregnancy. Psychoneuroendocrinology, **34**, 32-37 (2009)

20) Abell, J.G., Stalder, T., Ferrie, J.E., Shipley, M.J., Kirschbaum, C., Kivimäki, M. and Kumari, M.: Assessing cortisol from hair samples in a large observational cohort: The Whitehall II study. Psychoneuroendocrinology, **73**, 148-156 (2016)

21) Sauvé, B., Koren, G., Walsh, G., Tokmakejian, S. and Van Uum, S.H.: Measurement of cortisol in human hair as a biomarker of systemic exposure. Clin. Invest. Med, **30**, 183-191 (2007)

22) Wester, V.L., van der Wulp, N.R., Koper, J.W., de Rijke, Y.B. and Van Rossum, E.F.: Hair cortisol and cortisone are decreased by natural sunlight. Psychoneuroendocrinology, 72, 94-96 (2016)

23) De Berker, D.A., André, J. and Baran, R.: Nail biology and nail science. Int. J. Cosmet. Sci, **29**, 241-275 (2007)

24) Warnock, F., McElwee, K., Seo, R.J., McIsaac, S., Seim, D., Ramirez-Aponte, T., Macritchie, K.A. and Young, A.H.: Measuringcortisol and DHEA in fingernails: a pilot study. Neuropsychiatr.Dis. Treat, **6**, 1-7 (2010)

25) Gupta, G.R., Dhruw, V.K., Athawal, B.K., Siddiqui, P., Yousuf Agrawal, H.K. and Chandra, H.: Human nail growth pat-tern and medicolegal aspect. J. Indian Acad. Forensic Med, **27**, 87-91 (2005)

26) Izawa, S., Miki, K., Tsuchiya, M., Mitani, T., Midorikawa, T., Fuchu, T., Komatsu, T. and Togo, F.: Cortisol level measurements in fingernails as a retrospective index of hormone production. Psychoneuroendocrinology, **54**, 24-30 (2015)

27) 太田充彦，八谷寛：職業性心理社会的要因と視床下部―下垂体―副腎皮質系ホルモンの関連．産業ストレス研究，**24**，205-211（2017）

28) Izawa, S., Matsudaira, K., Arisaka, M., Miki, K. and Tsuchiya, M.: Psychosocial work environment and cortisol in fingernails among white-color workers. The 6th ICOH International Conference on Work Environment and Cardiovascular Diseases, Abstract Book, 112 (2013)

29) Higashi, T., Yamagata, K., Kato, Y., Ogawa, Y., Takano, K., Nakaaze, Y., Iriyama, T., Min, J.Z. and Ogawa, S.: Methods for determination of fingernail steroids by LC/MS/MS and differences in their contents between right and left hands. Steroids, **109**, 60-65 (2016)

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Assessments of accumulated stress-related hormones by using hair and fingernail samples: A perspective on job stress research

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Abstract Cortisol, one of stress-related hormones, has been considered a mediator linking psychosocial stress and various diseases such as coronary heart disease and depressive disorder. This review introduced assessments of cortisol by using hair and fingernail samples. Cortisol levels in hair and fingernail samples may retrospectively represent hormone productions during a few months and weeks, respectively. Only a few studies previously investigated the associations between psychosocial stress in work environments and cortisol in hair and fingernail samples. Further studies are needed because hair and fingernail samples could largely contribute to exploring the biological links between job stress and health.

Job Stress Res., 24 (2), 213-218 (2017)

経営層を動かす！ エビデンスのあるキラートーク

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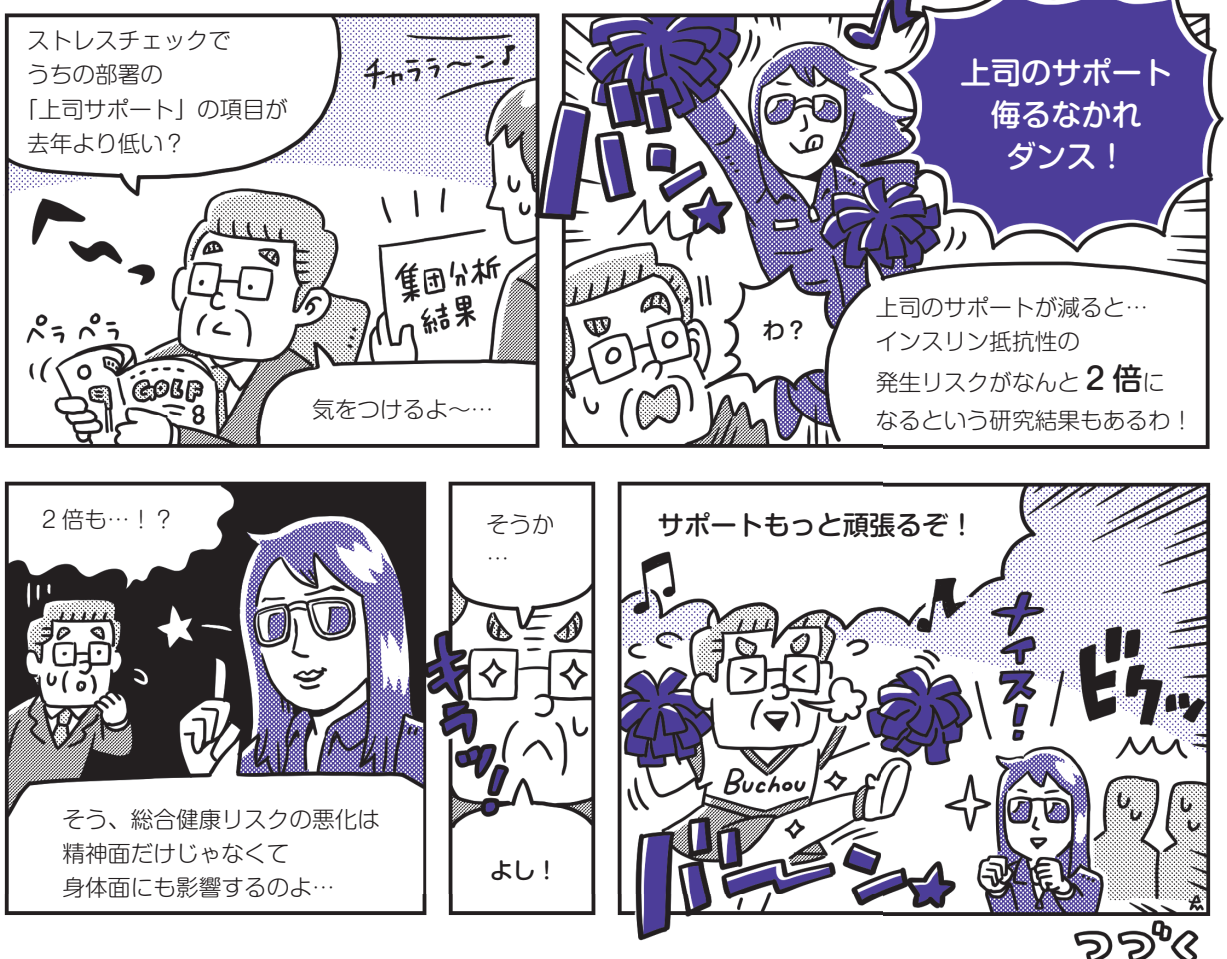
第5回

上司のサポートが減ると、インスリン抵抗性の発生リスクが2倍に！？

キラ・キラ美

社員想いの産業看護職。経営層の健康管理への意識を変えるべく、エビデンスのあるキラートークを『007』のジェームズ・ボンドばりに撃ち放つ。



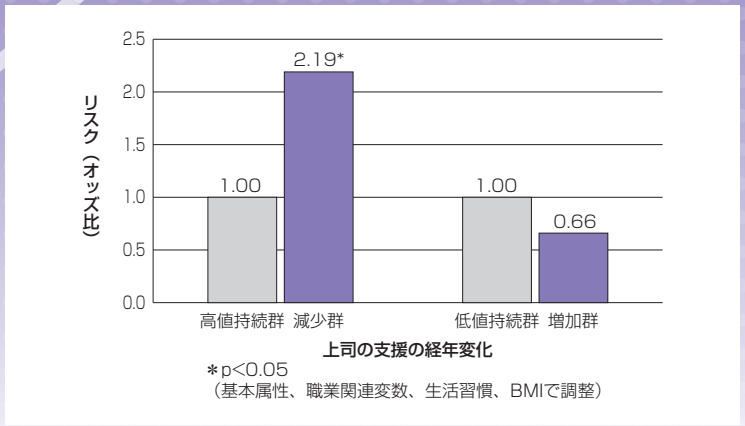


図1 上司の支援の経年変化によるインスリン抵抗性発生のオッズ比[2)]

総合健康リスクは職場にどんな影響を及ぼすか

2015年12月より法制化されたストレスチェック制度において、個人や職場のストレス状態を測定する尺度として、「職業性ストレス簡易調査票（Brief Job Stress Questionnaire；BJSQ）」[1)]の使用が厚生労働省より推奨されています。BJSQでは、「仕事のストレス判定図」[1)]を用いて、仕事の量的負担、仕事のコントロール、上司・同僚の支援の各得点から、職場ごとの総合健康リスク（健康障害の発生リスクがどの程度あるかを数値化したもの）を算出することができます。

すでにBJSQを使用してストレスチェックを実施し、安全衛生委員会等でフィードバックを済ませた方も多いと思いますが、総合健康リスクが高いこと（または低いこと）が、将来的にどのような影響を職場に及ぼすのか、説明に苦労されたのではないでしょうか。

科学的根拠を交えることでより効果的なフィードバックに

総合健康リスクが高い状態、つまり、仕事の要求度が高く、仕事のコントロールが低く、上司・同僚の支援が少ない状態は、「高ストレイン状態／孤立ストレイン状態」と定義され、心血管疾患、脳血管疾患、精神疾患等の発症リスクを高めることが国内外の調査で明らかになっています。

今回ご紹介する研究[2)]では、BJSQで測定した職場環境の経年変化が、糖尿病発症の前段階であるインスリン抵抗性にどのような影響を与えるかについて検討しています。某企業グループに勤務する男性労働者1,815名を対象に行った3年間の縦断研究において、ベースライン時の職場環境と3年後の職場環境を比較し、職場環境が変化することによるインスリン抵抗性の発生リスクを算出しています。その結果、「上司の支援が減った群は上司の支援が良好なままの群に比べて、インスリン抵抗性の発生リスクが2倍になる」ことが明らかになりました（**図1**）。つまり、ある年のストレスチェック結果で、「上司の支援が高い」と判定されたとしても、高く保ち続けなければ、将来の糖尿病発症リスクが上昇する可能性が示唆されています。

総合健康リスクの悪化は、精神面のみならず、血糖値や血圧のような身体面へも影響することについても念頭に置きながら、説明する必要があります。ストレスチェック結果をフィードバックする際、今回ご紹介したような科学的根拠を交えることで、職場環境を良好に保つ動機づけになるのではないでしょうか。

◉参考・引用文献

1) 労働省．労働省平成11年度「作業関連疾患の予防に関する研究」労働の場におけるストレス及びその健康影響に関する研究報告書．2000, 411p.

2) Hino, A. Inoue, A. et al. Changes in the psychosocial work characteristics and insulin resistance among Japanese male workers: a three-year follow-up study. J. Occup. Health. 58 (6) , 2016, 534-62.

植物性フラボノイドによる交感神経―副腎髄質系機能への作用*

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*Effects of plant flavonoids on functions of the sympatho-adrenal medullary system

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Key Words: Adrenal medulla, Catecholamine, Flavonoids, Nicotinic acetylcholine receptor, Stress

はじめに

日常摂取する食品の中には、多くの植物性フラボノイドが存在している。例えば、果物、野菜、種子、ワイン、お茶、そして伝統的中国医学や漢方に含まれる生薬等で、大豆食品のダイゼインやゲニスティン、また漢方等で陳皮として用いられる蜜柑の果皮成分でノビレチンなどがそうである。このフラボノイドの種類には、5000 種以上の多種多様な化合物が存在するが [1)]、私達が日頃摂食するのは大きく分けて６種類（フラボン、フラボノール、イソフラボン、フラバノン、フラバノール、アントシアニジン）(図 1)である [2)]。最近の研究によると、これらのフラボノイドには、加齢や生活習慣病に起因する心血管系疾患、糖尿病、そしてある種の癌等のリスクを軽減させるという薬理学的に興味ある可能性が報告され、注目を集めている [2)]。

一方、私達の体は、外界からの刺激、例えば気温の変化や精神的ストレス等に対して体の機能や状態を一定に保とうとする働き（生体の恒常性）がある。この働きを担うのが自律神経で、内臓や血管の収縮･弛緩や内分泌ホルモンの分泌など、ほとんどの体の臓器は自律神経すなわち交感・副交感両神経系により調節を受けている [3)]。しかしながら、強いストレスを長期に受けると、体はそれ

に反応して、交感神経や副腎髄質から大量のノルアドレナリン(NA)やアドレナリンというカテコールアミン（CA）を持続的に放出し、自律神経の機能不全を誘い、最終的には胃潰瘍等の消化性疾患や心筋梗塞、脳卒中等の重大な疾患を引き起こす[4]。本稿では植物性フラボノイドの CA 動態に及ぼす影響について、主として培養ウシ副腎髄質細胞を用いて検討した私達の最近の研究について概説する。

交感神経系のモデルとしての培養ウシ副腎髄質細胞

生体内に存在する CA は、主として副腎髄質、交感神経及び中枢神経系のクロマフィン細胞において産生されるが、その中で、最も大量に CA を産生し貯蔵する臓器は、副腎髄質である。この副腎髄質細胞において、生理的刺激であるアセチルコリン(ACh)による CA 分泌には少なくとも 2 つの異なるイオンチャネルが関与している(図２)[5]。すなわち、ニコチン性 ACh (nACh) 受容体ーイオンチャネル及び電位依存性 Ca^{2+}チャネルである。ACh 刺激により nACh 受容体ーイオンチャネルを介する Na^{+}流入は、細胞膜の脱分極を起こし、その結果、電位依存性 Ca^{2+}チャネルを活性化して Ca^{2+}流入を引き起こす。そして細胞内 Ca^{2+}濃度が上昇することにより最終的に CA 分泌が促進する。一方、ACh により律速酵素であるチロシン水酸化酵素が活性化され、CA 生合成が促進される[6]。また、放出された NA は、NA トランスポーターにより再取り込みされる。上述のように副腎髄質細胞でのイオンチャネルを介する CA 分泌や生合成の調節は、交感神経や中枢 NA 神経におけるそれらに類似しており、これまでに交感神経系のモデルとして多くの臨床薬物の作用機序の解明に用いられて来た[7]。

植物性フラボノイドによる CA 動態に及ぼす影響

（1）ダイゼインおよびノビレチンの CA 生合成･分泌における 2 相性反応

アジアの国々 、とりわけ日本や中国等において大豆は伝統的に豆腐などの食品として消費されている[8]。この大豆にはダイゼインなどのイソフラボン類が多く含まれ、非ステロイド構造でありながら、エストロゲン類似性の化学構造を基本骨格に有していることから植物性エストロゲンとも言われる。ダイゼインは大豆食品の中に配糖体（ダイジン）として含まれ、体内の腸内細菌等で代謝さることにより生成される。この大豆イソフラボンが骨粗鬆症、更年期障害、高コレステロール血症、前立腺癌等のリスクを低下させる可能性が報告されている[9]。

培養ウシ副腎髄質細胞をダイゼインと反応させると、濃度依存的(10-1000 nM)に[^{14}C]チロシンからの ^{14}C-CA 生合成を 15-20% 程度促進した[10] (図 3A)。

一方、ダイゼイン(1.0 μM) は、生理的刺激の ACh による CA 生合成の促進作用を抑制した。さらに、ダイゼイン(1.0-100 μM) は、ACh による CA 分泌も抑制した[10] (図 3B)。この結果から、ダイゼインの低濃度(10-1000 nM) は CA 生合成を促進するが、高濃度(1.0-100 μM)では、ACh による CA 分泌や生合成をむしろ抑制することが示された。日本の成人の平均血中ダイゼイン濃度が数 100 nM という報告[11]があり、さらに 1 日数回大豆食品を食べる人であれば数μM に達したという報告[12]もある。これらのことを考えると、CA 生合成や分泌に及ぼすダイゼインの濃度は、大豆食品を日常的に摂取する人であれば到達可能な濃度と考えられる。

従来、漢方治療において蜜柑の果皮を乾燥したものを陳皮として用いられて来た。その果皮成分でポリメトキシフラボンであるノビレチンは、神経栄養学的作用や抗痴呆効果等が報告[13]されている。培養ウシ副腎髄質細胞においてノビレチン(1.0-100 μM) は、それ単独で CA 分泌および $^{45}Ca^{2+}$流入を濃度依存的に促進した[14]。一方、ACh による CA 分泌、$^{22}Na^{+}$流入や $^{45}Ca^{2+}$流入を濃度―依存的 (1.0-100 μM) に抑制した[14]。さらに、ノビレチン (0.3-10 μM) は nACh 受容体(α3β4) を発現させたアフリカツメガエル卵母細胞において ACh 誘発電流を可逆的に(図 4A)、また濃度依存的(図 4B)に抑制した[14]。以上より、ノビレチンは直接的に nACh 受容体に作用し ACh による Na^{+}流入を抑制し、電位依存性 Ca^{2+}チャネルを介する Ca^{2+}流入を阻害し、その結果 CA 分泌を抑制したものと思われる。

（2）ゲニステインによる NA トランスポーターへの作用

NA トランスポーターは、交感神経終末に発現し NA の作用を空間的および時間的に制御する。すなわち、NA トランスポーターは細胞外へ放出された NA の再取り込みを介して神経伝達を終結させる。この NA の再取り込みの実験材料として、NA トランスポーターを発現しているヒトの神経芽(SK-N-SH) 細胞を用いた。ゲニスティンは、ダイゼインとともに大豆成分に含まれるイソフラボンであり、植物性エストロゲンである。

ゲニスティン (0.1-10 μM) は、ベル型 (bell-shaped) の濃度依存性の[^{3}H] NA の取り込みを促進した(図 5)[15]。一方、植物性エストロゲンであるクメストロールや同じイソフラボンであるダイゼインには、このような作用は見られなかった。ゲニスティンは、チロシンキナーゼ阻害作用の広いスペクトル[16]を有しているが、ダイゼインにはこのような阻害作用はない。そこで、細胞膜チロシンキナーゼ阻害剤として tyrophostin 25 を、また細胞質可溶性型 src ファミリィーのチロシンキナーゼ阻害剤の PP2 やハービマイシンの作用を調べた結果、前者は NA の取り込みを促進したが、後者は影響しなかった。以上の結果から、ゲニスティ

ンは細胞膜型チロシンキナーゼを阻害剤することにより、NA トランスポーター活性を促進しているものと考えられた。

（3）イカリソウ成分イカリソサイド A の CA 生合成· 分泌における抑制作用

生薬「淫洋藿」はインポテンツ、不妊症、半身不随、リウマチ、運動麻痺、筋肉の痙攣、疼痛等に用いられている [17]。淫洋藿（イカリソウ）の活性成分の一つであるイカリソサイド A はイソプレン側鎖のついたフラボノイドでフラボノール化合物である。イカリソウには、その他にイカリン、エピメジン C、エピメドサイド A などのフラボノール化合物が存在する。これらフラボノール化合物において、イカリソサイド A のみが ACh による CA 分泌、$^{22}Na^{+}$流入や $^{45}Ca^{2+}$流入を濃度―依存的に抑制した [18]。構造活性相関からすると、イカリソサイド A のみが 7 位に水酸基があり、他の 3 化合物の 7 位は糖鎖であることからこの構造的な差による nACh 受容体機能への影響があるものと考察された。

植物性フラボノイドの生体機能への影響とその意義

私達は日常生活において、強いストレスや精神的興奮を受けると、その刺激やストレスを大脳皮質が感受し、それはその直下にある感情や情動を司る大脳辺縁系に影響し、さらに視床下部へ伝達される。従って、強いストレスや精神的興奮は、視床下部の中枢性自律神経、特に交感神経の興奮を引き起こしひいては、腹部交感神経より ACh が遊離されて副腎髄質に作用すると、そこからアドレナリンや NA 等の多量の CA が血中へ放出される(図 6)。生体内での適度な交感神経刺激は、私達の心臓や血管機能のバランスを維持する上で重要である [1]。しかしながら、過度なストレスや精神的興奮が長期間持続した場合、過剰な CA は血圧上昇や動脈硬化を引き起こし、心不全の悪化を誘発する。特に、過剰な CA 刺激と心不全の悪化との相関関係は多くの研究報告により支持されている [4,19,]。また、ストレスホルモンであるアドレナリンは、β_2 アドレナリン受容体を介して、 GTP 結合蛋白質を経由するシグナルを活性化し、最終的に癌抑制因子 p53 レベルを低下させ、DNA 損傷を引き起こすことが報告されている [20]。

植物性エストロゲンでフラボノイド化合物のダイゼインや蜜柑の果皮成分のノビレチンは、それら単独で CA 生合成や分泌を促進させた。この結果は、これらの化合物に、交感神経系を強化する薬理作用が示唆される。しかし大豆成分でイソフラボンのゲニステインは、NA トランスポーター機能を促進した。これは、シナプス間隙に遊離された NA の再取り込みを高めることにより、交感神経活動を抑制させることが示唆される。一方、生理的刺激である ACh の CA 生合成・分泌反応をダイゼイン、ノビレチン、イカリソサイド A は抑制した。この結果は、これら植物性フラボノイドは、ストレス反応による CA 分泌や生

合成の促進を抑制することを示唆している（図 6）[21]。今回の研究結果は、上記の植物性フラボノイドがストレスや精神的興奮による CA 過剰分泌からの心臓や血管系疾患に対して保護的作用を有する可能性を示しており、今後のさらなる研究が必要と思われる。

まとめ

植物性フラボノイドは、交感神経や副腎髄質機能に対して２相性の作用を示し、それ単独で促進又は抑制作用を、一方ストレス刺激下では抑制作用の可能性を示唆した。この事は、日常摂取している食品によっては、私達の体の機能、特に中枢での精神機能や末梢自律神経、特に交感神経系機能に多少なりとも影響している可能性があるが、その結論は今後、人における臨床研究の結果を待たなければならない。

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文献

1) Falcone Ferreyra ML, Rius SP, Casati P. Flavonoids: biosynthesis, biological functions, and biotechnological applications. Front Plant Sci 2012; 3: 1-15.
2) Lu MF, Xiao ZT, Zhang HY. Where do health benefits of flavonoids come from? Insights from flavonoid targets and their evolutionary history. Biochem Biophys Res Commun 2013; 434:701-4
3) Wesfall TC, Westfall DP. Adrenergic agonist and antagonists, In: Brunton LL, Chabner BC and Knollmann BC, editors. *Goodman & Gilman's the Pharmacological Basis of Therapeutics*, 12th edn, McGraw-Hill NY; 2011. p. 277-330.
4) Freedman NJ, Lefkowitz RJ. Anti-β_1-adrenergic receptor antibodies and heart failure: causation, not just correlation. J Clin Invest 2004; 113:1379-82.
5) Wada A, Takara H, Izumi F et al. Influx of ^{22}Na through acetylcholine receptor-associated Na channels: relationship between ^{22}Na influx, ^{45}Ca influx and secretion of catecholamines in cultured bovine adrenal medulla cells. Neuroscience 1985; 15: 283-92.

6) Yanagihara N, Wada A, Izumi F. Effects of α_2-adrenergic agonists on carbachol-stimulated catecholamine synthesis in cultured bovine adrenal medullary cells. Biochem Pharmacol 1987; 36: 3823-8.
7) Kajiwara K, Yanagita T, Nakashima Y, et al. Differential effects of short and prolonged exposure to carvedilol on voltage-dependent Na^+ channels in cultured bovine adrenal medullary cells. J Pharmacol Exp Ther 2002; 302: 212-8.
8) Kurzer MS, Xu X. Dietary phytoestrigens. Annu Rev Nutr 1997; 17: 353-381.
9) Murkies AL, Wilcox G, Davis SR. Clinical review 92: phytoestrogens. J Clinical Endocrinol Metab 1998; 83: 297-303.
10) Liu M, Yanagihara N, Toyohira Y, et al. Dual effects of daidzein, a soy isoflavone, on catecholamine synthesis and secretion in cultured bovine adrenal medullary cells. Endocrinol 2007;148: 5348-54.
11) Morton MS, Arisaka O, Miyake N, et al. Phytoestrogen concentrations in serum from Japanese men and women over forty years of age. J Nutr. 2002; 132: 3168-71.
12) King RA, Bursill DB. Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal in humans. Am J Clin Nutr. 1998; 67: 867-72.
13) Nagase H, Omae N, Omori A, et al. Nobiletin and its related flavonoids with CRE-dependent transcription-stimulating and neuritegenic activities. Genistein, a specific inhibitor of tyrosine-specific protein kinases. Biochem Biophys Res Commun 2005; 337: 1330-6.
14) Zhang H, Toyohira Y, Ueno S, et al. Dual effects of nobiletin, a citrus polymethoxy flavone, on catecholamine secretion in cultured bovine adrenal medullary cells. J Neurochem 2010; 114: 1030-8.
15) Toyohira Y, Ueno S, Tsutsui M, et al. Stimulatory effects of the soy phytoestrogen genistein on noradrenaline transporter and serotonin transporter activity. Mol Nutr Food Res 2010; 54: 516-24.
16) Akiyama T, Ishida J, Nakagawa S, et al. Genistein, a specific inhibitor of tyrosine-specific protein kinases. J Biol Chem 1987; 262: 5592-5.
17) Dou J, Liu Z, Liu S. Structure identification of a prenylflavonol glycoside from epimedium koreanum by electrospray ionization tandem mass spectrometry. Anal Sci 2006; 22: 449-52.
18) Li X, Toyohira Y, Horishita T, et al. Ikarisoside A inhibits

acetylcholine-induced catecholamine secretion and synthesis by suppressing nicotinic acetylcholine receptor-ion channels in cultured bovine adrenal medullary cells. Naunyn-Schmiedeberg's Arch of Pharmacol 2015; 388: 1259-69.
19) Goldstein DS. Catecholamine and stress. Endocr Regul 2003; 37: 69-80.
20) Hara MR, Kovacs JJ, Whalen EJ, et al. A stress response pathway regulates DNA damage through β_2-adrenoreceptors and β-arrestin-1. Nature 2011; 477: 349-53.
21) Yanagihara N, Zhang H, Toyohira Y, et al. New insights into the pharmacological potential of plant flavonoids in the catecholamine system. J Pharmacol Sci 2014; 124:123-8.

図の説明

図１　食品中に含まれる植物性フラボノイドの化学構造式[文献 2)を改変]

図２　培養ウシ副腎髄質細胞での CA 生合成· 分泌及び再取り込みにおける機序

図３　大豆成分ダイゼインによるカテコールアミン動態における 2 相性反応[文献 10)を改変]

図４　α3β4 nACh 受容体を発現させたアフリカツメガエル卵母細胞におけるノビレチンの抑制作用[文献 14)を改変]

図５大豆成分ゲニステインによる NA トランスポーター機能の促進作用[文献 15)を改変]

図６　植物性フラボノイドによるストレス応答下での生体 CA 過剰反応に対する抑制作用

過度のストレスや精神的興奮は大脳皮質を刺激し、それは大脳辺縁系、視床下部（自律神経の中枢）へと伝達される。その結果、腹部交感神経から ACh が遊離され、さらに副腎髄質からアドレナリン（Ad）等の CA が血中へ多量に放出される。植物性フラボノイドは ACh の作用を抑制することにより、CA の分泌過剰を抑制する。

BMJ Open

Psychosocial factors at work and inflammatory markers: protocol for a systematic review and meta-analysis

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ABSTRACT

Introduction Chronic inflammation may be a mediator for the development of cardiovascular disease (CVD), metabolic diseases and psychotic and neurodegenerative disorders. Meta-analytic associations between work-related psychosocial factors and inflammatory markers have shown that work-related psychosocial factors could affect the flexibility and balance of the immune system. However, few systematic reviews or meta-analyses have investigated the association between work-related psychosocial factors and inflammatory markers. Based on prospective studies, the present investigation will conduct a comprehensive systematic review and meta-analysis of the association between work-related psychosocial factors and inflammatory markers.

Methods and analysis The systematic review and meta-analysis will include published studies identified from electronic databases (PubMed, EMBASE, PsycINFO, PsycARTICLES, Web of Science and Japan Medical Abstracts Society) according to recommendations of the Meta-analysis of Observational Studies in Epidemiology guideline. Inclusion criteria are studies that: examined associations between work-related psychosocial factors and increased inflammatory markers; used longitudinal or prospective cohort designs; were conducted among workers; provided sufficient data for calculating ORs or relative risk with 95% CIs; were published as original articles in English or Japanese; and were published up to the end of 2017. Study selection, data extraction, quality assessment and statistical syntheses will be conducted by 14 investigators. Any inconsistencies or disagreements will be resolved through discussion. The quality of studies will be evaluated using the Risk of Bias Assessment Tool for Non-randomized Studies.

Ethics and dissemination The investigation study will be based on published studies, so ethics approval is not required. The results of this study will be submitted for publication in a scientific peer-reviewed journal. The findings may be useful for assessing risk factors for increased inflammatory markers in the workplace and determining future approaches for preventing CVD, metabolic diseases and psychotic and neurodegenerative disorders.

PROSPERO registration number CRD42018081553.

Strengths and limitations of this study

- This systematic review and meta-analysis will offer comprehensive understanding of the association between work-related psychosocial factors and inflammatory markers.
- The review will include a range of work-related psychosocial factors and focus on inflammatory markers.
- To ensure stronger evidence, the review will include only prospective studies.
- The findings of this review may be useful for assessing chronic inflammation as a risk factor for cardiovascular disease (CVD), metabolic diseases and psychotic and neurodegenerative disorders in the workplace as well as for determining future approaches for preventing CVD, metabolic diseases and psychotic and neurodegenerative disorders.
- Depending on the results, limitations could be confounding factors that may not have been adjusted for in the selected studies as well as low generalisability.

INTRODUCTION

Most adults spend around half of their waking hours at work, and so the workplace is an important setting to promote health and well-being. Increasing attention is being directed to work-related psychosocial factors, such as job strain,[1–5] effort–reward imbalance,[6] organisational justice[7–9] and workplace social capital[10]; there is a major focus on work stress.[2] These factors may affect cardiovascular disease (CVD), metabolic diseases and psychotic and neurodegenerative disorders through such mechanisms as prolonged overactivation and dysregulation of the autonomic nervous system and the hypothalamic–pituitary–adrenal cortex axis.[11–13]

Chronic inflammation has been suggested as a potential mediator for the development of CVD, metabolic diseases and psychotic and neurodegenerative disorders.[14–18]

Several studies have reported associations between adverse work-related psychosocial factors and increased levels of inflammatory markers. Inflammatory markers, including C reactive protein (CRP),[19–24] interleukin 6 (IL-6)[24 25] and tumour necrosis factor-α (TNF-α), have been implicated in coordinating atherosclerosis.[26] Previous meta-analyses[27 28] have identified the associations between psychosocial factors and inflammatory markers; however, the findings from those studies were not conclusive because of methodological heterogeneity (eg, conceptualisation or measurement of work-related psychosocial factors, sample compositions and statistical approaches).

Meta-analytic associations between work-related psychosocial factors and inflammatory markers indicate that such factors may affect the flexibility and balance of the immune system. Some meta-analyses have investigated inflammatory markers in relation to psychological stress[27–30] and unemployment[31]; however, few systematic reviews or meta-analyses have been conducted regarding the associations between work-related psychosocial factors and inflammatory markers. A systematic review of 56 studies by Nakata[32] suggested that work-related psychosocial factors were related to disrupted immune response. However, that study did not statistically synthesise the associations. To our knowledge, only one meta-analysis of the association between effort–reward imbalance and inflammatory markers (k=7, n=9952) found a negative association with immunity (r=−0.09; CI −0.14 to −0.05; p<0.001).[13] These systematic reviews and meta-analyses included cross-sectional studies. However, pooled associations between work-related psychosocial factors and inflammatory markers derived from prospective studies may provide more reliable evidence.

Based on published prospective studies, the present investigation will conduct a comprehensive systematic review and meta-analysis of the associations between work-related psychosocial factors and inflammatory markers. Inflammatory markers will include those that were previously investigated in terms of associations with psychosocial factors at work, including CRP, IL-6 and TNF-α. Our hypothesis is that adverse work-related psychosocial factors would increase inflammatory markers. Moreover, we will identify the work-related psychological factors that have the strongest associations with specific inflammatory markers.

METHODS AND ANALYSIS

Study design

This study protocol for a systematic review and meta-analysis of prospective studies follows the Preferred Reporting Items for Systematic Reviews and Meta-Analysis Protocols guideline.[33] Future findings will be reported according to the Meta-analysis of Observational Studies in Epidemiology (MOOSE) reporting guidelines.[34] This study protocol was registered with PROSPERO (CRD42018081553).

Eligibility criteria

Participants, exposures, comparisons and outcomes (PECO) of the studies included in this systematic review and meta-analysis will be defined as follows: (P) inclusion of all workers; (E) presence of adverse psychosocial factors at work; (C) absence of adverse psychosocial factors at work; and (O) increased inflammatory markers. Target participants will all be employees of participating companies. There will be no exclusion criteria related to employment status, job type or shift type. The study exposures (adverse psychosocial factors at work) will include a range of task and organisational characteristics and work conditions,[35] such as job strain,[1–5] low social support, effort–reward imbalance,[6] organisational injustice[7–9] and low workplace social capital.[10] Long working hours and shift work will also be included as target exposures. Inflammatory markers will include those investigated in terms of association with psychosocial factors at work in previous studies, including CRP, IL-6 and TNF-α.

Eligibility criteria for selection are the following studies that (1) were conducted to evaluate associations between psychosocial factors at work and inflammatory markers; (2) used longitudinal or prospective cohort designs; (3) were conducted among workers; (4) provided sufficient data for calculating coefficients of associations between psychosocial factors at work and inflammatory markers (γ, β), ORs, relative risks (RRs) or HRs with SEs or 95% CIs; (5) were published as original articles in English or Japanese; and (6) were published up to the end of 2017.

Information sources, search strategy and data management

A systematic search of published studies will be conducted using electronic databases: PubMed (MEDLINE), EMBASE, PsycINFO, PsycARTICLES, Web of Science and the Japan Medical Abstracts Society. Search terms will include words related to the PECO of eligible published studies. The proposed search strategy appears in online supplementary appendix 1. All identified studies will be managed in a Microsoft Excel file (Washington, USA). Before the study selection process, duplicated citations in the Excel file will be excluded by KW. Decisions on all studies will be recorded.

Study selection process

First, following the eligibility criteria, 14 investigators (HE, KW, EA, HA, YA, AI, RI, MI, KI, YK, NN, YO, ASa and KT) will independently conduct screening of identified titles and abstracts in pairs. Second, we will obtain full texts of all eligible studies. In the full-text review phase, the studies will be examined using a standardised form (see online supplementary appendix 2) to assess eligibility for inclusion in this review. The number of papers examined by each investigator will depend on the investigator's capacity. Any discrepancies in assessment will be recorded and the inter-rater reliability determined; such matters will be discussed among all the investigators until consensus is reached. We will directly contact the corresponding authors

of eligible studies if the results of the publication are unclear and may be related to multiple interpretations or if the reported results did not show data relevant to our study analysis. The reasons for excluding studies will be recorded. A flow chart will be prepared showing the entire review process.

Data extraction

Data will be extracted independently from the included studies by 14 investigators (HE, KW, EA, HA, YA, AI, RI, MI, KI, YK, NN, YO, ASa and KT) working in pairs using a standardised data extraction form. The data will be distributed according to the investigators' capacity. Any discrepancies or inconsistencies in the assessment will be recorded and the inter-rater reliability determined; such matters will be discussed among all the investigators until consensus is reached. The extracted data will include the following: year of publication; country where the study was conducted; number of participants at baseline and in the analysis; sampling framework; participants' demographic characteristics (ie, mean age, sex proportions and employment status); length of follow-up; follow-up rate; exposure and comparison variables (adverse psychosocial factors at work); outcome variables (inflammatory markers); number and proportion of participants with increased levels of inflammatory markers or mean scores and variances or SD of markers; and sufficient data for calculating the coefficients (β, γ), ORs, RRs or HRs with SEs or 95% CIs for the association between adverse psychosocial factors at work and inflammatory markers. If the included studies report multiple measures of association, we will attempt to select measures of association adjusted by demographic variables (eg, age, sex, education and marital status). If the studies report measures of association adjusted by lifestyle variables (eg, smoking, physical activity and sleep), we will as far as possible extract measures both with and without adjustment for lifestyle variables. To avoid overadjustment, measures of association adjusted for other adverse psychosocial factors at work or inflammatory markers will not be adopted. Sex-stratified coefficients will be selected if they are the only reported results. Any missing data from the studies will be obtained by contacting the relevant research team.

Assessment of study quality

Fourteen investigators (HE, KW, EA, HA, YA, AI, RI, MI, KI, YK, NN, YO, ASa and KT) will independently assess in pairs the quality of each included study using the internationally recognised Risk of Bias Assessment Tool for Non-randomized Studies (RoBANS).[36 37] The RoBANS was developed to determine the risk of bias of non-randomised studies; it comprises six domains: selection of participants; confounding variables; measurement of exposure; blinding of outcomes; incomplete outcome data; and selective outcome reporting. The risk of bias for each domain is classified as low, high or unclear risk. The number of papers assessed by each investigator will depend on their capacity. Any discrepancies in quality assessment among the investigators will be recorded and the inter-rater reliability determined; such matters will be discussed among all the investigators until consensus is reached.

Data synthesis and statistical methods

The included studies will be statistically synthesised in a meta-analysis to estimate pooled coefficients and 95% CIs, stratified by types of measures of association (β, γ, OR, RR and HR). If the included studies report ORs, RRs or HRs, we will calculate log-transformed ORs, RRs or HRs and determine SEs based on 95% CIs. These parameters will be used in the meta-analysis and for examining publication bias by means of a funnel plot and Egger's test with statistical software, R V.3.4.1.[38 39] We will employ a random-effects model[40] to summarise the results using R V.3.4.1 with the 'meta' and 'metafor' packages.[41]

For the main analysis, we will synthesise all types of psychosocial factors at work in the random-effects model. The results will be presented in a narrative format if a meta-analysis is not appropriate or possible, for example, if only two or fewer studies are eligible and included in the study. Heterogeneity will be assessed using the χ^2 test with Cochran's Q statistic, which is calculated by I^2 values,[42] assuming that I^2 values of 25%, 50% and 75% indicate low, medium and high heterogeneity, respectively.

Subgroup and sensitivity analyses will be conducted to compare the results across subgroups or under specific conditions when sufficient heterogeneity is detected. Major possible grouping characteristics will include types of exposure and outcome, participants' demographic characteristics (eg, sex, age, employment status, occupational groups) and study quality. Any subgroup differences will be reported, and our findings will be explained by considering these differences. Results with and without adjustment for lifestyle variables will be compared in another sensitivity analysis. If trends are observed between pooled associations and any grouping characteristics, meta-regression will be conducted using the 'metareg' function of R. A sensitivity analysis may be conducted for included studies where the RoBANS is classified as low risk. All extracted data and analysed results will be deposited by the corresponding author and made available for external reviewers and readers on request.

Patient and public involvement statement

This study will not involve any patients or study participants: this study protocol is for a systematic review and meta-analysis.

Ethics and dissemination

This study does not require ethical approval because the systematic review and meta-analysis will be based on previously published studies. The results will be

submitted for publication in a scientific peer-reviewed journal, according to the MOOSE guideline.[34]

Strengths and limitations

This systematic review and meta-analysis will be based on prospective studies and show the strongest evidence for the associations between psychosocial factors at work and inflammatory markers. The findings will highlight potential mediators and underlying mechanisms for the development of CVD owing to adverse psychosocial factors.

There are several likely limitations in this study, including confounding bias and low generalisability. If selected studies do not report demographic-adjusted associations, the findings will be distorted by the unobserved characteristics among the population. In addition, the findings will not be generalisable to populations not included in the selected studies.

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REFERENCES

1. Kivimäki M, Nyberg ST, Batty GD, *et al*. Job strain as a risk factor for coronary heart disease: a collaborative meta-analysis of individual participant data. *Lancet* 2012;380:1491–7.
2. Kivimäki M, Kawachi I. Work stress as a risk factor for cardiovascular disease. *Curr Cardiol Rep* 2015;17:630.
3. Fishta A, Backé EM. Psychosocial stress at work and cardiovascular diseases: an overview of systematic reviews. *Int Arch Occup Environ Health* 2015;88:997–1014.
4. Theorell T, Karasek RA. Current issues relating to psychosocial job strain and cardiovascular disease research. *J Occup Health Psychol* 1996;1:9–26.
5. Belkic KL, Landsbergis PA, Schnall PL, *et al*. Is job strain a major source of cardiovascular disease risk? *Scand J Work Environ Health* 2004;30:85–128.
6. Siegrist J. Adverse health effects of high-effort/low-reward conditions. *J Occup Health Psychol* 1996;1:27–41.
7. De Vogli R, Ferrie JE, Chandola T, *et al*. Unfairness and health: evidence from the Whitehall II Study. *J Epidemiol Community Health* 2007;61:513–8.
8. Elovainio M, Leino-Arjas P, Vahtera J, *et al*. Justice at work and cardiovascular mortality: a prospective cohort study. *J Psychosom Res* 2006;61:271–4.
9. Kivimäki M, Ferrie JE, Brunner E, *et al*. Justice at work and reduced risk of coronary heart disease among employees: the Whitehall II Study. *Arch Intern Med* 2005;165:2245–51.
10. Oksanen T, Kawachi I, Jokela M, *et al*. Workplace social capital and risk of chronic and severe hypertension: a cohort study. *J Hypertens* 2012;30:1129–36.
11. Kivimäki M, Virtanen M, Elovainio M, *et al*. Work stress in the etiology of coronary heart disease–a meta-analysis. *Scand J Work Environ Health* 2006;32:431–42.
12. Barth J, Schneider S, von Känel R. Lack of social support in the etiology and the prognosis of coronary heart disease: a systematic review and meta-analysis. *Psychosom Med* 2010;72:229–38.
13. Eddy P, Heckenberg R, Wertheim EH, *et al*. A systematic review and meta-analysis of the effort-reward imbalance model of workplace stress with indicators of immune function. *J Psychosom Res* 2016;91:1–8.
14. Lopez-Candales A, Hernández Burgos PM, Hernandez-Suarez DF, *et al*. Linking chronic inflammation with cardiovascular disease: from normal aging to the metabolic syndrome. *J Nat Sci* 2017;3:e341.
15. Siegrist J, Li J. Work stress and altered biomarkers: a synthesis of findings based on the effort-reward imbalance model. *Int J Environ Res Public Health* 2017;14:1373.
16. Ralston JC, Lyons CL, Kennedy EB, *et al*. Fatty acids and NLRP3 inflammasome-mediated inflammation in metabolic tissues. *Annu Rev Nutr* 2017;37:77–102.
17. Singh B, Chaudhuri TK. Role of C-reactive protein in schizophrenia: an overview. *Psychiatry Res* 2014;216:277–85.
18. Dopkins N, Nagarkatti PS, Nagarkatti M. The role of gut microbiome and associated metabolome in the regulation of neuroinflammation in multiple sclerosis and its implications in attenuating chronic inflammation in other inflammatory and autoimmune disorders. *Immunology*. In Press. 2018;154:178–85.
19. Eguchi H, Shimazu A, Kawakami N, *et al*. Source-specific workplace social support and high-sensitivity C-reactive protein levels among Japanese workers: A 1-year prospective cohort study. *Am J Ind Med* 2016;59:676–84.
20. Tsai SS, Lai CH, Shih TS, *et al*. High job strain is associated with inflammatory markers of disease in young long-haul bus drivers. *J Occup Health Psychol* 2014;19:336–47.
21. Emeny R, Lacruz ME, Baumert J, *et al*. Job strain associated CRP is mediated by leisure time physical activity: results from the MONICA/KORA study. *Brain Behav Immun* 2012;26:1077–84.
22. Xu W, Chen B, Guo L, *et al*. High-sensitivity CRP: possible link between job stress and atherosclerosis. *Am J Ind Med* 2015;58:773–9.
23. Almadi T, Cathers I, Hamdan Mansour AM, *et al*. The association between work stress and inflammatory biomarkers in Jordanian male workers. *Psychophysiology* 2012;49:172–7.
24. Elovainio M, Ferrie JE, Singh-Manoux A, *et al*. Organisational justice and markers of inflammation: the Whitehall II study. *Occup Environ Med* 2010;67:78–83.
25. Nakata A, Irie M, Takahashi M. Source-specific social support and circulating inflammatory markers among white-collar employees. *Ann Behav Med* 2014;47:335–46.
26. Ridker PM, Lüscher TF. Anti-inflammatory therapies for cardiovascular disease. *Eur Heart J* 2014;35:1782–91.
27. Herbert TB, Cohen S. Stress and immunity in humans: a meta-analytic review. *Psychosom Med* 1993;55:364–79.
28. Segerstrom SC, Miller GE. Psychological stress and the human immune system: a meta-analytic study of 30 years of inquiry. *Psychol Bull* 2004;130:601–30.

29. Steptoe A, Hamer M, Chida Y. The effects of acute psychological stress on circulating inflammatory factors in humans: a review and meta-analysis. *Brain Behav Immun* 2007;21:901–12.
30. Marsland AL, Walsh C, Lockwood K, *et al*. The effects of acute psychological stress on circulating and stimulated inflammatory markers: A systematic review and meta-analysis. *Brain Behav Immun* 2017;64:208–19.
31. Hughes A, Kumari M, McMunn A, *et al*. Unemployment and inflammatory markers in England, Wales and Scotland, 1998-2012: meta-analysis of results from 12 studies. *Brain Behav Immun* 2017;64:91–102.
32. Nakata A. Psychosocial job stress and immunity: A systematic review. In: Yan Q, ed. *Psychoneuroimmunology: methods and protocols*. Totowa, NJ: Humana Press, 2012:39–75.
33. Moher D, Shamseer L, Clarke M, *et al*. Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. *Syst Rev* 2015;4:1.
34. Stroup DF, Berlin JA, Morton SC, *et al*. Meta-analysis of observational studies in epidemiology: a proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. *JAMA* 2000;283:2008–12.
35. Semmer NK. Job stress interventions and the organization of work. *Scand J Work Environ Health* 2006;32:515–27.
36. Kim SY, Park JE, Lee YJ, *et al*. Testing a tool for assessing the risk of bias for nonrandomized studies showed moderate reliability and promising validity. *J Clin Epidemiol* 2013;66:408–14.
37. Wells G, Shea B, O'Connell D. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomized studies in meta-analyses. Secondary The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyses. 2014. http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp
38. Watanabe K, Imamura K, Kawakami N. Working hours and the onset of depressive disorder: a systematic review and meta-analysis. *Occup Environ Med* 2016;73:877–84.
39. Sakuraya A, Watanabe K, Kawakami N, *et al*. Work-related psychosocial factors and onset of metabolic syndrome among workers: a systematic review and meta-analysis protocol. *BMJ Open* 2017;7:e016716.
40. Hunter JE, Schmidt FL. Fixed Effects vs. Random effects meta-analysis models: implications for cumulative research knowledge. *Int J Sel Assess* 2000;8:275–92.
41. Team RC. *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing, 2014.
42. Higgins JPT, Thompson SG. Quantifying heterogeneity in a meta-analysis. *Stat Med* 2002;21:1539–58.

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A Pilot Study of Healthy Living Options at 16 Truck Stops Across the United States

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Abstract

Purpose: There is a growing body of evidence that the built environment influences diet and exercise and, as a consequence, community health status. Since long-haul truck drivers spend long periods of time at truck stops, it is important to know if this built environment includes resources that contribute to the emotional and physical well-being of drivers.

Setting: The truck stop environment was defined as the truck stop itself, grocery stores, and medical clinics near the truck stop that could be accessed by a large truck or safely on foot.

Design: Researchers at the National Institute for Occupational Safety and Health (NIOSH) developed and utilized a checklist to record the availability of resources for personal hygiene and comfort, communication and mental stimulation, health care, safety, physical activity, and nutrition at truck stops.

Subjects: The NIOSH checklist was used to collect data at a convenience sample of 16 truck stops throughout the United States along both high-flow and low-flow truck traffic routes.

Measures: The checklist was completed by observation within and around the truck stops.

Results: No truck stops offered exercise facilities, 94% lacked access to health care, 81% lacked a walking path, 50% lacked fresh fruit, and 37% lacked fresh vegetables in their restaurant or convenience store.

Conclusion: The NIOSH found that most truck stops did not provide an overall healthy living environment.

Keywords

truck stop, obesity, nutrition, exercise, safety

Purpose

It is generally accepted within the medical and public health communities that diet and exercise are 2 important determinants of individual health.[1,2] Furthermore, there is a growing body of evidence that the built environment influences these determinants and, as a consequence, community health status. The built environment is generally defined as the man-made surroundings such as buildings, transportation systems, parks, and other man-made structures.[3]

Some studies report a negative relationship between body mass index (BMI) and access to a supermarket[4,5] and a positive relationship between BMI and access to either convenience stores[6] or fast-food restaurants.[7,8] A more recent study using a longitudinal regression model demonstrated that increases in density of both supermarkets and commercial physical activity facilities reduced BMI by 5.46 to 7.36 lbs/ft^2 among men.[9]

Truck drivers, by virtue of their occupation, are often constrained for long periods of time to specific built environment, such as loading docks, truck stops, trucking terminals, highway rest areas, and truck cabs. Drivers spend time in these locations not only during their working day but also during off-duty periods since parking for large trucks can be difficult to find

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at other locations. Long-haul truck drivers (LHTDs) carry freight on delivery routes that require sleep periods away from home. A recent survey of LHTDs found that 62.9% of drivers sleep at home less than 7 days each month.[10] As a consequence, they often spend long periods confined to truck stops when they are working. This makes the truck stop environment integral to the daily lives of LHTDs.

For people to make changes in behavior that improve their health requires support for the change at the individual, environment, and social levels.[11] For truck drivers, the truck stop defines an important environment level of support for individuals to make choices related to diet and exercise. Studies indicate that the truck drivers' work environment provides few opportunities for healthy food and exercise.[12-14] Given the time truck drivers are restricted to the truck stop environment, it is not surprising that studies have found truck drivers are less likely to exercise regularly or make healthy dietary choices than the general population,[12,15-17] or that a recent survey of LHTDs found that 89% of truck drivers had a BMI greater than 25.[10] The availability of healthy food and exercise options in the truck stop environment is a critical element for supporting truck drivers' ability to improve their health.

To better understand the truck stop environment, researchers from the National Institute for Occupational Safety and Health (NIOSH) developed a checklist designed to record resources available at truck stops that might contribute to the emotional and physical well-being of drivers. The checklist expands previously reported information through the inclusion of items describing the availability of nutritious food in restaurants and convenience stores at and near truck stops. Using the checklist, NIOSH researchers collected data at a convenience sample of 16 truck stops across the United States along both high-flow and low-flow highway segments. Data for this study were collected concurrently with quality assurance site visits by NIOSH staff during the National Survey of Long-Haul Truck Driver Injury and Health (LHTDS), a nationwide survey of LHTDs.[10] In this article, the authors use data collected with the checklist, as well as selected data collected as part of the LHTDS, to describe resources available to LHTDs for personal hygiene and comfort, communication and mental stimulation, health care, safety, physical activity, and nutrition in the truck stop environment.

Methods

Setting

For this study, the truck stop environment was defined as the truck stop itself, along with restaurants, grocery stores, and medical clinics near the truck stop that could be accessed safely on foot or accessed by tractor-trailers, also known as 18-wheelers. Tractor-trailers are representative of the size and weight of trucks typically used by long-haul drivers. Information on truck stop indoor and outdoor amenities, convenience store healthy food options, and restaurant/fast-food healthy food options were collected by NIOSH researchers from October to December 2010.

Design

The approach for this truck stop study was to collect data on truck stop amenities at a portion of the 32 truck stops used in the LHTDS. This allowed NIOSH researchers to collect information on truck stops amenities at the same time that they were conducting quality assurance audits of the LHTDS. The truck stops in this amenity study were derived from the methodology used to generate the LHTDS, however; LHTDS sample weights cannot be used to generate national estimates for the truck stop amenities reported in this study. Since the truck stops in this amenity study are a subset of the truck stops in the LHTDS, a brief description of the sample strategy LHTD survey is appropriate. More details on the LHTDS sampling strategy and design are available in the article by Sieber et al.[10] The sample of truck drivers in the LHTDS was selected in 3 stages, the first 2 of which selected the sampled truck stops and the final stage selected the sampled truck drivers. In the first stage, limited-access highway segments were stratified by geographic region and truck traffic volume. Truck traffic volume was defined as either high flow (12 500 or more trucks/day) or low flow (less than 12 500 trucks/day). The number of high-flow state highway segments in each geographical region was selected proportional to the length in miles of limited-access highway in that region. Low-flow state highway segments were selected with probability proportional to that state's population. Once highway segments were selected, a national truck stop list was used to select those stops that had a restaurant and at least 5 paved parking spaces[18]; the selection probability was proportional to the number of parking spaces. The sample pool of qualifying truck stops included 1490 independent truck stops and 918 chain truck stops, from which the LHTDS collected truck driver information at 32 truck stops.

Sample

The 16 stops visited by NIOSH researchers for the truck stop amenity study were a convenience sample selected based on limited travel funds, proximity to NIOSH facilities, and ability to combine multiple truck stop visits into single travel events. Of the 16 stops in the study, 3 were independent truck stops and 13 belonged to 3 different truck stop chains (chain 1: $n = 7$, chain 2: $n = 4$, chain 3: $n = 2$); the percentage of truck stops on high- and low-flow highway segments was similar to the full LHTDS sample. The geographic distribution of the 16 truck stops is shown in Table 1 and Figure 1. When high-flow samples are compared with the LHTDS, the truck stop amenity study had proportionately fewer truck stops in the Central and Great Lakes regions and proportionately more in the Northeast and South regions. Four of the 6 low-flow trucks stops from the LHTDS were included in the truck stop amenity study. Most (75%) of the truck stops in the amenity study were located along high-flow truck traffic routes. The number of overnight truck parking spots ranged between 20 and 800, with an average 231 spaces per stop. Average parking space occupancy was 47% during the day and 82% at night. The number of drivers

Table 1. Sample Geographic Distribution for the LHTDS and the Pilot Study of Healthy Living Options at 16 Truck Stops Across the United States.

High-Flow Sample Strata

Geographic Region	Pilot Study Sample Distribution		LHTDS Sample Distribution		Total Miles in Region	
	Number	Percent	Number	Percent	Number	Percent[a]
Central	1	8	6	24	5013	22
Great Lakes	2	16	6	24	4075	18
North East	2	16	3	12	2275	10
South	5	42	7	28	7012	30
West	2	16	4	16	4566	20
Subtotal	12		26		22 941	

Low-Flow Sample Strata

State (Geographic Region)	Pilot Study Sample Distribution		LHTDS Sample Distribution		State Population	
	Number	Percent	Number	Percent	Number	Percent[b]
Minnesota	1	25	1	17%	5 266 214	2
Ohio	0	0	1	17%	11 542 645	4
Louisiana	0	0	1	17%	4 497 076	1
Arkansas	1	25	1	17%	2 889 450	1
California	2	50	2	33%	36 961 664	12
Subtotal	4		6			
Total	16		32			

Abbreviation: LHTDS, National Survey of long-haul truck drivers.
[a]Percentage of total limited-access highway miles in the United States.
[b]Percentage of US population.

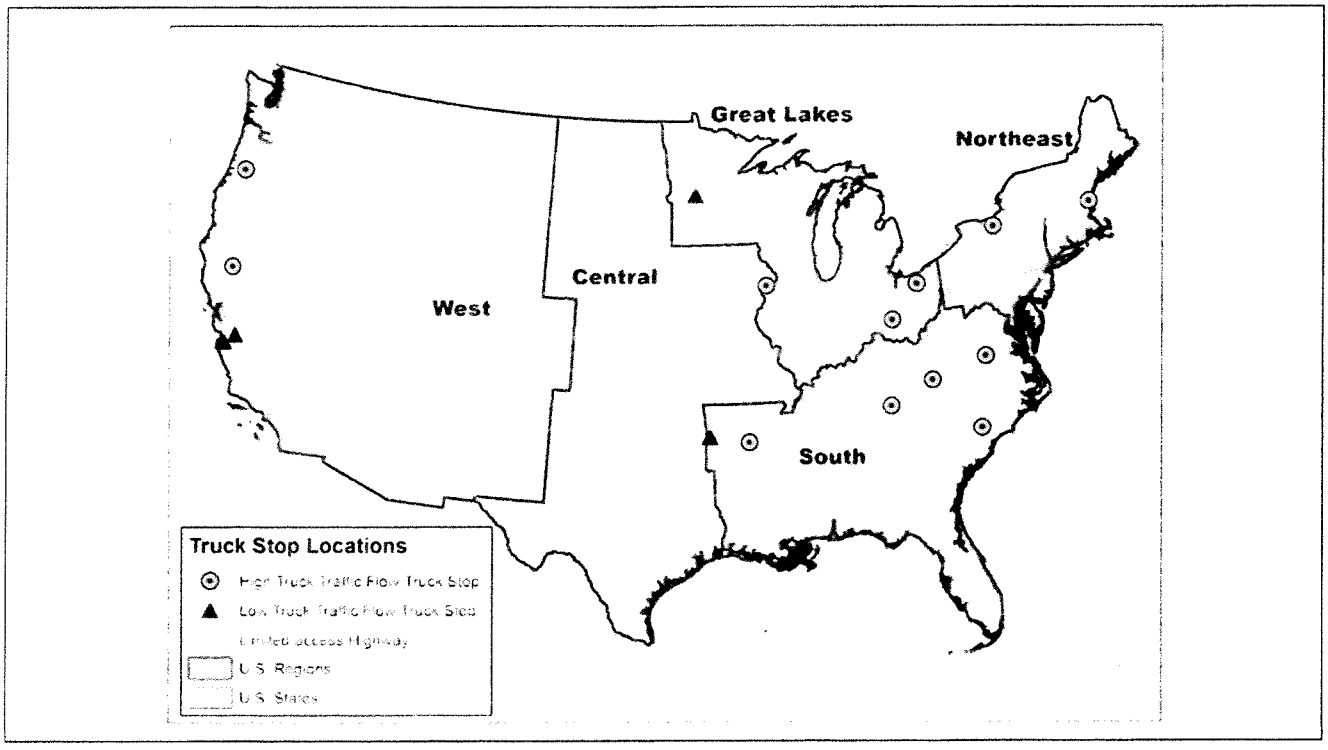

Figure 1. Geographical Distribution of the 16 Truck Stops in the United States.

visiting the truck stop varied between 10 and 4000 drivers on weekdays and between 5 and 2500 drivers on weekends.

Measures

At all 32 truck stops in the LHTDS, information was obtained on truck stop characteristic by interviewing the truck stop owner or manager. Data on these characteristics were extracted from the LHTDS for the 16 truck stops for this article. Results abstracted from the LHTDS for this article include the availability of motels/hotels, Internet kiosks, wireless Internet, and parking lots that allowed only trucks to enter, while remaining results pertaining to other living environment characteristics were collected by NIOSH researchers via direct observation using a checklist developed by NIOSH. Truck stops were visited over a 3-day period during daylight and nighttime time periods. The checklist took approximately 4 hours to complete over the 3 days.

The checklist consisted of 5 sections: truck stop indoor amenities, truck stop outdoor amenities, convenience store healthy food options, energy products, and restaurant/fast-food healthy food options, with space for comments from the data collector if they needed to further describe the environment. The data collector recorded the presence of a checklist item by checking or circling it on the form, or in the case of energy products, recording the number of different products available. Energy products were defined as any beverage, shot, or pill that claimed it would increase the consumer's energy or alertness. Energy products were tallied by brand; the different varieties within brand were not captured. To reduce interrater variability, 2 NIOSH researchers independently collected information on the checklist at the first 2 truck stops visited. At both truck stops, the researchers reviewed and discussed each element of their ratings to assure all future checklists were completed in a consistent manner. No further interrater variability assessment was done to further validate the survey instrument.

Facilities were considered to be nearby if they could safely be accessed by foot or tractor-trailer and seen from the truck stop. Parking lots were considered to be well lit by NIOSH researchers if functioning lighting was observed positioned throughout the parking lot including parking lot edges.

The availability of nutritious food was ascertained by NIOSH researchers in the truck stop convenience store by observing displayed items and in truck stop restaurants by viewing the menus and salad bars. In the convenience store, healthy foods were defined as (1) fresh fruit or vegetables; (2) packaged (frozen, canned, or dried) fruit if it did not have added sugar or fat; and (3) packaged vegetables, entrees (canned/frozen meals), and prepared snacks if they met or exceeded the US Food and Drug Administration (FDA) guidelines for low-fat and low-sodium foods, which require 3 g of fat or less and 140 g of sodium or less per reference amounts customarily consumed.[19]

Restaurant menus did not contain dietary information. Restaurant foods were considered healthy if they were advertised as cooked with little or no added oil (ie, baked, broiled, steamed, or poached); healthy animal proteins included white-meat poultry, shellfish, or any fish, including salmon; healthy salads needed to have greens other than iceberg lettuce that is considered to have low nutritional value; and healthy vegetarian dishes included fresh vegetables and needed to be low in sugar and be foods other than white potatoes or white rice, which have high glycemic loads and are considered to be of low nutritional value. While not an exhaustive list, we felt these options were the healthy choices most likely to be available.

All data are reported as percentages. Data extracted from the LHTDS were analyzed using the PROC/FREQ procedure generated using (SAS/STAT) software, copyright (2002-2010) SAS Institute Inc., Cary, NC, USA.

Table 2. Percentage of Truck Stops With Selected Physical Amenities at 16 Truck Stops Across the United States.

Amenity	Number of Stops	Percent
Hygiene/comfort		
Showers	16	100
Laundry	13	81
Motel/hotel on-site or nearby	11	69
Driver lounge	11	69
Truck hookups (shore power)	6	38
Barber or hair salon	1	6
Connectivity		
Wi-Fi	13	81
Internet kiosk	7	44
Nutrition		
Full-service restaurant on-site	11	69
Full-service restaurant nearby	2	13
Fast-food available on-site	9	56
Fast-food available nearby	3	19
Truck-accessible grocery nearby	2	13
Health care		
Health clinic on-site	1	6
Truck-accessible medical clinic nearby	0	0
Safety		
Parking area poorly lit[a]	9	60
Parking area limited to trucks	1	6
Physical activity		
Walking path	3	19
Designated exercise area	0	0

[a]n = 15 truck stops, 1 truck stop was only visited during daylight hours.

Results

Table 2 shows the physical amenities available at the 16 checklist truck stops. Only showers were universally available; more than 81% lacked a walking path, grocery store, or a barber/hair salon; 94% lacked any type of health-care facilities accessible to the drivers. Most truck stops had a laundry, motel/hotel, driver lounge, full-service restaurant, and/or fast-food available either at the truck stop or nearby. None of the truck stops visited offered designated exercise facilities. Only 6% had

Table 3. Available Healthy Food Options at 16 Truck Stops Across the United States.

Healthy Food Option	Number of Truck Stops	Percent
Restaurant (full-service + fast-food combined) healthy food option		
Healthy animal protein available	15	94
White-meat poultry	14	88
Salmon	6	38
Fish (excluding salmon)	8	50
Shellfish	2	13
Healthy vegetable/vegetarian dish available	12	75
Fresh salads (excluding iceberg lettuce)	9	56
Low-fat/low-sugar vegetarian dishes (excluding white rice and white potatoes)	5	31
Both healthy animal protein and healthy vegetable/vegetarian dish available	12	75
No healthy option available	1	6
Convenience store healthy food option		
Low-fat/low-sodium prepared snacks[a]	7	44
Healthy fruit available	12	75
Fresh fruit	8	50
Frozen/canned/dried Fruit (no sugar added or fat)	7	44
Healthy vegetable or prepared entrée available[a]	1	6
Fresh vegetables	1	6
Frozen/canned/dried vegetables[a]	0	0
Frozen/canned prepared entrees[a]	0	0
Both healthy fruit and vegetable/entrée available	1	6
No healthy option available (including snacks)	3	19
Fresh vegetable available in either restaurant or convenience store	10	63

[a]Three gram or less fat and 140 g or less sodium per serving.

parking areas that restricted nontruck drivers and few had adequate lighting in the truck parking area.

All of the truck stops had at least 1 convenience store and some form of restaurant (either full-service or fast-food). Where there was more than 1 restaurant option, healthy food availability was assessed at all restaurants using a single checklist. Table 3 shows the percentage of the 16 truck stops with specific healthy food options available at restaurants (full-service and/or fast-food combined) and convenience stores. Truck stop restaurants offered a healthy animal protein at 94% of stops and a healthy vegetable at 75% of stops; the most common healthful food options available in restaurants were white-meat poultry (88%) and vegetable/vegetarian dish (75%). Convenience stores offered a healthy fruit at 75% of truck stops, healthy snacks at 44% of stops, and a healthy vegetable at only 6% of stops; none of them offered a healthy prepared entrée.

The number of energy shot and pill brands offered at the 16 truck stops was tabulated. The average number of energy brands available was 15 (range: 5-30). The average number of energy shot and pill brands at each truck stop was 6 (range: 0-12; data not shown).

Discussion

Our pilot study found that while the truck stop environment generally provided opportunities for personal hygiene such as showers and laundries, and connectivity via wireless Internet, we consider them to be deficient for healthy living options. The environment rarely provided opportunities to obtain health care, lacked healthy food choices when there is no time to stop for a restaurant meal, and rarely provided options that encouraged physical activity. Furthermore, safety in the parking lot was less than optimal, as almost all stops allowed anyone to drive into the parking area, and lighting was often inadequate, which may discourage exercise around the parking area.

The truck stop environment did not provide ready access to medical care. Our pilot study found that 94% of the truck stops visited did not have a health-care clinic on-site or nearby. Results from the LHTDS showed that 18.3% of the drivers delayed or did not receive needed health-care treatment in the past 12 months.[10] Other studies have shown that truck drivers had challenges finding health care, were more likely to self-medicate, and were dissatisfied overall with health-care access while on the road due to parking lot and driveway accessibility limitations for large trucks with trailers.[17,20-22]

The truck stop environment generally did not help patrons meet the US Department of Agriculture (USDA) guidelines for diet. For average adults needing 2200 calories daily, the USDA Food Pattern recommends 2 cups of fruit and 3 cups of vegetables every day.[23] To help Americans meet this recommendation, the Dietary Guidelines Advisory Committee suggested improving the availability of fresh produce; yet, 38% of truck stops did not carry any fresh vegetables in either their restaurant or convenience store.[2] Healthy food was more likely to be available in the truck stop restaurants, although 25% of these did not have both a healthy animal protein and healthy vegetarian dish on the menu. Restaurant menus did not contain specific dietary information. Studies have shown that given a choice, drivers would choose healthy food options but may not know which food options are truly healthy.[24,25]

Thirty percent of truck drivers report "sometimes" or "often" being given an unrealistically tight delivery schedule.[26] Furthermore, around 59% of drivers are paid by the mile, meaning that they don't make money unless they are driving, and 46% are penalized for a late pick up or delivery.[27] These time and financial pressures can compel drivers to rely on the convenience store rather than taking time for a sit-down meal. Convenience store meal options were the only quick food option at 25% of the stops we visited where no fast-food restaurant was available. Only 1 of the 16 convenience stores offered a healthy vegetable, and none offered a healthy entrée. Fruit was easier to find, either fresh or frozen/canned/dried

with no sugar added or fat, although 25% were lacking this as well. These results are similar to a study that found 8 truck stops on the East Coast to be "not at all supportive" of healthful eating.[14] Grocery stores have greater availability and quality of healthy food choices than restaurants and convenience stores[28]; however, truck-accessible grocery stores were rare in our study, the time required to shop for and prepare these foods may be difficult for truck drivers, and trucks are not always outfitted with refrigerators or microwaves.

Obesity is a significant problem among truck drivers. A diet low in added sugars, moderate fat intake, and adequate physical activity can help individuals to prevent or reverse obesity, which, along with a diet low in sodium, reduces the risk of hypertension, stroke, and heart disease.[23] The LHTDS study found that more than one-quarter of long-haul drivers reported a diagnosis of hypertension and 89% had a BMI greater than 25.[10] Lack of physical activity is a significant contributor to obesity. The Physical Activity Guidelines for Americans recommends that adults engage in moderate-intensity aerobic activities for at least 150 min/week or of vigorous-intensity aerobic activities for at least 75 min/week.[29] The LHTDS measured the number of days in the previous week that LHTDs engaged in moderate to vigorous physical activity (MVPA) for at least 30 minutes at a time; Sieber et al[10] found that 27% of LHTDS participants did not engage in any MVPA the previous week. Birdsey et al[15] found that only 26% of male and 20% of female LHTDS participants engaged in MVPA 5 or more days/week. These physical activity levels are similar to other surveys where approximately 10% of truck drivers claimed vigorous activity 3 times a week.[12,17,30]

The lack of safe walking areas combined with poor lighting and hazards posed by walking around moving vehicles are strong deterrents for truck drivers who might otherwise utilize their time at the truck stop obtaining exercise. In our pilot study, it was rare to find a walking path (including sidewalks), and there were no designated exercise areas at any of our 16 stops; few truck stops had adequate lighting (40%), and only 6% had parking areas solely for trucks. Studies have shown that drivers sometimes walk around the parking area for exercise, but they run the risk of being struck by vehicles, especially at night in lots with inadequate lighting.[31,32] As with our study, Apostolopoulos et al[14] also found that truck stops (n = 8) were "not at all supportive" of active living. The truck stop environment should be designed to encourage physical activity of truck drivers.

Truck stop parking lots are vulnerable to crime, which jeopardizes drivers' safe and undisturbed rest.[33,34] All but 1 stop in our study allowed anyone to access the truck parking area, and 9 (60%) of the 15 stops were poorly lit after dark. Truck stops could improve safety and reduce disturbance by increasing their lighting and restricting access of the truck parking area to only truck drivers. One study suggested that increasing lighting and security at truck stops may reduce solicitation of truck stop patrons by sex workers and drug dealers.[35] Some research has shown improved lighting can reduce crime in parking areas.[36,37]

In recent years, some truck stops, including some from our study, have increased healthy options on restaurant menus and provided walking areas and exercise facilities.[38,39] Additionally, at least 1 truck manufacturer has developed an optional "gym" for their trucks. As the Federal Motor Carrier Safety Administration has proposed a mandatory sleep study for any driver with a BMI ≥35, drivers may welcome any opportunity to help them achieve a healthy weight.[40]

Strengths and Limitations

This pilot study was conducted in conjunction with a large nationally representative survey of LHTDs to characterize the truck stop environment, which is an integral part of the LHTD's daily work life. We developed a simple checklist and used well-defined FDA product labeling and definitions to allow quick and accurate data collection on a subset of truck stops while conducting quality control visits for LHTDS.[19] This allowed us to collect valuable data about the truck stop environment without drawing resources away from the main truck driver survey.

Limitations to our study include the small number of truck stops that NIOSH researchers were able to visit due to limited resources. The small sample size and the convenience nature of the truck stop selection process mean the results are not representative of all truck stops in the country. For example, in our study, independent truck stops represented 19% of the visited truck stops, but 62% of truck stops in the United States. Forty-four percent of the truck stops visited were from a single truck stop chain, but that truck stop chain only represents 4.6% of the truck stops in the sample frame. Nevertheless, our study is strengthened by the wide geographic distribution of the 16 selected truck stops, which suggests our results are not limited to a single state or small geographic area. A larger-scale survey should be conducted to fully understand the magnitude of health and wellness barriers at truck stops. The stratified sample strategy used in the LHTDS could be used to select a representative sample of truck stops for such a full-scale study of healthy options at truck stops.

Other limitations are related to the checklist and how it was administered. The lighting around truck stop parking areas was rated by visual observation and not objectively with specialized equipment, and noise was not assessed at all. Restaurant menus did not contain similar dietary information to the packaged food in the convenience store; therefore, available healthy options could only be tabulated through visual observations not through actual dietary information. Lack of dietary information on truck stop menus is a challenge not only for researchers conducting this study but also for truck drivers who might like to make healthier eating choices. The checklist for available food items was not comprehensive and did not include items like whole-grain breads, vegetable proteins, portion size, or a measure of the number or diversity of healthy items available. The checklist also did not capture any other food or beverage options such as fried food, coffee, or sugary drinks. Checklist data were subject to observer bias and inconsistency between

observers. To achieve better consistency, a standard checklist was used and both researchers independently used the checklist at 2 truck stops, discussing and reconciling differences among their checklists while still at each of the 2 truck stops. Future research should evaluate checklist instrument for interrater reliability and validity and implement a similar sampling strategy to the LHTDS to achieve more nationally representative results.

Conclusions

Many LHTDs spend multiple nights away from home when they are working and are subsequently dependent on the built environment of the truck stop to provide for their daily needs. The current pilot study found that most truck stops did not provide an overall healthy living environment, as only 6% of the truck stops offered fresh vegetables in convenience stores and no truck stop offered drivers designated exercise facilities. This pilot study underscores the limited access to healthy food and exercise options for this working population at the truck stops evaluated and raises important questions about the adequacy of the truck stop environment for promoting a healthy lifestyle among LHTDs. Widespread efforts to provide healthy food and opportunities for safe physical activity at truck stops are needed for the health of these workers.

So What? Implications for Health Promotion Practitioners and Researchers

What is already known on this topic?

Truck stops are integral to the daily lives of long-haul truck driver (LHTDs) since these workers spend long periods away from home confined to facilities that can accommodate their large vehicles. It is accepted that diet and exercise contribute to the overall health. Long-haul drivers have a high prevalence of obesity, and research has demonstrated an association between built environment and body mass index.

What does this article add?

This study assessed the healthy living options at a sample of truck stops geographically dispersed throughout the United States and found few opportunities for safe physical activity and healthy eating.

What are the implications for health promotion practice or research?

Understanding the built environment of truck stops can inform federal agencies and industry leaders in their efforts to help truck drivers improve and maintain their health.

Authors' Note

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health (NIOSH). This author worked at NIOSH, DSR, at the time of data collection

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References

1. DHHS. Physical Activity Guidelines Advisory Committee Report, 2008. In: Committee PAGA, ed. Washington, DC: U.S. Department of Health and Human Services; 2008:683.
2. USDA. Report of the Dietary Guidelines Advisory Committee on the dietary guidelines for Americans, 2010: to the Secretary of Agriculture and the Secretary of Health and Human Services. Washington, D.C.: United States Department of Agriculture, Agricultural Research Service; 2010.
3. Papas M, Alberg A, Ewing R, Helzisouer K, Gary T, Klassen A. The built environment and obesity. *Epidemiol Rev.* 2007;29: 129-143.
4. Powell LM, Han E, Chaloupka FJ. Economic contextual factors, food consumption, and obesity among U.S. adolescents. *J Nutr.* 2010;140(6):1175-1180.
5. Morland KB, Evenson KR. Obesity prevalence and the local food environment. *Health Place.* 2009;15(2):491-495.
6. Morland K, Diez Roux AV, Wing S. Supermarkets, other food stores, and obesity: the atherosclerosis risk in communities study. *Am J Prev Med.* 2006;30(4):333-339.
7. Mehta NK, Chang VW. Weight status and restaurant availability a multilevel analysis. *Am J Prev Med.* 2008;34(2):127-133.
8. Li F, Harmer PA, Cardinal BJ, et al. Built environment, adiposity, and physical activity in adults aged 50-75. *Am J Prev Med.* 2008; 35(1):38-46.
9. Boone-Heinonen J, Diez-Roux A, Goff D, et al. The neighborhood energy balance equation: does neighborhood food retail environment + physical activity environment = obesity? The CARDIA study. *PLoS One.* 2013;8(12):1-11.
10. Sieber WK, Robinson CF, Birdsey J, et al. Obesity and other risk factors: the national survey of U.S. long-haul truck driver health and injury. *Am J Ind Med.* 2014;57(6):615-627.

11. McLeroy KR, Steckler A, Bibeau D, Glanz K. An ecological perspective on health promotion programs. *Health Educ Q.* 1988;15(4):351-377.
12. McCullagh J. Fat and fitness - in for the long haul. *Occup Health.* 2005;57(7):24-26.
13. Apostolopoulos Y, Sönmez S, Shattell M, Haldeman L, Strack R, Jones V. Barriers to truck drivers' healthy eating: environmental influences and health promotion strategies. *J Workplace Behav Health.* 2011;26(2):122-143.
14. Apostolopoulos Y, Shattell MM, Sönmez S, Strack R, Haldeman L, Jones V. Active living in the trucking sector: environmental barriers and health promotion strategies. *J Phys Act Health.* 2012; 9(2):259-269.
15. Birdsey J, Sieber W, Chen G, et al. National survey of U. S. long haul truck driver health and injury (LHTDS): health behaviors. *JOEM.* 2015;57(2):210-216.
16. VanderBeek A. World at work: truck drivers. *Occup Environ Med.* 2012;69(4):291-295.
17. Korelitz J, Fernandez A, Uyeda V, Spivey G, Browdy B, Schmidt R. Health habits and risk factors among truck drivers visiting a health booth during a trucker trade show. *Am J Health Promot.* 1993;8(2):117-123.
18. Vos Rd, Brice T. *National Truck Stop Directory: The Trucker's Friend.* Clearwater, FL: TR Publications; 2008.
19. FDA. A Food Labeling Guide: Guidance for Industry (9. Appendix A: Definitions of Nutrient Content Claims); 2013. Web site. http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/LabelingNutrition/ucm064911.htm. Accessed September 13, 2016.
20. Layne DM, Rogers B, Randolph SA. Health and gender comparisons in the long-haul trucking industry: a pilot study. *AAOHN J.* 2009;57(10):405-413.
21. Reed DB, Cronin JS. Health on the road: issues faced by female truck drivers. *AAOHN J.* 2003;51(3):120-125.
22. Solomon AJ, Doucette JT, Garland E, McGinn T. Healthcare and the long haul: Long distance truck drivers - a medically underserved populations. *Am J Ind Med.* 2004;46(5):463-471.
23. USDA, DHHS. *Dietary Guidelines for Americans, 2010.* 7th ed. Washington, D.C.: U.S. Government Printing Office; 2010.
24. Whitfield Jacobson PJ, Prawitz AD, Lukaszuk JM. Long-haul truck drivers want healthful meal options at truck-stop restaurants. *J Am Diet Assoc.* 2007;107(12):2125-2129.
25. Gill PE, Wijk K. Case study of a healthy eating intervention for Swedish lorry drivers. *Health Educ Res.* 2004;19(3): 306-315.
26. McCartt AT, Hellinga LA, Solomon MG. Work schedules of long-distance truck drivers before and after 2004 hours-of-service rule change. *Traffic Inj Prev.* 2008;9(3): 201-210.
27. Monaco K, Olsson L, Hentges J. Hours of sleep and fatigue in motor carriage. *Contemp Econ Pol.* 2005;23(4):615-624.
28. Pereira RF, Sidebottom AC, Boucher JL, Lindberg R, Werner R. Assessing the food environment of a rural community: baseline findings from the heart of New Ulm project, Minnesota, 2010-2011. *Prev Chronic Dis.* 2014;11:E36.
29. DHHS. 2008 Physical activity guidelines for Americans: be active, healthy, and happy! Washington, DC: U.S. Dept. of Health and Human Services; 2008.
30. Turner LM, Reed DB. Exercise among commercial truck drivers. *AAOHN J.* 2011;59(10):429-436.
31. Anonymous. Two truck drivers killed in separate accidents at same truck stop. *WBIR 10.* January 19, 2011.
32. Weigel J. Truck driver killed in Memphis truck stop lot identified. *WLKY.* WLKY.com; 2014.
33. Bloom S. Two charged in truck stop crime spree. *WWBT NBC 12.* July 03, 2012.
34. Anonymous. County attorney sues truck stop for allowing crime. *Your Houston News.* February 22, 2014.
35. Apostolopoulos Y, Sonmez S, Shattell M, Kronenfeld J. Sex work in trucking milieux: "lot lizards," truckers, and risk. *Nurs Forum.* 2012;47(3):140-152.
36. Farrington DP, Welsh BC. Improved street lighting and crime prevention. *Justice Quarterly.* 2002;19(2):313-342.
37. Bachner JP. Effective security lighting. *J Secur Admin.* 1985;9(1): 59-73.
38. Marcum E. Pilot opening fitness centers for truck drivers. *Knoxville News Sentinel.* 2013.
39. TA and Petro. *Stay Health on the Highway.* September 23, 2015. Web site. http://www.ta-petro.com/amenities/stayfit. Accessed September 19, 2016.
40. FMCSA. Department of Transportation Federal Motor Carrier Safety Administration. Proposed Recommendations on Obstructive Sleep Apnea. 77:23794-23797. Federal Register; 2012.

Accept Rapid Communications Poster Abstract

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Welcome!
On behalf of the Scientific Program Committee, we look forward to welcoming you to Santiago and the 2018 International Congress of Behavioral Medicine (ICBM, www.icbm2018.cl). Thank you very much for submitting your rapid communications scientific abstract to the 2018 ICBM.

We were thrilled to receive such a significant number of outstanding submissions this year. Thanks to your efforts we have created a very exciting scientific program representing the forefront of behavioral medicine research and innovations from around the world, taking place against the backdrop of social and cultural activities in Santiago, Chile.

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Abstract title:Work-related psychosocial risk factors and hepatic abnormalities among Japanese male workers
Poster session: 15:15 – 15:45, Friday, November 16, 2018
Location of session: Volcanes Foyer, Hotel Intercontinental Santiago
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Work-related psychosocial risk factors and hepatic abnormalities among Japanese male workers

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Key words: hepatic abnormalities, psychosocial factors at work, Japan

Introduction & Purpose: Most liver cirrhosis among Japanese men, except for type B or type C hepatitis, is caused by excessive alcohol consumption. Many research studies have identified associations between psychosocial factors at work and alcohol consumption; therefore, those factors may be a risk for hepatic abnormalities. This study investigated the effects of psychosocial factors at work on biological indices of hepatic abnormalities among Japanese male workers.

Methods: A 1-year follow-up prospective study was conducted between 2012 and 2013 at a manufacturing company in western Japan. At baseline, 4,085 male workers answered the Brief Job Stress Questionnaire measuring job stressors and social supports. Among these workers, 3,841 underwent blood examination at follow-up to determine abnormal levels of biological indices of hepatic abnormalities, defined as AST (GOT) > 40 IU/L, ALT (GPT) > 35 IU/L, and gamma-GTP > 70 IU/L. After excluding 193 participants who had one or more missing answers in the questionnaire at baseline, data from the remaining 3,648 workers were used in statistical analyses. Multiple logistic regression was performed to elucidate the effects of job stressors and social supports on liver function tests. In all analyses, age, current medical history of hepatic disorders, smoking habits, alcohol consumption, exercise habits, sleep hours, and BMI were considered as control variables.

Results: High Qualitative Overload (OR = 1.61, 95% CI 1.13 – 2.29, $p<0.01$) and low Suitable Jobs (OR = 1.98, 95% CI 1.18 – 3.34, $p<0.05$) were associated with abnormal levels of gamma-GTP. Low Physical Demands was inversely associated with abnormal levels of AST (GOT) (OR = 2.36, 95% CI 1.41 – 3.96, $p<0.05$), ALT (GPT) (OR = 2.30, 95% CI 1.81 – 2.91, $p<0.001$) and gamma-GTP (OR = 2.49, 95% CI 1.79 – 3.47, $p<0.001$).

Conclusions: Japanese male workers who had high qualitative overload or felt low suitability for their jobs may have had impaired liver function, especially gamma-GTP, after a 1-year period.

Acknowledgement: This study was supported by Industrial Disease Clinical Research Grants (No. 160701) from Japan Ministry of Health, Labour, and Welfare.

ORIGINAL ARTICLE

Protective Role of Myelocytic Nitric Oxide Synthases against Hypoxic Pulmonary Hypertension in Mice

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Abstract

Rationale: Nitric oxide (NO), synthesized by NOSs (NO synthases), plays a role in the development of pulmonary hypertension (PH). However, the role of NO/NOSs in bone marrow (BM) cells in PH remains elusive.

Objectives: To determine the role of NOSs in BM cells in PH.

Methods: Experiments were performed on 36 patients with idiopathic pulmonary fibrosis and on wild-type (WT), nNOS (neuronal NOS)$^{-/-}$, iNOS (inducible NOS)$^{-/-}$, eNOS (endothelial NOS)$^{-/-}$, and n/i/eNOSs$^{-/-}$ mice.

Measurements and Main Results: In the patients, there was a significant correlation between higher pulmonary artery systolic pressure and lower nitrite plus nitrate levels in the BAL fluid. In the mice, hypoxia-induced PH deteriorated significantly in the n/i/eNOSs$^{-/-}$ genotype and, to a lesser extent, in the eNOS$^{-/-}$ genotype as compared with the WT genotype. In the n/i/eNOSs$^{-/-}$ genotype exposed to hypoxia, the number of circulating BM-derived vascular smooth muscle progenitor cells was significantly larger, and transplantation of green fluorescent protein–transgenic BM cells revealed the contribution of BM cells to pulmonary vascular remodeling. Importantly, n/i/eNOSs$^{-/-}$-BM transplantation significantly aggravated hypoxia-induced PH in the WT genotype, and WT-BM transplantation significantly ameliorated hypoxia-induced PH in the n/i/eNOSs$^{-/-}$ genotype. A total of 69 and 49 mRNAs related to immunity and inflammation, respectively, were significantly upregulated in the lungs of WT genotype mice transplanted with n/i/eNOSs$^{-/-}$-BM compared with those with WT-BM, suggesting the involvement of immune and inflammatory mechanisms in the exacerbation of hypoxia-induced PH caused by n/i/eNOSs$^{-/-}$-BM transplantation.

Conclusions: These results demonstrate that myelocytic n/i/eNOSs play an important protective role in the pathogenesis of PH.

Keywords: bone marrow; pulmonary vascular remodeling; right ventricular hypertrophy; vascular smooth muscle progenitor cell

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

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At a Glance Commentary

Scientific Knowledge on the Subject: Nitric oxide (NO), synthesized by NOSs (NO synthases), plays a role in the development of pulmonary hypertension (PH). However, the role of NO/NOSs in bone marrow (BM) cells in PH remains to be clarified.

What This Study Adds to the Field: Here we show that transplantation of BM cells from mice lacking all NOSs aggravates hypoxia-induced PH in wild-type (WT) mice, and transplantation of BM cells from the WT mice ameliorates hypoxia-induced PH in the NOSs$^{-/-}$ mice, demonstrating a protective role of myelocytic NOSs in the pathogenesis of PH. Our findings provide novel insights into the cellular and molecular basis of PH.

Pulmonary hypertension (PH) is a progressive disease of the pulmonary microvasculature, leading to right ventricular failure and premature death. A recent large clinical study in a community-based general population of 10,314 subjects has reported that the prevalence of PH is 9.1% (1), indicating that PH is a common disease. Despite recent therapeutic advances, the prognosis for patients with PH is still poor. In the Assessing the Spectrum of Pulmonary hypertension Identified at a Referral Centre (ASPIRE) registry, the 3-year survival rates for idiopathic PH (group 1), for PH due to left heart disease (group 2), for PH due to lung diseases and/or hypoxia (group 3), for chronic thromboembolic PH (group 4), and for miscellaneous PH (group 5) were 68%, 73%, 44%, 71%, and 59%, respectively (2). These survival rates are similar to or even worse than the 3-year survival rate for all cancers (69.5%) (3). Although several possible pathogenetic factors, including mutations in the *BMPR2* (bone morphogenic protein receptor 2) gene, a deficiency in prostacyclin and nitric oxide (NO) release, and an excess in endothelin-1 and its receptor expression, have been believed to be involved in the development of PH (4), the precise mechanisms remain to be elucidated.

NO is synthesized from L-arginine by a family of NOSs (NO synthases; nNOS [neuronal NOS], iNOS [inducible NOS], and eNOS [endothelial NOS]), all of which isoforms are expressed in human lung tissues under both physiological and pathological conditions (5–7). nNOS is located in the airway epithelium, vascular endothelium, vascular smooth muscle cells (VSMCs), and nerve cells; iNOS is expressed in the airway epithelium, vascular endothelium, VSMCs, and alveolar macrophages; and eNOS is localized in the vascular endothelium and VSMCs (8–10). There has been no previous study that characterizes the role of nNOS in PH. The detrimental role of iNOS in hypoxic PH has been reported by using an iNOS inhibitor (11), whereas the opposing protective role of iNOS has been shown in iNOS$^{-/-}$ mice (12). The beneficial role of eNOS in hypoxic PH has been indicated in eNOS$^{-/-}$ and eNOS-transgenic mice (13, 14), although conflicting results in the eNOS$^{-/-}$ mice have also been reported (15).

The role of the n/i/eNOSs in their entirety in PH has been examined in pharmacological studies with nonselective NOSs inhibitors, such as N^{ω}-nitro-L-arginine methyl ester (L-NAME). Although several studies have shown that the nonselective NOSs inhibitors potentiate pulmonary artery pressure (PAP) in animals with hypoxia-induced PH, there are also publications reporting a lack of such potentiation (16). It has been indicated that acute administration of L-NAME increased PAP in perfused lungs of rats with hypoxia-induced PH, but this effect was not reversed by administration of L-arginine, indicating that the effect of L-NAME was not mediated by NOSs inhibition (16). Thus, because of the nonspecificity of nonselective NOSs inhibitors, the role of NOSs in the pathogenesis of PH still remains unclear. In this study, we addressed this issue in our mice lacking all NOSs (17). Recent studies have reported that myeloid abnormalities are associated with patients with PH (18), that 13% to 48% of patients with bone marrow (BM) abnormalities (myeloproliferative disorders) suffer from PH (19), and that transplantation of BM cells with *BMPR2* gene mutation causes PH in wild-type (WT) mice, suggesting the role of BM cells in the development of PH (20). However, no study has ever addressed the role of myelocytic NOSs in PH. On the basis of the aforementioned research, we investigated the role of NOSs, particularly myelocytic NOSs, in the development of PH. Some of the results of the study have been previously reported in the form of an abstract (21).

Methods

Subjects

This study was approved by the Institutional Review Board of the University of Occupational and Environmental Health. *See* the online supplement for further details.

Pulmonary Artery Systolic Pressure Measured by Echocardiography

Pulmonary artery systolic pressure (PASP) was estimated by echocardiography (22, 23) (*see* online supplement).

Animals

This study was approved by the Ethics Committee of Animal Care and Experimentation, the University of Occupational and Environmental Health (*see* online supplement).

Hypoxia Exposure

The mice were exposed to normoxia (21% O_2) or hypoxia (10% O_2) for 3 weeks (*see* online supplement).

BAL Fluid Sampling

BAL fluid (BALF) sampling was performed as we previously reported (24) (*see* online supplement).

Nitrite plus Nitrate Measurement

NOx (nitrite plus nitrate) concentrations were assessed by the Griess method, as we previously reported (17) (*see* online supplement).

Western Blot Analysis

Western blot analysis was performed as we previously reported (25) (*see* online supplement).

Hemodynamics

Right ventricular systolic pressure (RVSP) was measured with a pressure transducer (Millar Instruments) under anesthesia with sevoflurane (*see* online supplement).

Morphology

Hematoxylin and eosin or elastic van Gieson staining or α-SMA (α-smooth muscle actin) immunostaining was performed as we previously reported (26) (*see* online supplement).

Treatment with Isosorbide Dinitrate or Sodium Nitrate

Isosorbide dinitrate (ISDN) (0.6 mg/dl; Eisai) or sodium nitrate (2, 5, and 45 mmol/L; Wako) was administered orally in the triple n/i/eNOS$^{-/-}$ mice from 3 days before to 3 weeks after the hypoxic exposure (27) (*see* online supplement).

Bleomycin Treatment

Bleomycin (8.0 mg/kg/d; Nippon Kayaku) was intraperitoneally administered in the WT and triple n/i/eNOSs$^{-/-}$ mice for 10 consecutive days, and PH evaluation was performed at 14 days after the last administration (28) (*see* online supplement).

BM-derived VSMC Progenitor Cells

After red blood cells were removed from anticoagulated blood by erythrocyte lysis, Sca-1 (stem cell antigen-1)$^{+}$/c-kit^{-}/Lin (lineage)$^{-}$ cells were counted with flow cytometry (Sony), as we previously reported (29) (*see* online supplement).

Stromal Cell–derived Factor-1α

SDF-1α (stromal cell–derived factor-1α) levels were measured with an immunoassay kit (R&D Systems) (*see* online supplement).

BM Transplantation

BM transplantation was performed as we previously reported (30) (*see* online supplement).

Fluorescent Staining

Immunofluorescence for α-SMA and DAPI was analyzed by confocal laser scanning microscopy (*see* online supplement).

Micro Computed Tomographic Analysis

A micro computed tomographic analysis was performed under sevoflurane anesthesia (RIGAKU) (*see* online supplement).

RNA Sequencing

Complementary DNA libraries were sequenced on an HiSeq 2500 platform (Illumina) (*see* online supplement).

Statistical Analyses

Results are expressed as mean ± SEM. Statistical analyses were performed by Student's *t* test, or ANOVA followed by Bonferroni test (*see* online supplement).

Results

An Inverse Correlation between PASP and NOx or Nitrate Levels in BALF in Patients with Idiopathic Pulmonary Fibrosis

We performed detailed examinations, including bronchoscopy and Doppler echocardiography, in 303 subjects who were suspected to be suffering from interstitial lung diseases, to determine a diagnosis. We instructed current smokers to refrain from smoking for 2 weeks before the examinations to minimize the influence of smoking on the examinations. We did not measure a urinary or blood level of cotinine, which is a metabolite of nicotine and a marker of current smoking status. We finally studied 36 consecutive patients with idiopathic pulmonary fibrosis (IPF). The baseline characteristics of the patients are shown in Table E1 in the online supplement. In the patients, PASP estimated by Doppler echocardiography was significantly and negatively correlated with BALF NOx levels and BALF nitrate levels, but not with BALF nitrite levels (Figure 1). There were no significant correlations between the BALF NOx, nitrate, or nitrite levels and BALF cellular profiles, lung function, serum lactate dehydrogenase or Krebs von den Lungen-6 levels, or partial pressure of oxygen in arterial blood (Figure E1).

Lower Plasma and BALF NOx Levels in n/i/eNOSs$^{-/-}$ Mice Exposed to Hypoxia

We then conducted a basic study in which WT, single NOS$^{-/-}$, and triple n/i/eNOSs$^{-/-}$ mice were exposed to either normoxia or hypoxia for 3 weeks. After the normoxic exposure, the plasma NOx levels were significantly lower in the nNOS$^{-/-}$, iNOS$^{-/-}$, eNOS$^{-/-}$, and n/i/eNOSs$^{-/-}$ mice and lowest in the n/i/eNOSs$^{-/-}$ mice as compared with the WT mice (Figure 2A), and the BALF NOx levels were significantly reduced in the iNOS$^{-/-}$, eNOS$^{-/-}$, and n/i/eNOSs$^{-/-}$ mice and lowest in the n/i/eNOSs$^{-/-}$ mice (Figure 2B). As compared with the normoxic exposure, the hypoxic exposure did not significantly affect the plasma NOx levels in any of the mice (Figure 2A) but significantly increased the BALF NOx levels in the WT mice (Figure 2B). After both the normoxic and hypoxic exposures, the NOS$^{-/-}$ and n/i/eNOSs$^{-/-}$ mice were devoid of expression of genetically disrupted NOS and n/i/eNOSs, respectively (Figures 2C–2F). As compared with the normoxic exposure, the hypoxic exposure tended to increase expression levels of genetically intact NOSs in the lung in all the genotypes and significantly upregulated iNOS levels in the eNOS$^{-/-}$ mice and eNOS levels in the iNOS$^{-/-}$ mice (Figures 2C–2F).

Upregulation of arginase induces NO deficiency and NOS uncoupling via metabolizing L-arginine, and arginase expression is increased in the lung of

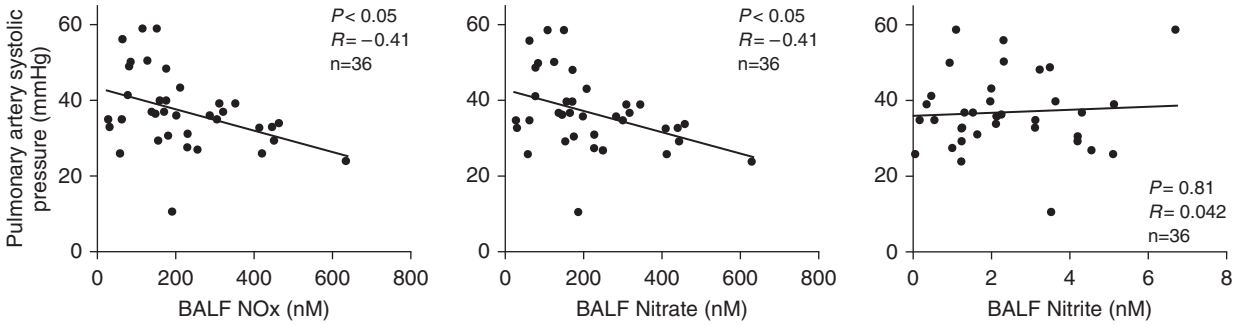

Figure 1. An inverse correlation between pulmonary artery systolic pressure and NOx (nitrite plus nitrate), nitrate, or nitrite levels in BAL fluid (BALF) in patients with idiopathic pulmonary fibrosis. Pulmonary artery systolic pressure estimated by Doppler echocardiography was significantly and negatively correlated with NOx and nitrate concentrations in BALF in patients with idiopathic pulmonary fibrosis.

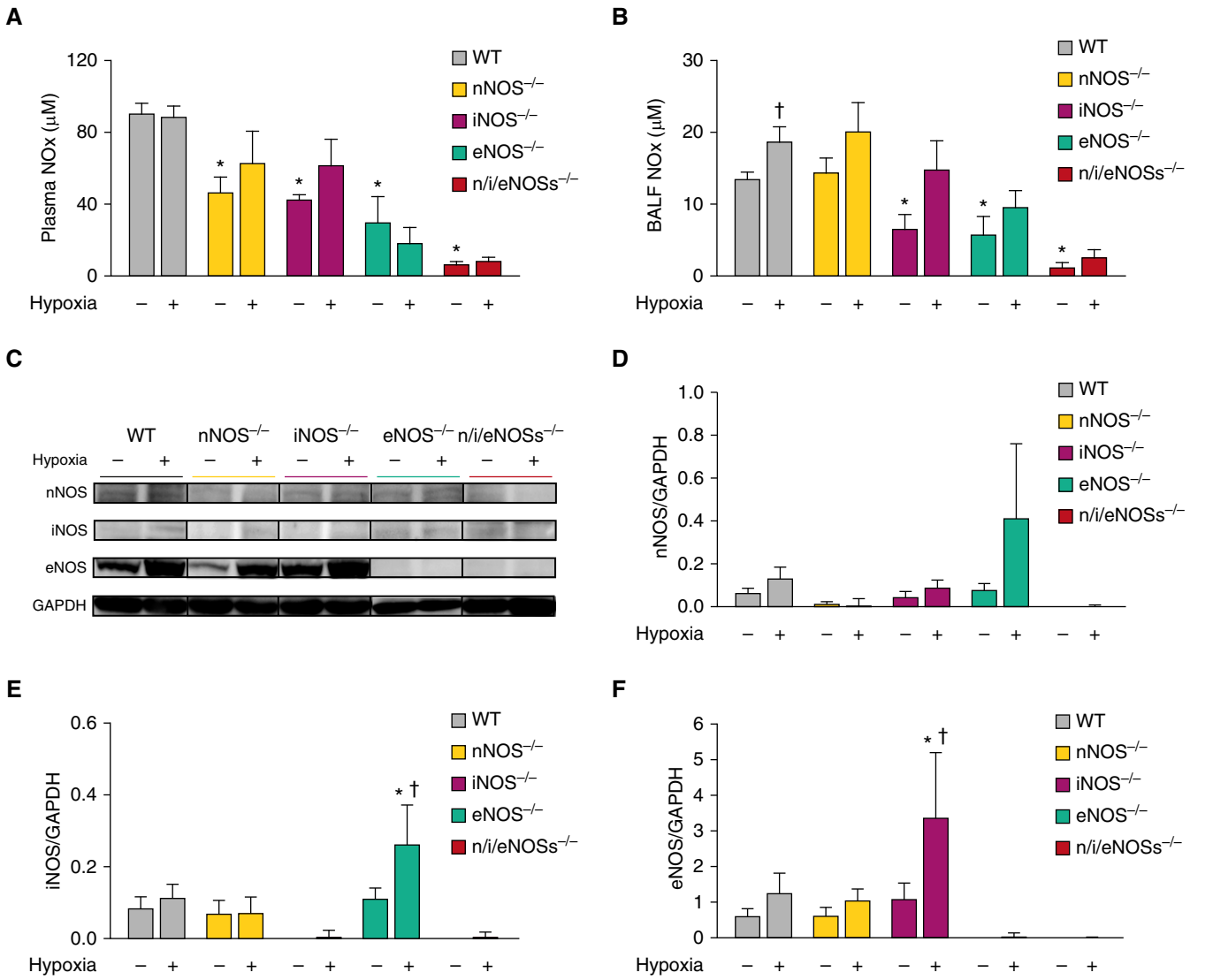

Figure 2. NOx (nitrite plus nitrate) levels in plasma and BAL fluid (BALF) and NOS (nitric oxide synthase) expression levels in the lung in wild-type (WT), single NOS$^{-/-}$, and triple neuronal/inducible/endothelial NOS$^{-/-}$ (n/i/eNOSs$^{-/-}$) mice exposed to normoxia or hypoxia. (*A* and *B*) NOx levels in the plasma ($n = 7$–10) and BALF ($n = 4$–7) after the normoxic and hypoxic exposure. *$P < 0.05$ versus WT mice. †$P < 0.05$ versus normoxia. (*C*–*F*) NOS isoform expression levels in the lung after the normoxic and hypoxic exposure ($n = 4$–5). *$P < 0.05$ versus WT mice after the hypoxic exposure. †$P < 0.05$ versus normoxia.

patients with idiopathic PH (31). The hypoxic exposure significantly augmented arginase-1 levels in the lung in all the genotypes, and there were no significant differences in the hypoxia-induced arginase-1 levels among the five genotypes (Figure E3).

Reduced Survival in n/i/eNOSs$^{-/-}$ Mice Exposed to Hypoxia

All the mice lived after the normoxic exposure, whereas after the hypoxic exposure, the survival rate was significantly worse in the n/i/eNOSs$^{-/-}$ mice and, to a lesser extent, in the eNOS$^{-/-}$ mice than in the WT mice (Figure 3A). After the hypoxic exposure, but not after the normoxic exposure, body weight was also significantly lower in the n/i/eNOSs$^{-/-}$ mice and, to a lesser degree, in the eNOS$^{-/-}$ mice than in the WT mice (Figure E2). A postmortem analysis indicated severe PH in all of the dead n/i/eNOSs$^{-/-}$ mice, and no other pathological findings that could explain the cause of death were observed in any of the mice (data not shown).

Accelerated Hypoxia-induced PH in n/i/eNOSs$^{-/-}$ Mice

We next compared the extent of hypoxia-induced PH among the five genotypes. After the normoxic exposure, there were no significant differences in RVSP measured by right heart catheterization (an index of PASP), in the weight ratio of the right ventricle to the left ventricle plus the interventricular septum

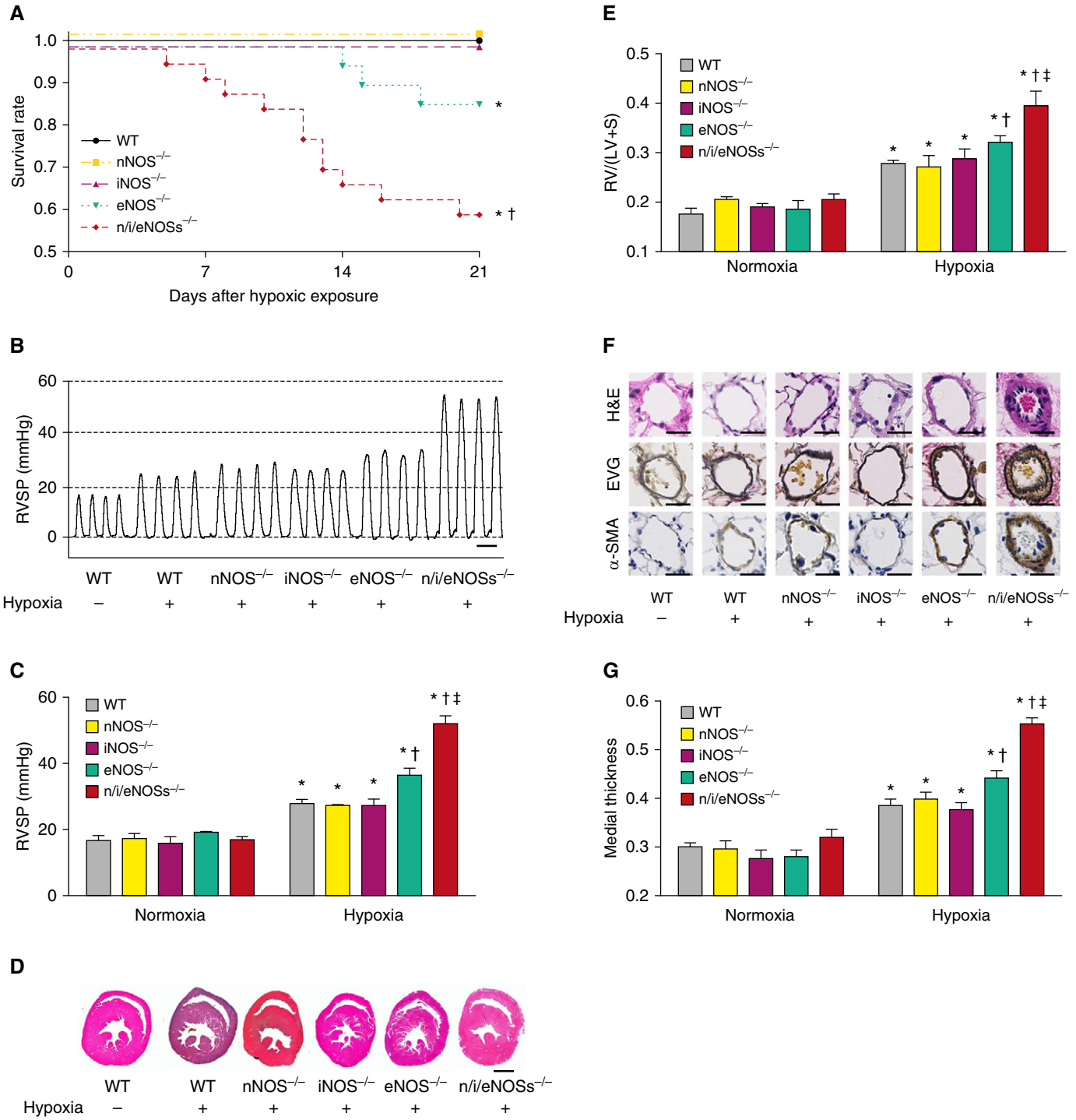

Figure 3. Survival rate, right ventricular systolic pressure (RVSP), right ventricular hypertrophy, and pulmonary vascular remodeling in wild-type (WT), single NOS (nitric oxide synthase)$^{-/-}$, and triple neuronal/inducible/endothelial NOS$^{-/-}$ (n/i/eNOSs$^{-/-}$) mice exposed to normoxia or hypoxia. (*A*) Survival rate after the hypoxic exposure ($n = 14$–34). *$P < 0.05$ versus WT; †$P < 0.05$ versus eNOS$^{-/-}$. (*B*) Representative tracing of RVSP (scale bar = 0.1 s). (*C*) RVSP after the normoxic and hypoxic exposure. (*D*) Hematoxylin and eosin (H&E) staining of heart cross-sections. Scale bar = 1 mm. (*E*) Weight ratio of the right ventricle to the left ventricle plus the interventricular septum [RV/(LV + S)] after the normoxic and hypoxic exposure. *$P < 0.05$ versus normoxia; †$P < 0.05$ versus WT; ‡$P < 0.05$ versus eNOS$^{-/-}$. (*F*) H&E, elastic van Gieson (EVG), and α-SMA (α-smooth muscle actin) staining of small pulmonary arteries. Scale bars = 50 μm. (*G*) Medial thickness of small pulmonary arteries (50–150 μm in diameter) ($n = 5$–10). *$P < 0.05$ versus normoxia; †$P < 0.05$ versus WT; ‡$P < 0.05$ versus eNOS$^{-/-}$.

[RV/(LV + S)] (an index of RV hypertrophy), or in the medial thickness of the small pulmonary arteries (an index of pulmonary vascular remodeling) in the five genotypes (Figures 3B–3G). As compared with the normoxic exposure, the hypoxic exposure significantly increased the RVSP, RV/(LV + S), and medial thickness in all the five genotypes. Importantly, when the extents of these increases were compared among the five genotypes, the RVSP, RV/(LV + S), and medial thickness were all significantly accelerated in the n/i/eNOSs$^{-/-}$ mice and, to a lesser extent, in the eNOS$^{-/-}$ mice as compared with the WT mice (Figures 3B–3G). Prominent muscularization of the media of the small pulmonary arteries was seen in the n/i/eNOSs$^{-/-}$ mice exposed to hypoxia (Figure 3F).

Because our original clinical question started with the patients with IPF, we also used a bleomycin model that shows pulmonary fibrosis and PH. Bleomycin-induced increases in PH parameters were similarly more enhanced in the n/i/eNOSs$^{-/-}$ than in the WT mice (Figure E6).

An NO Donor and Nitrate Reverse Hypoxia-induced PH in n/i/eNOSs$^{-/-}$ Mice

We studied the effect of NO supplementation with ISDN or sodium nitrate on hypoxia-induced PH in the n/i/eNOSs$^{-/-}$ mice. Simultaneous oral treatment with ISDN or 5 mmol/L sodium nitrate significantly restored low plasma NOx levels and improved reduced survival, weight loss, and increases in the RVSP, RV/(LV + S), and medial thickness in the n/i/eNOSs$^{-/-}$ mice exposed to hypoxia (Figures E4 and E5).

Involvement of BM Cells in the Development of Hypoxia-induced PH in n/i/eNOSs$^{-/-}$ Mice

We next examined whether BM cells were involved in the development of hypoxia-induced PH in the triple n/i/eNOSs$^{-/-}$ mice. After the normoxic exposure, there was a comparable number of circulating BM-derived VSMC progenitor cells between the WT and n/i/eNOSs$^{-/-}$ mice, as assessed by flow cytometric Sca-1^{+}/c-kit^{-}/Lin^{-} cells (29), whereas

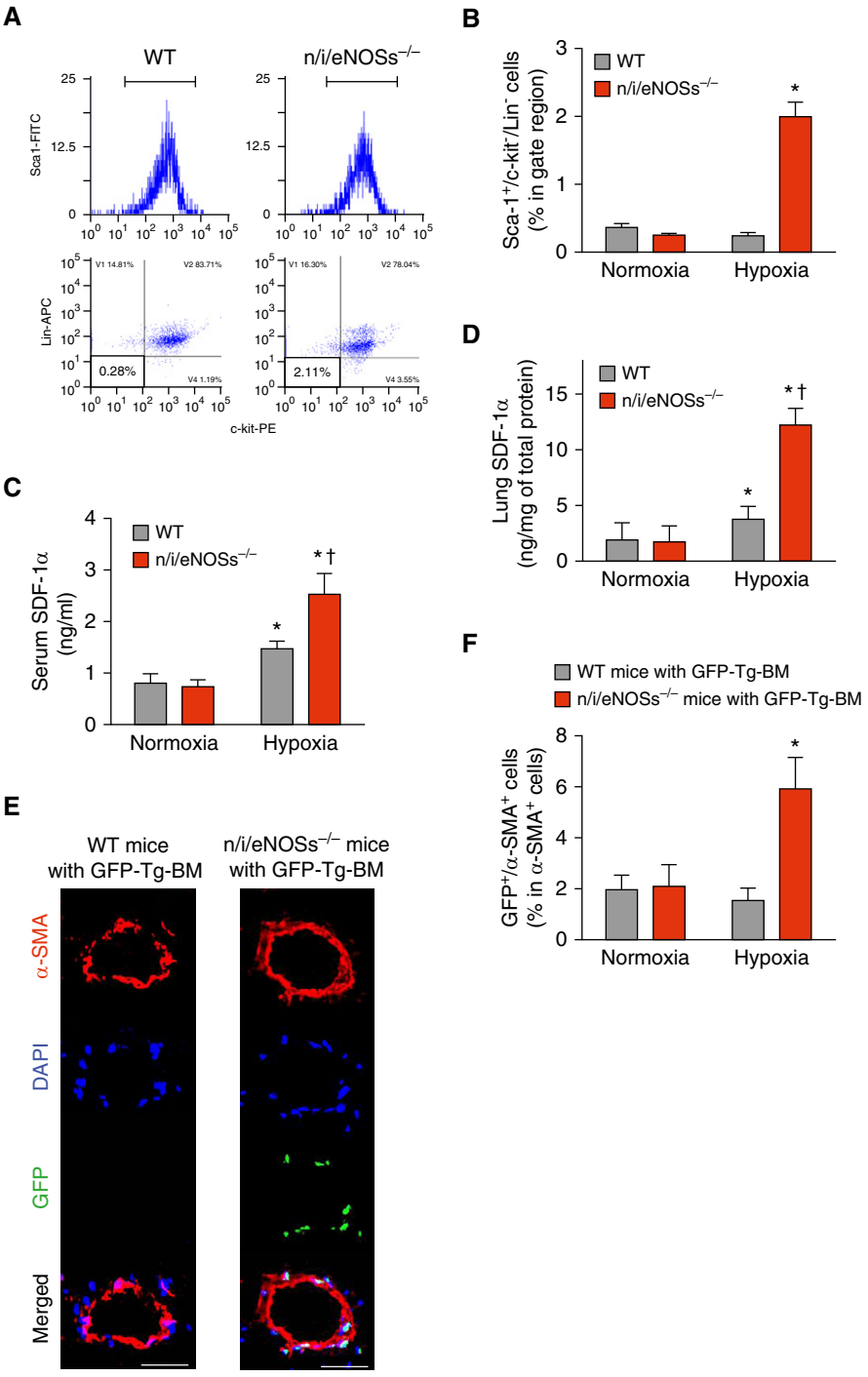

Figure 4. Involvement of bone marrow (BM) cells and SDF-1α (stromal cell–derived factor-1α) in hypoxia-induced pulmonary hypertension in neuronal/inducible/endothelial NOS (nitric oxide synthase)$^{-/-}$ (n/i/eNOSs$^{-/-}$) mice. (*A* and *B*) Circulating Sca-1 (stem cell antigen-1)$^{+}$/c-kit^{-}/Lin (lineage)$^{-}$ cells (interpreted as BM-derived vascular smooth muscle cell progenitor cells) assessed by flow cytometry ($n = 5$). *$P < 0.05$ versus normoxia. (*C* and *D*) Serum and lung SDF-1α levels ($n = 5$). *$P < 0.05$ versus normoxia; †$P < 0.05$ versus wild-type (WT) mice. (*E*) Immunofluorescent staining in the lung of WT and n/i/eNOSs$^{-/-}$ mice transplanted with BM cells isolated from GFP (green fluorescent protein)–transgenic (GFP-Tg) mice after the hypoxic exposure. Apparent GFP-positive green fluorescence and GFP/α-SMA (α-smooth muscle actin)–double-positive white fluorescence were observed ($n = 5$). DAPI shows nuclear staining. Scale bars = 50 μm. (*F*) The number of GFP/α-SMA–double-positive cells (% in total α-SMA–positive cells). *$P < 0.05$ versus normoxia. APC = activated protein C; FITC = fluorescein isothiocyanate; PE = phycoerythrin.

after the hypoxic exposure, there was a significant increase only in the n/i/eNOSs$^{-/-}$ mice, but not in the WT mice (Figures 4A and 4B). After the normoxic exposure, the serum and lung SDF-1α levels were similar between the two genotypes, whereas after the hypoxic exposure, both levels were significantly higher in the n/i/eNOSs$^{-/-}$ than in the WT mice (Figures 4C and 4D).

After the hypoxic exposure, little GFP (green fluorescent protein)-positive green fluorescence was seen in the lungs of WT mice transplanted with GFP-transgenic (GFP-Tg) BM, whereas apparent GFP-positive green fluorescence and GFP/α-SMA–double-positive white fluorescence were detected in the lungs of n/i/eNOSs$^{-/-}$ mice transplanted with GFP-Tg-BM (Figure 4E). Quantitative analysis indicated that, after the hypoxic exposure, there were significantly more lung GFP/α-SMA–double-positive cells in the n/i/eNOSs$^{-/-}$ mice, but not in the WT mice (Figure 4F).

n/i/eNOSs$^{-/-}$-BM Transplantation Exacerbates Hypoxia-induced PH

On the basis of these findings, we explored the role of n/i/eNOSs in BM cells. As compared with WT mice transplanted with WT-BM, in WT mice with n/i/eNOSs$^{-/-}$-BM, the plasma NOx levels were significantly reduced by 51.6%, and the RVSP, RV/(LV + S), and medial thickness were all significantly increased (Figures 5A–5D). Furthermore, as compared with n/i/eNOSs$^{-/-}$ mice transplanted with n/i/eNOSs$^{-/-}$-BM, in n/i/eNOSs$^{-/-}$ mice with WT-BM, the plasma NOx levels were significantly higher by 476.0%, and the RVSP, RV/(LV + S), and medial thickness were all significantly decreased, suggesting the involvement of n/i/eNOSs in BM cells (Figures 5A–5D). As compared with the WT mice transplanted with WT-BM, in the n/i/eNOSs$^{-/-}$ mice with WT-BM, the PH parameters were significantly enhanced, and as compared with n/i/eNOSs$^{-/-}$ mice with n/i/eNOSs$^{-/-}$-BM, in the WT mice with n/i/eNOSs$^{-/-}$-BM, the PH parameters were significantly lessened, suggesting the involvement of n/i/eNOSs in non-BM cells (Figures 5A–5D). n/i/eNOSs in BM and non-BM cells appeared to almost equally contribute to the development of hypoxia-induced PH. eNOS$^{-/-}$-BM or iNOS$^{-/-}$-BM transplantation did not significantly affect hypoxia-induced PH in the WT mice (Figures E7 and E8).

Micro–computed tomography analysis indicated that RV ejection fraction was significantly lower in the WT mice transplanted with n/i/eNOSs$^{-/-}$-BM than in those with WT-BM, without affecting LV ejection fraction or lung volume (Figures 5E and 5F).

Mechanisms for Exacerbation of Hypoxia-induced PH Caused by n/i/eNOSs$^{-/-}$-BM Transplantation

We performed mRNA sequencing and analyzed 13,748 mRNAs enrolled in the reference mouse genome mm10 database. Among them, there was different expression of 2,469 mRNAs between the lungs of WT mice transplanted with WT-BM and those with n/i/eNOSs$^{-/-}$-BM after the hypoxic exposure, with statistically significant differences ($P < 0.05$) and with more than 1.2-fold changes ($n = 4$ each); 1,024 and 1,445 mRNAs were significantly up- and downregulated, respectively, in the lungs of WT mice transplanted with n/i/eNOSs$^{-/-}$-BM compared with those with WT-BM.

Gene ontology analysis indicated that, in the upregulated mRNAs, the significant biological process terms included "immune system process," "immune response," "innate immune response," and "inflammation response," suggesting the involvement of immunity and inflammation (Figure 6A and Table E2). In the downregulated mRNAs, the significant biological process terms included "protein folding," "translation," and "DNA repair," making these results difficult to interpret (Table E3).

Canonical pathway analysis showed that the significant terms included "nuclear factor of activated T cells signaling," "endothelin-1 signaling," "Wnt signaling," "FLT3 signaling," "Notch signaling," "B cell receptor signaling," "T helper cell signaling," and "thrombin signaling" (Figure 6B). These signaling pathways are relevant to "immunity" and "inflammation." Representative genes, including the endothelin-converting enzyme 1 and the endothelin type B receptor, are shown in Figure 6C. Mechanistic network analysis showed that the significant downstream signaling terms included "immune response of cells" and "response of antigen presenting cells," again suggesting the involvement of "immunity" (Figure 6D).

In the lungs of WT mice transplanted with n/i/eNOSs$^{-/-}$-BM compared with those with WT-BM, 69 and 49 mRNAs categorized as "immunity" and "inflammation," respectively, were significantly increased (Figures 7A and 7B). Representative genes, including complement C3, IL6 receptor α, and angiotensin II type 1a receptor, are shown in Figure 7C.

We evaluated the extent of inflammation in the lung by counting the number of Mac-2–positive inflammatory cells. As compared with the WT mice transplanted with WT-BM, the number of Mac-2–positive inflammatory cells (mostly macrophages) in the lung were significantly increased in the WT mice with n/i/eNOSs$^{-/-}$-BM and in the n/i/eNOSs$^{-/-}$ mice with WT-BM to a similar extent, and in the n/i/eNOSs$^{-/-}$ mice with n/i/eNOSs$^{-/-}$-BM to a greater extent after the hypoxic exposure (Figures 7D and 7E).

Discussion

Inverse Association between PASP and NOx Levels in BALF in Humans

We demonstrated in this study that higher PASP was associated with lower BALF NOx levels in patients with IPF (group 3 PH). In agreement with this evidence, it has been reported that PASP is negatively correlated with NOx levels in BALF, airway gases, and plasma of patients with idiopathic PH (group 1 PH) (32, 33). These results indicate that defective NO production is present under PH conditions. On the basis of this clinical outcome, we performed the basic study.

Systemic n/i/eNOSs Deficiency Promotes Development of Hypoxia-induced PH in Mice

As compared with the WT genotype, the extents of hypoxia-induced PH were comparable in the nNOS$^{-/-}$ and iNOS$^{-/-}$ genotypes but worsened modestly in the eNOS$^{-/-}$ genotype and markedly in the n/i/eNOSs$^{-/-}$ genotype, suggesting the protective roles of eNOS and n/i/eNOSs, but not of nNOS or iNOS, in the

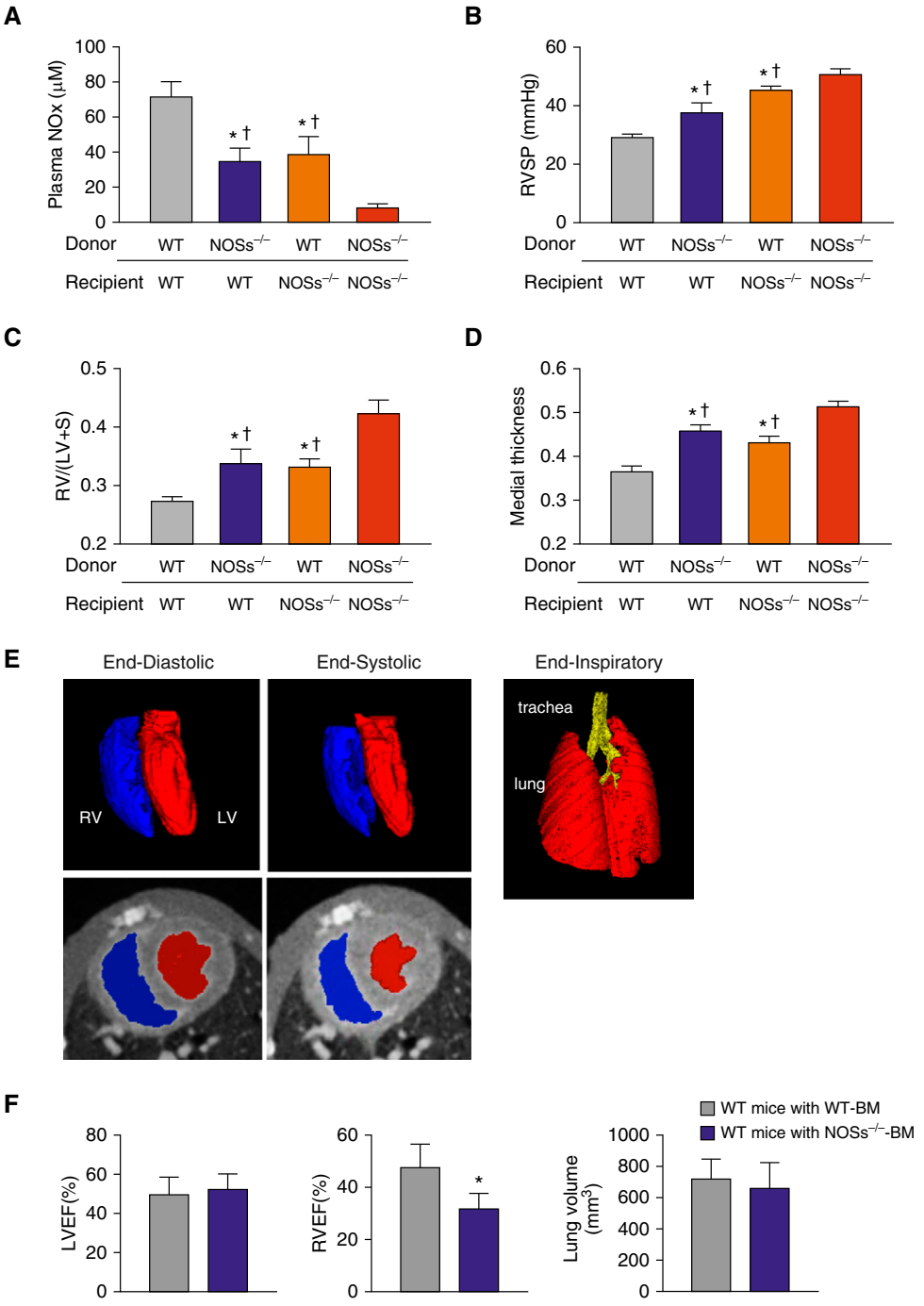

Figure 5. Plasma NOx (nitrite plus nitrate) levels, hypoxia-induced pulmonary hypertension (PH), and cardiac function in wild-type (WT) and neuronal/inducible/endothelial NOS (nitric oxide synthase)$^{-/-}$ (n/i/eNOSs$^{-/-}$) mice transplanted with either WT or n/i/eNOSs$^{-/-}$ bone marrow (BM) cells. (*A–D*) Plasma NOx levels after the hypoxic exposure and hypoxia-induced PH ($n = 5$–6). *$P < 0.05$ versus WT mice transplanted with WT-BM; †$P < 0.05$ versus n/i/eNOSs$^{-/-}$ mice transplanted with n/i/eNOSs$^{-/-}$-BM. (*E*) Micro–computed tomography images. (*F*) Left ventricular ejection fraction (LVEF), right ventricular ejection fraction (RVEF), and lung volume after the hypoxic exposure ($n = 4$). *$P < 0.05$. RV/LV + S = weight ratio of the RV to LV plus the interventricular septum; RVSP = right ventricular systolic pressure.

development of PH. eNOS in non-BM cells, but not in BM cells, seems to substantially contribute to the protective effects of n/i/eNOSs. All these abnormalities in the n/i/eNOSs$^{-/-}$ genotype were inhibited by NO supplementation with ISDN and sodium nitrate, confirming that the observed phenomena were indeed caused by defective NO production. The n/i/eNOSs$^{-/-}$ mice showed the absence of expression of all NOSs in the lung

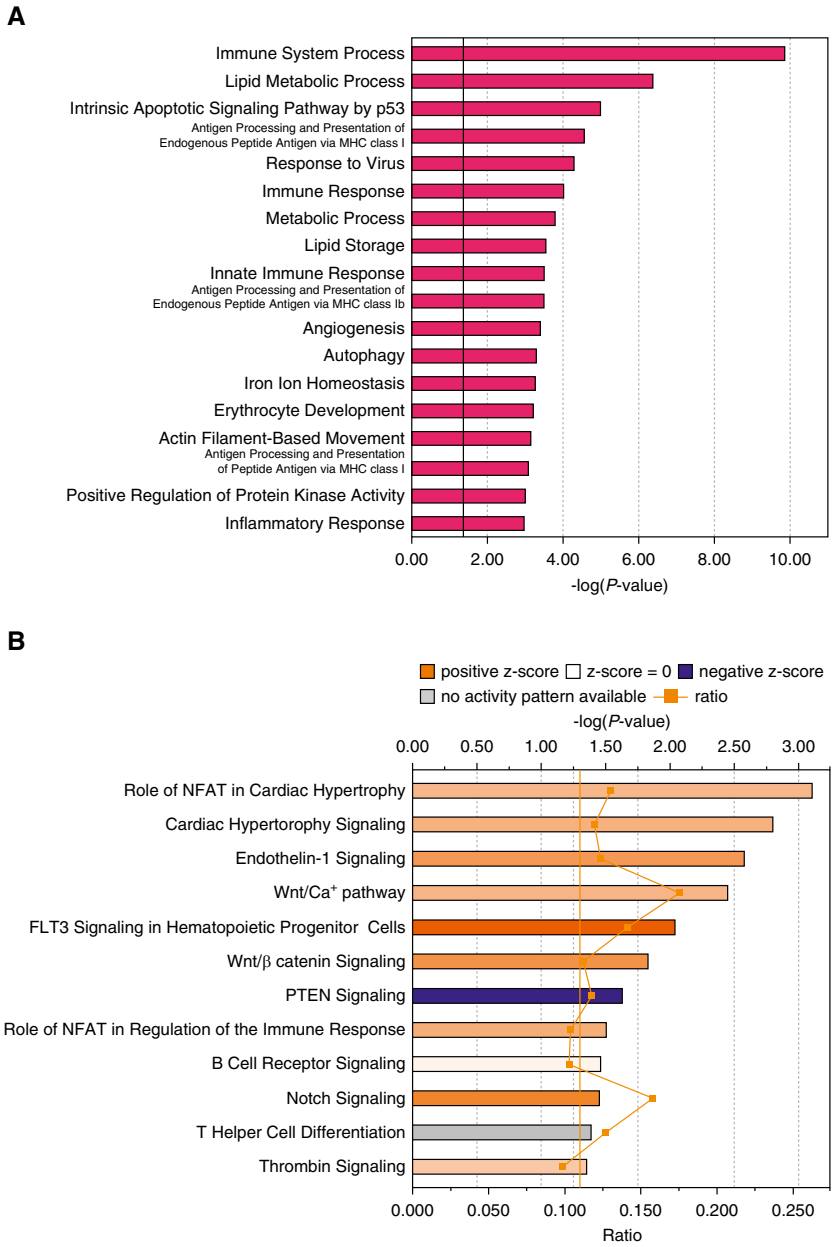

Figure 6. Systemic pathway analysis of differentially expressed genes between the lungs of wild-type (WT) mice transplanted with WT bone marrow (BM) and those with neuronal/inducible/endothelial NOS (nitric oxide synthase)$^{-/-}$ (n/i/eNOSs$^{-/-}$)-BM. mRNA sequencing was performed with a next-generation sequencer, and 2,469 mRNAs were differentially expressed between the lungs of WT mice transplanted with WT-BM and those with n/i/eNOSs$^{-/-}$-BM after the hypoxic exposure with statistically significant differences ($P < 0.05$) and with more than 1.2-fold changes ($n = 4$ each); 1,024 and 1,445 mRNAs were significantly up- and downregulated, respectively, in the lungs of WT mice transplanted with n/i/eNOSs$^{-/-}$-BM. (*A*) Significant biological process terms detected by gene ontology analysis of the software of database for annotation, visualization, and integrated discovery (DAVID). (*B*) Significant terms detected by the software of ingenuity pathways analysis (IPA). Bars indicate −log(*P* value), and orange and blue bars stand for up- and downregulated pathways, respectively. The line represents the ratio of the number of significantly expressed mRNAs to the number of genes listed in each pathway. The DAVID and IPA software concordantly indicated that the n/i/eNOSs$^{-/-}$-BM transplantation increased genes related to "immunity" and "inflammation." (*C*) Representative genes in significant terms detected by the canonical analysis of IPA software. *$P < 0.05$. (*D*) Significant terms detected by downstream effect analysis. ARHGEF = Rho guanine nucleotide exchange factor; BCL = B-cell leukemia/lymphoma; CD28 = CD28 antigen; DOCK8 = dedicator of cytokinesis 8; ECE-1 = endothelin-converting enzyme 1; ET-B = endothelin receptor type B; FGF-R = fibroblast growth factor receptor; FLT3 = FMS-like tyrosine kinase; FLT3L = FLT3 ligand; FPKM = fragments per kilobase of exon per million mapped fragments; HLA = major histocompatibility complex, class I; Ifnar = interferon α and β receptor subunit 1; IRF7 = interferon regulatory factor 7; MAPK = mitogen-activated protein kinase; MAVS = mitochondrial antiviral signaling protein; MEF = myocyte enhancer factor; MERTK = c-mer proto-oncogene tyrosine kinase; MHC = major histocompatibility complex; NFAT = nuclear factor of activated T cells; PARP9 = poly(ADP-ribose) polymerase family member 9; PDPK =

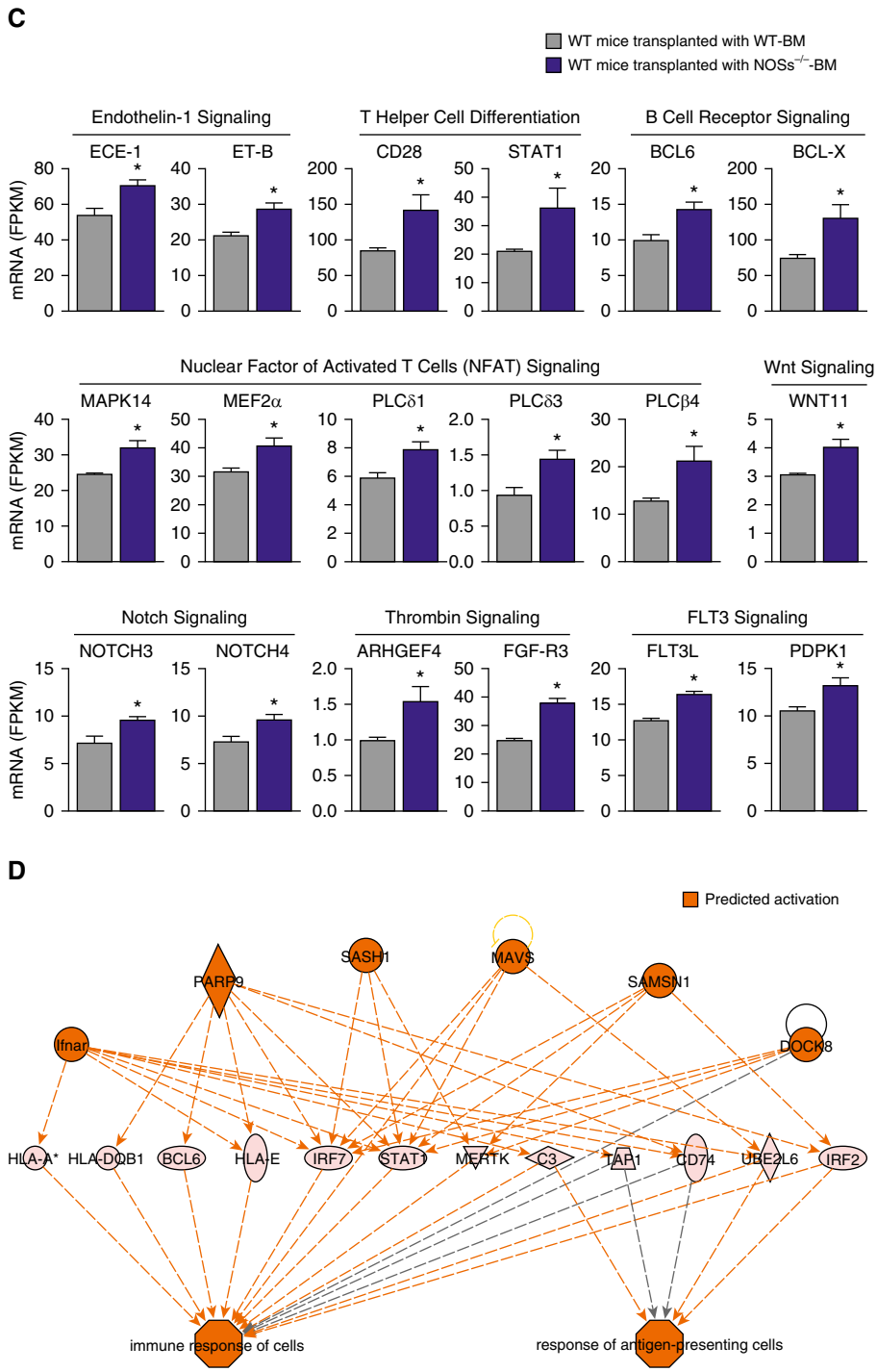

Figure 6. (Continued). 3-phosphoinositide–dependent protein kinase; PLC = phospholipase C; PTEN = phosphatase and tensin homolog; SASH1 = SAM and SH3 domain containing 1; SAMSN1 = SAM domain, SH3 domain and nuclear localization signals 1; STAT = signal transducer and activator of transcription; TAP1 = transporter 1, ATP-binding cassette, sub-family B; UBE2L6 = ubiquitin-conjugating enzyme E2L 6; WNT = wingless-type MMTV integration site family member.

and considerably reduced the BALF NOx levels, whereas the eNOS$^{-/-}$ mice exhibited a significant and insignificant increase in expression levels of iNOS and nNOS, respectively, in the lung after the hypoxic exposure along with fairly well-preserved BALF NOx levels. These results may explain in part why the n/i/eNOSs$^{-/-}$ mice developed more severe hypoxia-induced PH than the eNOS$^{-/-}$ mice. Because we did not use double NOSs$^{-/-}$ mice, the relative contribution of iNOS and nNOS to this mechanism is unknown.

Bleomycin-induced PH was also more aggravated in the n/i/eNOSs$^{-/-}$ than in the WT mice, as was the case for hypoxia-induced PH, suggesting that the n/i/eNOSs play a protective role in a broad range of PH types.

Myelocytic n/i/eNOSs Deficiency Promotes Development of Hypoxia-induced PH in Mice

A large number of circulating BM-derived VSMC progenitor cells and enhanced plasma and lung SDF-1α levels were noted in the n/i/eNOSs$^{-/-}$ genotype exposed to hypoxia. It has been reported that BM-derived mononuclear cells differentiate into VSMC progenitor cells and contribute to vascular lesion formation (34, 35) and that the CXC chemokine SDF-1α is a pivotal chemotactic factor of the BM-derived VSMC progenitor cells (36). It is therefore likely that SDF-1α–induced recruitment of circulating BM-derived VSMC progenitor cells was involved in the pulmonary vascular remodeling observed in the n/i/eNOSs$^{-/-}$ genotype exposed to hypoxia. We obtained further direct evidence of GFP/α-SMA–double-positive white fluorescence in the thickened media of the small pulmonary arteries in the n/i/eNOSs$^{-/-}$ mice transplanted with GFP-Tg-BM after the hypoxic exposure. It is thus evident that BM cells were involved in the development of hypoxia-induced PH in the n/i/eNOSs$^{-/-}$ genotype.

On the basis of these results, we explored the role of n/i/eNOSs in BM cells in the development of PH. There were lower levels of plasma NOx in the WT genotype transplanted with n/i/eNOSs$^{-/-}$-BM than in those with WT-BM. Surprisingly, approximately 50% of systemic NO production appears to be derived from BM cells. n/i/eNOSs$^{-/-}$-BM transplantation deteriorated the hypoxia-induced PH in the WT genotype, and WT-BM transplantation ameliorated the hypoxia-induced PH in the n/i/eNOSs$^{-/-}$ genotype. This is the first experimental demonstration

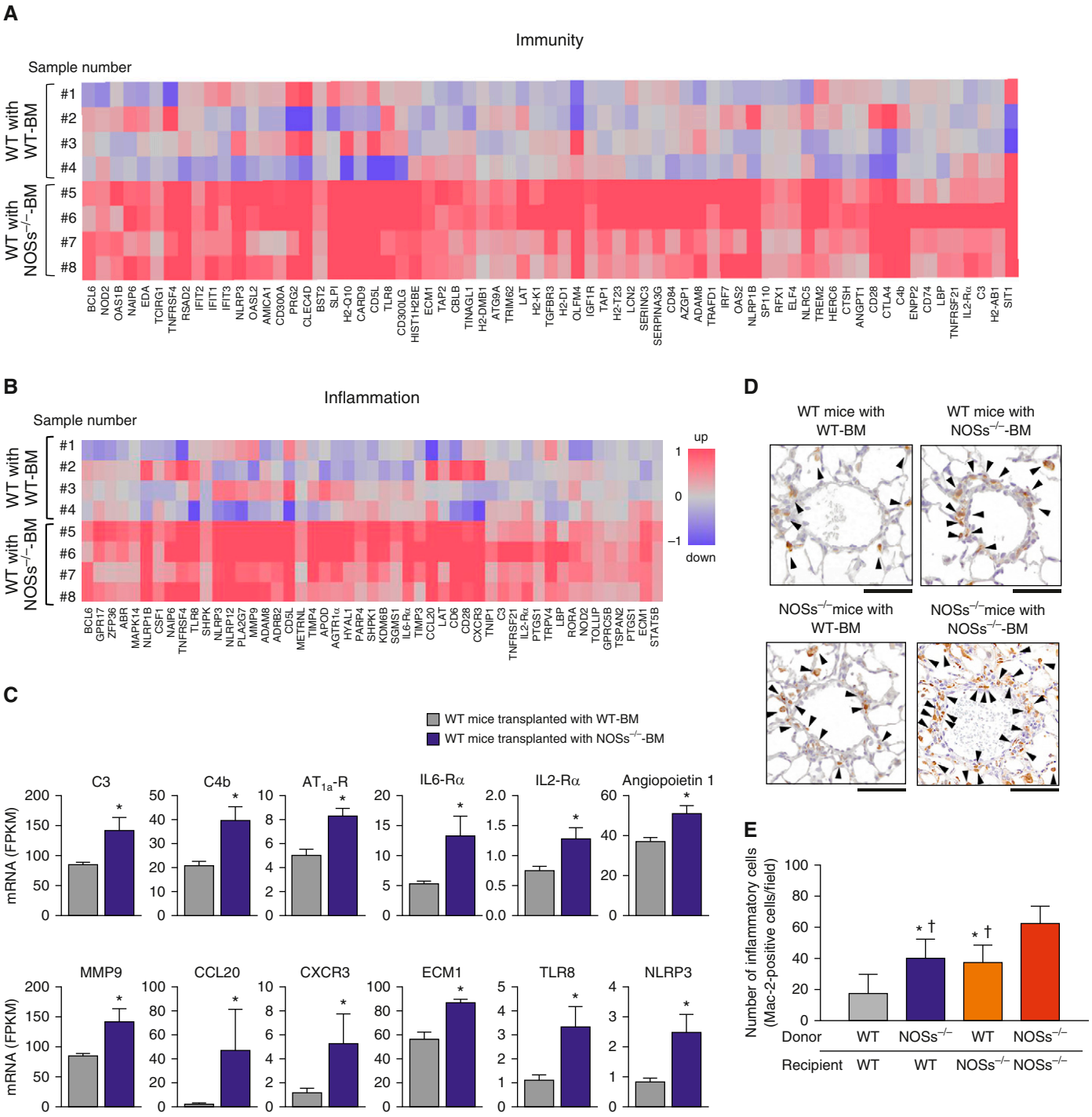

Figure 7. Differentially expressed immunity- and inflammation-related genes and inflammatory cell infiltration between the lungs of wild-type (WT) mice transplanted with WT bone marrow (BM) and those with neuronal/inducible/endothelial NOS (nitric oxide synthase)$^{-/-}$ (n/i/eNOSs$^{-/-}$)-BM. (*A* and *B*) Heat maps of differentially expressed mRNAs categorized as immunity and inflammation in the Subio platform. (*C*) Representative genes in the differentially expressed mRNAs. *$P < 0.05$. (*D*) Representative Mac-2 immunostaining in the lung after the hypoxic exposure. Arrowheads indicate Mac-2–positive cells. Scale bar = 50 μm. (*E*) The number of Mac-2–positive inflammatory cells in the lung after the hypoxic exposure ($n = 4$). *$P < 0.05$ versus WT mice transplanted with WT-BM; †$P < 0.05$ versus n/i/eNOSs$^{-/-}$ mice with n/i/eNOSs$^{-/-}$-BM. AT_{1a}-R = angiotensin II type 1a receptor; C = complement component; CCL = chemokine (C-C motif) ligand; CXCR = chemokine (C-X-C motif) receptor; ECM = extracellular matrix protein; FPKM = fragments per kilobase of exon per million mapped fragments; IL6-Rα = IL6 receptor α; MMP = matrix metalloproteinase; NLRP3 = NLR family, pyrin domain containing 3; TLR = toll-like receptor.

showing that myelocytic n/i/eNOSs exert inhibitory effects against the development of PH. The n/i/eNOSs$^{-/-}$-BM transplantation reduced RV ejection fraction but did not affect LV ejection fraction in the WT genotype. It is thus possible that the observed alterations were not due to left heart failure (group 2 PH).

Immune and Inflammatory Mechanisms Mediate the Promotion of Hypoxia-induced PH Caused by Myelocytic n/i/eNOSs Deficiency

We performed mRNA sequencing using a next-generation sequencer to examine the mechanisms involved in the deterioration of hypoxia-induced PH caused by myelocytic n/i/eNOSs deficiency. Intriguingly, even though the lung tissues were derived from the same WT mice, n/i/eNOSs$^{-/-}$-BM transplantation significantly changed many genes of the 2,469 mRNAs as compared with WT-BM transplantation. These results further support the important role of myelocytic n/i/eNOSs in the development of PH. The software analyses concordantly indicated that the n/i/eNOSs$^{-/-}$-BM transplantation increased the expression of genes related to "immunity" and "inflammation." Consistent with these results, as compared with WT mice transplanted with WT-BM, the number of inflammatory cells was increased in the WT mice with n/i/eNOSs$^{-/-}$-BM and in the n/i/eNOSs$^{-/-}$ mice with WT-BM to a similar extent, and in the n/i/eNOSs$^{-/-}$ mice with n/i/eNOSs$^{-/-}$-BM to a greater extent, suggesting the involvement of inflammation in the exacerbation of hypoxia-induced PH caused by n/i/eNOSs deficiency in BM and non-BM cells.

Conclusions

We were able to demonstrate that higher PASP was associated with lower BALF NOx levels in patients with IPF, and that the presence or absence of n/i/eNOSs in BM cells modulated the severity of hypoxia-induced PH in mice via the immune and inflammatory pathways, indicating a novel protective role of n/i/eNOSs, specifically in BM cells, in the pathogenesis of PH. Our findings should contribute to a better understanding of the cellular and molecular basis of this fatal pulmonary-vascular disorder. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

References

1. Strange G, Playford D, Stewart S, Deague JA, Nelson H, Kent A, *et al*. Pulmonary hypertension: prevalence and mortality in the Armadale echocardiography cohort. *Heart* 2012;98:1805–1811.
2. Hurdman J, Condliffe R, Elliot CA, Davies C, Hill C, Wild JM, *et al*. ASPIRE registry: assessing the Spectrum of Pulmonary hypertension Identified at a REferral centre. *Eur Respir J* 2012;39: 945–955.
3. National Cancer Institute. Surveillance, epidemiology, and end results (SEER) program website, cancer statistics review [accessed 2016 Sept 12]. Available from: http://seer.cancer.gov.
4. Chin K, Channick RN. Pulmonary hypertension. In: Broaddus VC, Mason RJ, Ernst JD, King JTE, Lazarus SC, Murray JF, *et al*., editors. Murray & Nadel's textbook of respiratory medicine, 6th ed. Philadelphia: Elsevier; 2015. pp. 1031–1049.
5. Brindicci C, Kharitonov SA, Ito M, Elliott MW, Hogg JC, Barnes PJ, *et al*. Nitric oxide synthase isoenzyme expression and activity in peripheral lung tissue of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2010;181:21–30.
6. Tsutsui M, Shimokawa H, Otsuji Y, Yanagihara N. Pathophysiological relevance of NO signaling in the cardiovascular system: novel insight from mice lacking all NO synthases. *Pharmacol Ther* 2010;128: 499–508.
7. Tsutsui M, Tanimoto A, Tamura M, Mukae H, Yanagihara N, Shimokawa H, *et al*. Significance of nitric oxide synthases: lessons from triple nitric oxide synthases null mice. *J Pharmacol Sci* 2015;127:42–52.
8. Buchwalow IB, Podzuweit T, Bocker W, Samoilova VE, Thomas S, Wellner M, *et al*. Vascular smooth muscle and nitric oxide synthase. *FASEB J* 2002;16:500–508.
9. Kobzik L, Bredt DS, Lowenstein CJ, Drazen J, Gaston B, Sugarbaker D, *et al*. Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localization. *Am J Respir Cell Mol Biol* 1993;9: 371–377.
10. Seimetz M, Parajuli N, Pichl A, Veit F, Kwapiszewska G, Weisel FC, *et al*. Inducible NOS inhibition reverses tobacco-smoke-induced emphysema and pulmonary hypertension in mice. *Cell* 2011;147:293–305.
11. Hampl V, Bíbová J, Banasová A, Uhlík J, Miková D, Hnilicková O, *et al*. Pulmonary vascular iNOS induction participates in the onset of chronic hypoxic pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L11–L20.
12. Fagan KA, Tyler RC, Sato K, Fouty BW, Morris KG Jr, Huang PL, *et al*. Relative contributions of endothelial, inducible, and neuronal NOS to tone in the murine pulmonary circulation. *Am J Physiol* 1999;277: L472–L478.
13. Ozaki M, Kawashima S, Yamashita T, Ohashi Y, Rikitake Y, Inoue N, *et al*. Reduced hypoxic pulmonary vascular remodeling by nitric oxide from the endothelium. *Hypertension* 2001;37:322–327.
14. Steudel W, Scherrer-Crosbie M, Bloch KD, Weimann J, Huang PL, Jones RC, *et al*. Sustained pulmonary hypertension and right ventricular hypertrophy after chronic hypoxia in mice with congenital deficiency of nitric oxide synthase 3. *J Clin Invest* 1998;101: 2468–2477.
15. Quinlan TR, Li D, Laubach VE, Shesely EG, Zhou N, Johns RA. eNOS-deficient mice show reduced pulmonary vascular proliferation and remodeling to chronic hypoxia. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L641–L650.
16. Hampl V, Herget J. Role of nitric oxide in the pathogenesis of chronic pulmonary hypertension. *Physiol Rev* 2000;80:1337–1372.
17. Morishita T, Tsutsui M, Shimokawa H, Sabanai K, Tasaki H, Suda O, *et al*. Nephrogenic diabetes insipidus in mice lacking all nitric oxide synthase isoforms. *Proc Natl Acad Sci USA* 2005;102: 10616–10621.
18. Farha S, Asosingh K, Xu W, Sharp J, George D, Comhair S, *et al*. Hypoxia-inducible factors in human pulmonary arterial hypertension: a link to the intrinsic myeloid abnormalities. *Blood* 2011;117: 3485–3493.
19. Adir Y, Elia D, Harari S. Pulmonary hypertension in patients with chronic myeloproliferative disorders. *Eur Respir Rev* 2015;24:400–410.
20. Yan L, Chen X, Talati M, Nunley BW, Gladson S, Blackwell T, *et al*. Bone marrow-derived cells contribute to the pathogenesis of pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2016; 193:898–909.
21. Tsutsui M, Ogoshi T, Kido T, Yamada S, Wang K, Toyohira Y, *et al*. Protective role of myelocytic nitric oxide synthases in hypoxic pulmonary hypertension in mice [abstract]. *Circulation* 2017;136: A17700.
22. Brittain EL, Duncan MS, Chang J, Patterson OV, DuVall SL, Brandt CA, *et al*. Increased echocardiographic pulmonary pressure in HIV-infected and uninfected individuals in the Veterans Aging Cohort Study. *Am J Respir Crit Care Med* 2018;197:923–932.
23. Kircher BJ, Himelman RB, Schiller NB. Noninvasive estimation of right atrial pressure from the inspiratory collapse of the inferior vena cava. *Am J Cardiol* 1990;66:493–496.

24. Sakamoto N, Mukae H, Fujii T, Kakugawa T, Kaida H, Kadota J, *et al*. Soluble form of Fas and Fas ligand in serum and bronchoalveolar lavage fluid of individuals infected with human T-lymphotropic virus type 1. *Respir Med* 2004;98:213–219.
25. Kina-Tanada M, Sakanashi M, Tanimoto A, Kaname T, Matsuzaki T, Noguchi K, *et al*. Long-term dietary nitrite and nitrate deficiency causes the metabolic syndrome, endothelial dysfunction and cardiovascular death in mice. *Diabetologia* 2017;60:1138–1151.
26. Morisada N, Nomura M, Nishii H, Furuno Y, Sakanashi M, Sabanai K, *et al*. Complete disruption of all nitric oxide synthase genes causes markedly accelerated renal lesion formation following unilateral ureteral obstruction in mice in vivo. *J Pharmacol Sci* 2010;114:379–389.
27. Furuno Y, Morishita T, Toyohira Y, Yamada S, Ueno S, Morisada N, *et al*. Crucial vasculoprotective role of the whole nitric oxide synthase system in vascular lesion formation in mice: involvement of bone marrow-derived cells. *Nitric Oxide* 2011;25:350–359.
28. Noguchi S, Yatera K, Wang KY, Oda K, Akata K, Yamasaki K, *et al*. Nitric oxide exerts protective effects against bleomycin-induced pulmonary fibrosis in mice. *Respir Res* 2014;15:92.
29. Uchida T, Furuno Y, Tanimoto A, Toyohira Y, Arakaki K, Kina-Tanada M, *et al*. Development of an experimentally useful model of acute myocardial infarction: 2/3 nephrectomized triple nitric oxide synthases-deficient mouse. *J Mol Cell Cardiol* 2014;77:29–41.
30. Sasaguri Y, Wang KY, Tanimoto A, Tsutsui M, Ueno H, Murata Y, *et al*. Role of histamine produced by bone marrow-derived vascular cells in pathogenesis of atherosclerosis. *Circ Res* 2005;96:974–981.
31. Xu W, Kaneko FT, Zheng S, Comhair SA, Janocha AJ, Goggans T, *et al*. Increased arginase II and decreased NO synthesis in endothelial cells of patients with pulmonary arterial hypertension. *FASEB J* 2004; 18:1746–1748.
32. Kaneko FT, Arroliga AC, Dweik RA, Comhair SA, Laskowski D, Oppedisano R, *et al*. Biochemical reaction products of nitric oxide as quantitative markers of primary pulmonary hypertension. *Am J Respir Crit Care Med* 1998;158:917–923.
33. Zhang R, Wang XJ, Zhang HD, Sun XQ, Zhao QH, Wang L, *et al*. Profiling nitric oxide metabolites in patients with idiopathic pulmonary arterial hypertension. *Eur Respir J* 2016;48:1386–1395.
34. Sata M, Saiura A, Kunisato A, Tojo A, Okada S, Tokuhisa T, *et al*. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. *Nat Med* 2002;8:403–409.
35. Shimizu K, Sugiyama S, Aikawa M, Fukumoto Y, Rabkin E, Libby P, *et al*. Host bone-marrow cells are a source of donor intimal smooth-muscle-like cells in murine aortic transplant arteriopathy. *Nat Med* 2001;7:738–741.
36. Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, *et al*. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nat Med* 2004;10:858–864.

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Angiotensin II promotes pulmonary metastasis of melanoma through the activation of adhesion molecules in vascular endothelial cells.

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Author information

Abstract

Hypertension is considered as one of the cancer progressive factors, and often found comorbidity in cancer patients. Renin-angiotensin system (RAS) plays an important role in the regulation of blood pressure, and angiotensin II (Ang II) is well known pressor peptide associated with RAS. Ang II has been reported to accelerate progression and metastasis of cancer cells. However, its precise mechanisms have not been fully understood. In this study, we sought to elucidate the mechanisms by which Ang II exacerbates hematogenous metastasis in mouse melanoma cells, focusing the adhesion pathway in vascular endothelial cells. For this purpose, B16/F10 mouse melanoma cells, which do not express the Ang II type 1 receptor (AT1R), were intravenously injected into C57BL/6 mice. Two weeks after cell injection, the number of lung metastatic colonies was significantly higher in the Ang II-treated group (1 μg/kg/min) than in the vehicle-treated group. The AT1R blocker valsartan (40 mg/kg/day), but not the calcium channel blocker amlodipine (5 or 10 mg/kg/day), significantly suppressed the effect of Ang II. In endothelium-specific Agtr1a knockout mice, Ang II-mediated acceleration of lung metastases of melanoma cells was significantly diminished. Ang II treatment significantly increased E-selectin mRNA expression in vascular endothelial cells collected from lung tissues, and thus promoted adherence of melanoma cells to the vascular endothelium. Ang II-accelerated lung metastases of melanoma cells were also suppressed by treatment with anti-E-selectin antibody (20 mg/kg). Taken together, Ang II-treatment exacerbates hematogenous cancer metastasis by promoting E-selectin-mediated adhesion of cancer cells to vascular endothelial cells.

KEYWORDS: Adhesion molecules; Angiotensin II; E-selectin; Lung metastasis; Vascular endothelial cells

RHEUMATOLOGY

Letter to the Editor (Case report)

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Autoantibodies to the survival of motor neuron complex in a patient with necrotizing autoimmune myopathy

Rheumatology key message

- Autoantibodies to the survival motor neuron complex may be a biomarker of necrotizing autoimmune myopathy.

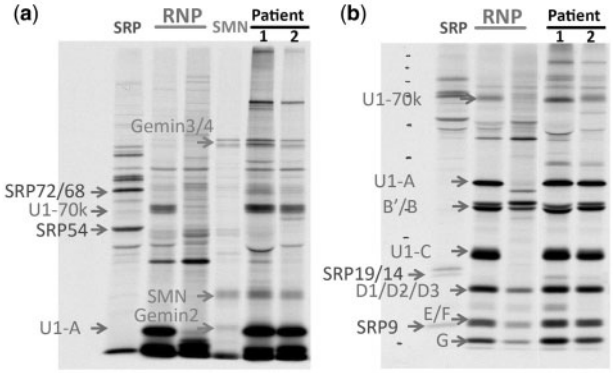
SIR, necrotizing autoimmune myopathies (NAMs) are a subset of autoimmune inflammatory myopathies characterized by proximal muscle weakness and distinctive pathology that is usually associated with autoantibodies to signal recognition particles (SRPs) and HMG-CoA reductase (HMGCR). [1–4]. We report the case of a previously healthy 27-year-old female with a 5 month history of arthritis and RP diagnosed by her general practitioner in an outpatient setting. At that time, her workup consisted of an RF that was slightly elevated at 22 kU/l (normal <20 kU/l), but anti-CCP and CRP were within normal limits. No therapeutic intervention was prescribed at that time and no further workup was completed until she presented to hospital 5 months later following gastroenteritis of undetermined aetiology accompanied by mild weakness. At that time her ANA was highly positive on HEp-2 substrate (titre 1:5120) with coarse nuclear speckled and nuclear dots staining patterns. Further testing revealed a high anti-U1-RNP titre and elevated creatine kinase (1729 U/l). A electromyogram in three separate muscle groups showed normal recruitment, no denervation and normal motor units with no myopathic units noted. One area of a single positive sharp wave was noted in the iliopsoas muscle. At presentation, her medications included birth control pills and occasional naproxen for joint pain. She had no known exposures to statins. Her only relevant family history included maternal Graves' disease. She was treated with prednisone 15 mg orally twice daily and HCQ and completely recovered in 4 days. Pulmonary function testing during this admission showed a reduction in her diffusing capacity of carbon monoxide at 75% of the predicted value. She was admitted to hospital 30 days later with progressive proximal muscle weakness and respiratory distress. A CT scan of her chest was reported as unremarkable, with no evidence of pulmonary embolism or interstitial lung disease. A muscle biopsy showed diffusely scattered necrotizing and regenerating myofibres, histiocytic but minimal lymphocytic inflammation, upregulation of major histocompatibility complex class 1 and sarcolemmal deposition of complement (C5b-9), compelling evidence for the diagnosis of NAM. She received high-dose pulse corticosteroids and IVIG, but decompensated on her sixth day of admission and required intubation for respiratory failure. Her creatine kinase rose to >23 000 U/l. Plasmapheresis and CYC were initiated, but despite maximal intervention, she was diagnosed with disseminated intravascular coagulation, developed pulseless electrical activity cardiac arrest and died on day 14 after her admission. At autopsy, findings were again consistent with NAM and also showed myocardial necrosis without a particular vascular territory as well as a transmural acute ulcer in the terminal ileum consistent with ischaemia.

Additional serology was negative for SRP, HMGCR, PM/Scl-100/PM/Scl-75, SSA/Ro60, Ro52/TRIM21, dsDNA, Jo-1 (histidyl tRNA synthetase) and other myositis-related autoantibodies (PL-7, PL-12, Mi2, Mi2−α, Mi2-β, MDA5, NXP2, TIF1-γ, Ku, EJ, OJ). However, high-titre autoantibodies to the survival of motor neuron (anti-SMN) complex proteins and U1-RNP were identified by immunoprecipitation (IP) of metabolically labelled cell lysates (Fig. 1).

This case was unique with respect to both the anti-SMN autoantibodies found as well as the severity of the myopathy and rapid time course of decompensation. To our knowledge, there have been no publications of autoantibodies directed towards the SMN complex in NAM. A single previous publication reported autoantibodies to the SMN complex in patients with PM and PM/SSc overlap [5], but features of NAM were not reported. Defects in the SMN gene are associated with a spinal muscular atrophy genetic disorder [6]. Our patient also had antibodies to U1-RNP components and clinical features of MCTD (RP, arthritis, myositis and high-titre U1-RNP). Previous studies have indicated that 20% of patients with anti-U1-RNP as detected by RNA IP techniques had histological evidence of NAM [7], but because RNA IP was used to detect autoantibodies, anti-SMN would not have been detected in that study. It remains unclear as to why autoantibodies to specific intracellular antigens SRP, HMGCR and tRNA synthetase are so closely linked to predictable clinical features and what role (if any) these autoantibodies play in the development of these features and in the pathogenesis of the associated disease processes [8]. The questions of whether these autoantibodies are produced following initial tissue damage or cell lysis and/or antedate the clinical presentation of NAM requires further study.

Given the uniqueness of the clinical presentation of this patient and the IP results, we believe that, along with antibodies to SRP and HMGCR, autoantibodies to the SMN complex may serve as an additional biomarker for NAM. Since anti-SMNs are the subject of a single publication to date, where they were found in only ~5% of the PM sera studied [6], further concerted research of a large multicentre cohort is needed to investigate a link between these autoantibodies and NAM and other diagnostic and prognostic features.

Fig. 1 Immunoprecipitation of patient sera revealed anti-SMN reactivity



Radiolabeled human K652 cell lysates were prepared and then immunoprecipitated (IP) as previously described [5] with control human sera bearing antibodies to signal recognition particle (SRP), U1 and U2 RNP and SMN (SMN, gemin-2, -3 and -4). IP reactivity resolved on (**a**) 8% and (**b**) 12.5% gels. The 12.5% gels are used to provide better resolution of U1-RNP proteins. Sera collected from the patient before (lane 1) and after (lane 2) plasmapheresis demonstrated strong reactivity with U1-A, U1-70k, U1-C, D1/D2/D3, B/B′ and E, F and G proteins (U1RNP components) and remarkably high reactivity with SMN, gemin-2, -3 and -4, but not SRP72/69 or SRP54.

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References

1 Hengstman GJ, ter Laak HJ, Vree Egberts WT *et al*. Anti-signal recognition particle autoantibodies: marker of a necrotising myopathy. Ann Rheum Dis 2006;65:1635–8.

2 Basharat P, Christopher-Stine L. Immune-mediated necrotizing myopathy: update on diagnosis and management. Curr Rheumatol Rep 2015;17:72.

3 Musset L, Allenbach Y, Benveniste O *et al*. Anti-HMGCR antibodies as a biomarker for immune-mediated necrotizing myopathies: A history of statins and experience from a large international multi-center study. Autoimmun Rev 2016;15:983–93.

4 Pinal-Fernandez I, Mammen AL. Spectrum of immune-mediated necrotizing myopathies and their treatments. Curr Opin Rheumatol 2016;28:619–24.

5 Satoh M, Chan JY, Ross SJ *et al*. Autoantibodies to survival of motor neuron (SMN) complex in patients with polymyositis: immunoprecipitation of D, E, F, G proteins without other components of small nuclear ribonucleoproteins. Arthritis Rheum 2011;63:1972–8.

6 Hosseinibarkooie S, Schneider S, Wirth B. Advances in understanding the role of disease-associated proteins in spinal muscular atrophy. Expert Rev Proteomics 2017;14:581–92.

7 Suzuki S, Yonekawa T, Kuwana M *et al*. Clinical and histological findings associated with autoantibodies detected by RNA immunoprecipitation in inflammatory myopathies. J Neuroimmunol 2014;274:202–8.

8 Arouche-Delaperche L, Allenbach Y, Amelin D *et al*. Pathogenic role of anti-signal recognition protein and anti-3-hydroxy-3-methylglutaryl-CoA reductase antibodies in necrotizing myopathies: myofiber atrophy and impairment of muscle regeneration in necrotizing autoimmune myopathies. Ann Neurol 2017;81:538–48.

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Research paper

Detection of anti-mitochondrial antibodies by immunoprecipitation in patients with systemic sclerosis

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ABSTRACT

Objective: To describe a new immunoprecipitation pattern identified in Italian patients affected by systemic sclerosis (SSc), corresponding to the pyruvate dehydrogenase antigen complex recognized by anti-mitochondrial antibodies (AMA) in primary biliary cholangitis (PBC).

Methods: Autoantibodies in sera from 85 patients with SSc were tested by protein- and RNA-immunoprecipitation. Immunoprecipitation-Western blot was used to determine the identified proteins, and medical records re-evaluated for liver function tests and PBC.

Results: In 13/85 (15%) SSc sera, a unique set of 75-50-40-34 kD proteins that had not been previously reported, was noted. The four proteins were identified as the proteins X/E3BP, E1α, E1β, and E2/E3 of the pyruvate dehydrogenase antigen complex by immunoprecipitation-Western blot. From clinical record evaluation, 9/13 (69%) SSc patients with this new pattern were positive for AMA by routine indirect immunofluorescence, and 7/13 (54%) had a diagnosis of PBC, while 4/13 (31%) manifested no biochemical signs of cholestasis. Twelve of 13 patients with SSc and AMA by immunoprecipitation have a limited cutaneous form of SSc and anti-centromere antibodies.

Conclusions: We describe a pattern of 4 proteins in 15% of SSc patients, identified for the first time by protein-immunoprecipitation. This pattern corresponds to serum AMA against the pyruvate dehydrogenase antigen complex and it must be considered in the interpretation of protein-immunoprecipitation results.

1. Introduction

A large proportion of patients with systemic sclerosis (SSc) manifest a coexisting autoimmune condition, including primary biliary cholangitis (PBC) (Assassi et al., 2009; Rigamonti et al., 2006). As immunoprecipitation (IP) is currently one of the most sensitive techniques to detect new and known rare autoantibodies, a clear understanding of common patterns is necessary for proper interpretation of the results. Anti-mitochondrial autoantibodies (AMA) are the hallmark of PBC (Gershwin et al., 2005; Selmi et al., 2014; Selmi et al., 2004) and in clinical practice they may predate the clinical onset of disease (Gershwin et al., 2000; Kaplan & Gershwin, 2005) when tested with routine indirect immunofluorescence performed on tissue slides, while the use of alternative techniques such as ELISA for mitochondrial antigens still is not routinely used and it has unclear clinical significance (Cavazzana et al., 2011). PBC is associated with other autoimmune diseases in about 30% of patients, with SSc found in 7–12% of cases (Assassi et al., 2009; Rigamonti et al., 2006), even though in clinical practice a higher percentage of SSc patients may have biochemical liver abnormalities without clinical significance (Norman et al., 2009).

We herein used IP to test new and uncommon serum autoantibodies in SSc and in this screening analysis we observed that 15% of SSc sera manifest a novel IP pattern, comprising a set of 4 proteins corresponding to the E1α, E1β, protein X/E3BP, and E2/E3 subunits of the pyruvate dehydrogenase complex (PDC) recognized by AMA (McHugh et al., 1990; Miyachi et al., 1980; Fregeau et al., 1990). The prevalence of AMA by IP outnumbers what is observed in routine tests in our cohort of SSc patients and this new IP pattern should be known when interpreting IP data in SSc sera.

2. Patients and methods

2.1. Subjects

Eighty-five consecutive patients with SSc attending the outpatient Rheumatology clinic at Humanitas Research Hospital (Rozzano, Milan,

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Italy) between 2012 and 2016, were enrolled to the study. Controls included sera from 74 healthy subjects, 49 patients with polymyositis/dermatomyositis (PM/DM), 32 patients with undifferentiated connective-tissue disease (UCTD), and 2 patients with established PBC previously AMA-positive and negative for serum antinuclear antibody (ANA) without signs of rheumatic diseases. Internationally accepted criteria were used for the diagnosis of SSc (van den Hoogen, 2013), PM/DM (Targoff et al., 1997), UCTD (Mosca et al., 2014), and PBC (Bowlus & Gershwin, 2014) and we collected clinical and laboratory data at enrollment. SSc patients who did not fulfill the ACR/EULAR criteria were defined as sine SSc (Poormoghim et al., 2000) and very early diagnosis of SSc (VEDOSS) (Minier et al., 2014). Liver laboratory tests included in our study are aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (gammaGT), alkaline phosphatase (ALP), bilirubin (total, direct) and, when available, liver histology was also evaluated. The study was approved by the Institutional Review Board of the Humanitas Research Hospital and a signed informed consent was obtained from all subjects in accordance with the Declaration of Helsinki and its subsequent modifications.

3. Methods

3.1. Protein- and RNA-immunoprecipitation (IP)

Sera were obtained from whole blood through centrifugation at 2000*g* for 15 min, and then stored in − 20 °C freezer until use. Serum autoantibodies were screened by protein-IP using ^{35}S-methionine-labeled K562 cell extract followed by SDS-PAGE and autoradiography, and by RNA-IP using unlabeled K562 cell extract followed by urea-PAGE and silver staining (Ceribelli et al., 2012; Ceribelli et al., 2010). Autoantibodies were analyzed using reference sera obtained from the Autoantibody Standardization Committee (www.autoab.org) and from internal controls, and they were used for the correct interpretation of protein-IP bands for known ANA specificities. These reference sera help in determining protein-IP specificities for ANA in connective tissue diseases and are used also for SSc patients as in our cohort.

3.2. IP-Western Blot (IP-WB)

Sera with a novel IP pattern of a set of 4 proteins were tested by IP-WB. In detail, 50 μl of candidate sera were cross-linked with protein-A Sepharose beads and then immunoprecipitated with cell extract from 5×10^6 K562 cells/sample. Proteins were then fractionated by 8% SDS-PAGE and transferred to a nitrocellulose filter, probed with 1:500 of mouse polyclonal anti-human PDH E1α antibody (Novus Biologicals, Littleton, CO, USA) for a 41 kD protein identification, followed by horseradish peroxidase (HRP) goat anti-mouse IgG (1: 10,000 dilution; ThermoFisher, Waltham, MA, USA). The same procedure was used to identify the other bands of the complex: mouse anti-human PDH E1β (1: 500 dilution; Novus Biological, Littleton, CO, USA) for the protein of 34 kD; mouse anti-human PDH protein X/E3BP (1: 1000 dilution; Novus Biological, Littleton, CO, USA) for the 54 kD; mouse anti-human PDH E2/E3 proteins of 58 kD and 74 kD (1: 10,000 dilution; Abcam, Cambridge, UK) followed by goat anti-mouse IgG (ThermoFisher, Waltham, MA, USA). Development was performed by Immobilon Western Chemiluminescent HRP substrate (Millipore, Darmstadt, Germany) and acquired using ChemiDoc (Bio-Rad, California, USA).

3.3. Indirect immunofluorescence (IIF)

Antinuclear and cytoplasmic antibodies were tested by IIF on HEp-2 ANA slides (INOVA Diagnostics, San Diego, CA, USA) using a 1:80 dilution of human sera of patients and controls, followed by AlexaFluor488 AffiniPure F(ab′)2 fragment goat anti-human IgG, Fcγ fragment specific (Jackson Immunoresearch Europe Ltd., Suffolk, UK) as previously described (Ceribelli et al., 2012). Images were acquired on Olympus BX53 Upright fluorescence microscope. No immunofluorescence analysis on tissue slides was performed for AMA identification.

3.4. ELISA

Positive serum AMA identified by protein-IP (n = 13) were tested for confirmation by ELISA using the QUANTA Lite M2 EP (MIT3) IgG ELISA (INOVA Diagnostics, Inc., San Diego, USA) with the patented pMIT3 antigen, which consists of a recombinant protein containing the immunodominant epitopes of the 3 major AMA targets (Moteki et al., 1996), currently limited to research settings. ELISA was performed according to the manufacturer instructions, and results were shown as Units.

3.5. Statistical analysis

All comparisons were performed by Fisher's exact test using Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). All analyses were two-tailed and p values < 0.05 were considered as statistically significant.

4. Results

4.1. Demographic and laboratory data of the SSc cohort

We included 85 patients (81 women, mean age ± standard deviation 66 ± 15 years) with SSc and their sera were studied by IP. The age at SSc onset was 53 ± 15 years, and the mean follow-up was 106 months (range 4 to 408). From clinical record retrospective evaluation, nine patients with limited cutaneous SSc had a previous diagnosis of PBC with available liver biopsy, and in one patient an overlap with ANA-positive autoimmune hepatitis was found. In 5/9 (56%) PBC cases, the onset of chronic cholestasis preceded the diagnosis of SSc (range 1–24 years), while in 4/9 (44%) the diagnosis of PBC followed SSc by 3–24 years. Additional features of our SSc cohort are described in Table 1. Fourteen cases defined as sine SSc (Poormoghim et al., 2000) (n = 4) and very early diagnosis of SSc (VEDOSS, (Minier et al., 2014), n = 10) are not included in the statistical evaluation shown in Table 1, but two AMA-positive IP cases were identified in sine SSc patients defined by ACA-positivity, Raynaud's phenomenon and capillaroscopy alterations but no signs of skin fibrosis.

Table 1
Main features of the limited and diffuse cutaneous SSc cohort analyzed for AMA identification by protein-IP. Only p values < 0.05 are reported. Fourteen cases defined as sine SSc (n = 4) and VEDOSS (n = 10) are not included in the present table.

	Limited cutaneous SSc (n = 59)	Diffuse cutaneous SSc (n = 12)	p values
PBC cases, % (n.cases)	13 (8)	8 (1)	ns
Interstitial lung disease, % (n.cases)	25 (15)	67 (8)	0.014
Pulmonary hypertension, % (n.cases)	24 (14)	42 (5)	ns
AMA by IP, % (n.cases)	17 (10)	8 (1)	ns
ACA, % (n.cases)	59 (35)	33 (4)	ns
ANA nucleolar, % (n.cases)	10 (6)	0	ns
ANA homogeneous, % (n.cases)	8 (5)	0	ns
Anti-topo1/Scl-70, % (n.cases)	5 (3)	50 (6)	< 0.001
Anti-RNAPIII, % (n.cases)	0	8 (1)	ns

Abbreviations: ACA = anti-centromere antibodies; AMA = anti-mitochondrial antibodies; ANA = anti-nuclear antibodies; IP = immunoprecipitation; PBC = primary biliary cholangitis; RNAPIII = RNA polymerase III.

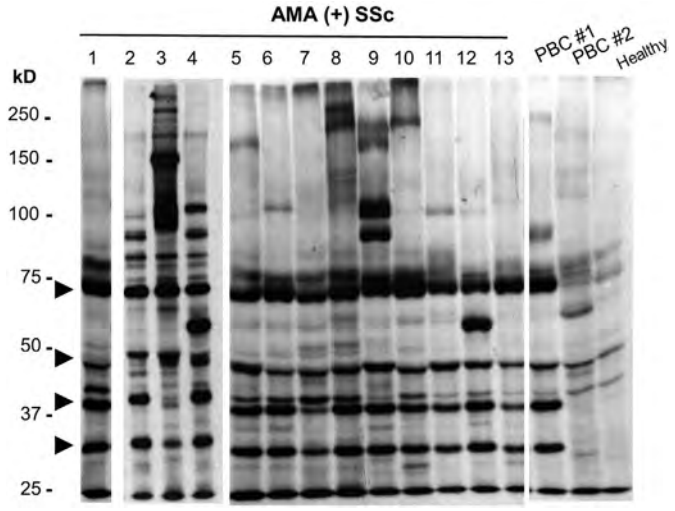

Fig. 1. IP pattern of AMA-positive patients. The protein-IP pattern of the 75-50-40-34 kD proteins (*black arrows*) of the mitochondrial complex antigen is shown in 13 SSc patients. IP results in 8% SDS-PAGE gel are shown. PBC #1 and PBC #2 represent two cases with isolated diagnosis of PBC and PBC #2 was known to have weak positivity for AMA by routine IIF on tissue slides and ELISA. Normal human serum is included as negative control.

4.2. Autoantibody analysis

By protein-IP we observed a new pattern with a set of 4 proteins of 75-50-40-34 kD molecular weight (Fig. 1) in 13/85 (15%) SSc sera, while no RNA band was detected by RNA-IP in these patients. The protein-IP pattern was observed also in the two AMA-positive PBC cases, and in one patient with UCTD without sign of liver disease suggestive for PBC, while no PM/DM manifested such pattern. We could identity each protein immunoprecipitated by these sera using IP-WB (Fig. 2) as the E1α, E1β, E2/E3 and protein X/E3BP subunits of the mitochondrial pyruvate dehydrogenase complex.

The main features of SSc cases with the new protein-IP profile are summarized in Table 2, and a comparison between AMA-positive and AMA-negative limited cutaneous SSc patients is described in Table 3. From a clinical viewpoint, 8/13 (61.5%) SSc patients with the AMA protein-IP profile we describe for the first time were positive for serum AMA by routine IIF on tissue slides, while 5/13 (38.5%) SSc patients tested negative and had no cytoplasmic staining was reported by routine IIF on Hep-2 slides; among these, 6/13 (46%) had a pre-existing diagnosis of PBC. In our cohort AMA-positive SSc patients have a limited cutaneous form and they are significantly associated with ACA positivity (Table 3), and only one AMA-positive case has diffuse cutaneous SSc.

The pMIT3 ELISA results were in agreement with the new protein-IP

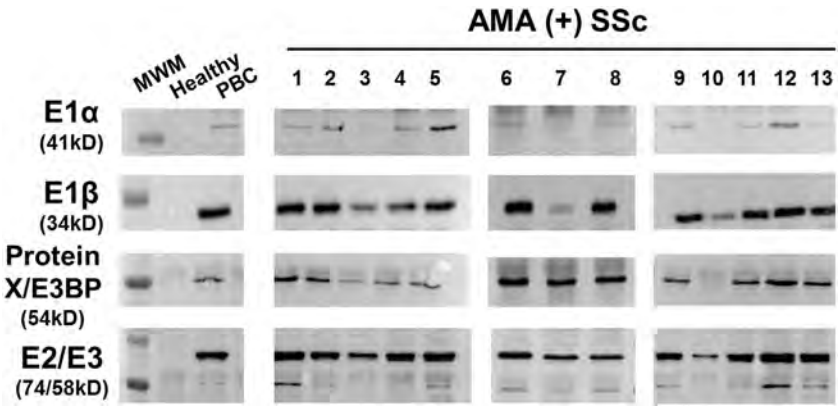

Fig. 2. IP-WB confirming the identity of the IP bands corresponding to the mitochondrial antigen complex components. The samples tested by IP-WB are the same shown in Fig. 1 for protein-IP, with positivity for the IP-AMA pattern as described, and their main features are described in Table 2. The molecular weight marker (MWM) is shown for reference, and IP-WB for each component confirms the identity of protein X/E3BP, E1α, E1β and E2/E3 corresponding to the 54 kD, 41 kD, 34 kD, 74/58 kD of the proteins shown in protein-IP, respectively.

Table 2
Demographic, clinical and laboratory features of SSc patients with detectable AMA by protein-IP.

Patient #	1	2	3	4	5	6	7	8	9	10	11	12	13
Demography													
Sex	F	F	F	F	F	F	F	F	F	F	F	F	M
Age, years	72	76	70	56	40	63	54	81	49	58	77	59	87
SSc	sine	L	L	L	L	L	L	sine	L	L	L	L	D
SSc year of onset	2005	2010	2009	–	2006	2007	2010	–	2006	2003	1981	2007	2001
PBC diagnosis, year	2004	–	2003	–	–	2006	–	–	2013 (+ AIH)	2006	2005	2011	1999
Liver cirrhosis	–	–	+	–	–	–	–	–	–	+	–	–	–
Autoantibodies													
ACA (+)	+	+	+	+	+	+	+	+	+	+	+	+	+
Other autoAbs	–	–	–	–	ANCA aPLs	–	–	–	–	Ro/SSA	–	–	Scl70
Laboratory													
High bilirubin	–	n/a	+	n/a	–	–	+	–	–	–	n/a	–	–
High gammaGT	–	n/a	+	+	–	+	–	–	+	+	+	+	–
High ALP	–	n/a	+	n/a	–	+	–	–	+	+	+	–	–
High AST/ALT	–	–	+	–	–	–	–	–	+	–	+	–	–
Therapy													
UDA therapy	+	–	+	–	–	+	–	–	+	+	+	+	+
Immunosuppressive therapy for PBC	–	–	–	–	–	–	–	–	PDN, AZA	PDN, AZA	–	–	– (PDN, AZA,CTX for SSc)

Abbreviations: ACPA = anti cyclic-citrullinated peptide antibodies; ALP = alkaline phosphatase; ALT = alanine aminotransferase; aPLs = anti-phospholipid antibodies; AIH = autoimmune hepatitis; AMA = anti-mitochondrial antibodies; ANCA = anti neutrophil cytoplasmic antibodies; AST = aspartate aminotransferase; AZA = azathioprine; CTX = cyclophosphamide; D = diffuse SSc; gammaGT = gamma glutamyltransferase; IIF = indirect immunofluorescence; L = limited SSc; MTX = methotrexate; n/a = not available; PDN = prednisone; SSc = systemic sclerosis; UDA = ursodeoxycholic acid.

Table 3
Comparison of main clinical and laboratory features in patients with limited cutaneous SSc based on serum AMA IP positivity. Two AMA-positive cases sine SSc and one AMA-positive diffuse SSc patient are not included in the present table. Only p values < 0.05 are reported.

	AMA positive $n = 10$	AMA negative $n = 49$	p value
Interstitial lung disease, %	8	28	ns
Pulmonary hypertension, %	0	26	ns
ACA, %	100	51	0.004
ANA nucleolar, %	0	12	ns
Anti-topo1/Scl-70, %	0	6	ns
Anti-RNAPIII, %	0	0	–

profile in 13/13 (100%) sera but we should note that this serology method is currently used only for research purposes. ACA-positive IIF pattern with cytoplasmic staining at different titer is present in 12/13 (92.3%) AMA-positive SSc cases, and in one case we had anti-cytoplasmic, nuclear speckled + nucleolar pattern by IIF (Fig. 3).

5. Discussion

Serum autoantibodies remain the most commonly used and helpful biomarker in the diagnosis and treatment of autoimmune diseases and may precede the clinical manifestations by several years, as reported in systemic lupus erythematosus (Arbuckle et al., 2003) and rheumatoid arthritis (Nielen et al., 2004). In some cases, however, serum autoantibodies such as ANA are detected in non-rheumatic conditions or may be falsely positive due to technical issues, particularly at low titer (Selmi et al., 2016), while other autoantibodies have strong clinical associations such as anti-topo I/Scl70 being frequently associated with diffuse cutaneous SSc (Pope & Johnson, 2015). Indeed, PBC and SSc represent unique conditions because they often coexist despite being considered rare (Assassi et al., 2009) and they manifest serum autoantibodies with high specificity, represented by AMA and ACA, respectively.

The diagnosis of PBC in patients with rheumatic diseases may be challenging until the clinical and laboratory expression of the disease, although it has been suggested that AMA-positive asymptomatic subjects will eventually develop PBC and that an early use of ursodeoxycholic acid may improve prognosis (Kikuchi et al., 2009). The serological scenario of SSc is further complicated by the recent description of several rare autoantibodies associated with SSc (Kayser & Fritzler, 2015). These new autoantibodies are identified first by IP in research laboratories and then validated by other techniques to be ultimately used in clinical practice, as in the case of recently identified autoantibodies in PM/DM (Ceribelli et al., 2017). Based on this rationale, identifying and sharing common patterns by IP is necessary to allow the testing of new and rare autoantibodies, and to improve the interpretation of IP results.

We herein report for the first time the IP pattern of serum AMA in SSc and the prevalence of PBC-associated autoantibodies even in the absence of biochemical sign of cholestasis, through the identification of a set of 4 proteins that correspond to the subunits of the pyruvate dehydrogenase complex. The conventional pattern of AMA detected in sera from PBC was first described by techniques such as immunoblot (Frazer et al., 1985; Alderuccio et al., 1986) and showed the reactivity of PBC sera with components of the PDC complex, namely E1α/β, E2, E3 and the protein X later identified as E3BP (Fussey et al., 1989). The novelty of our finding is based on the description for the first time of a protein-IP pattern that corresponds to the antigenic subunits recognized by AMA, which has never been described before. This identity of the

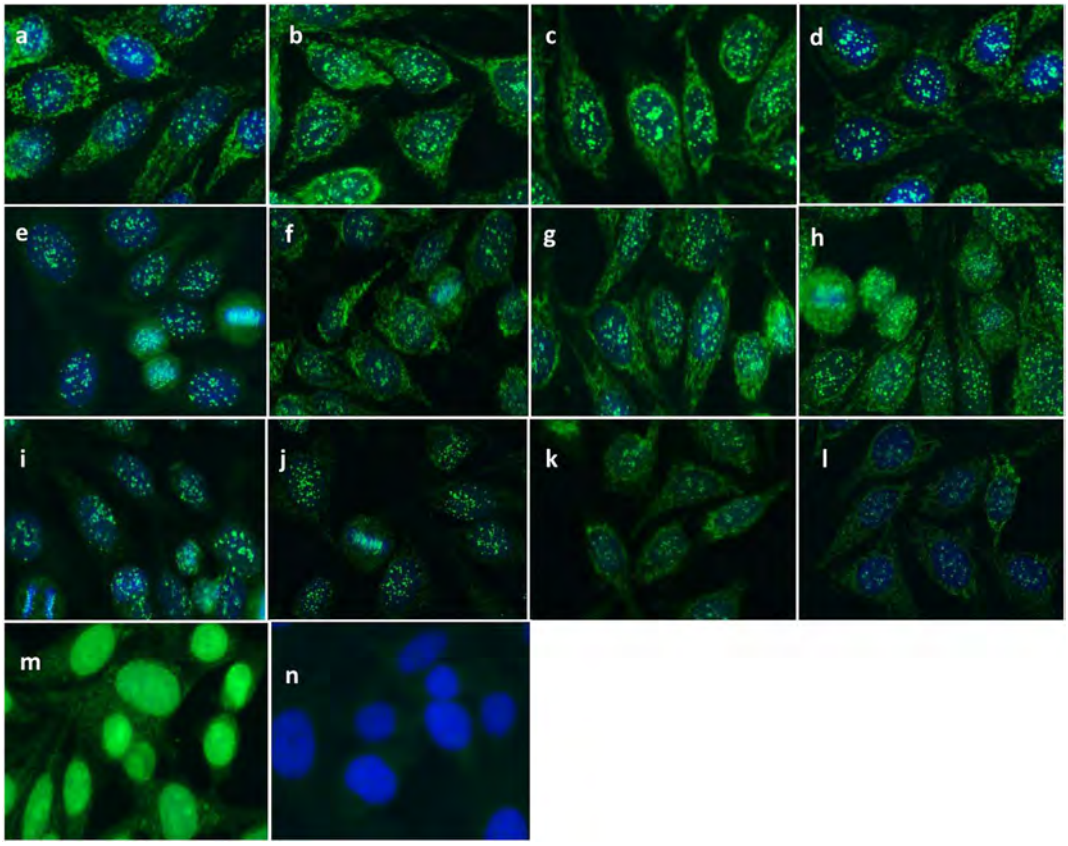

Fig. 3. Indirect immunofluorescence of AMA-positive SSc cases detected by protein-IP. HEp-2 ANA slides were used to analyze the immunofluorescence pattern of AMA-positive SSc cases (a to m), or normal human serum (n). Serum dilution, 1:80. ACA-positive with cytoplasmic staining at different titer is present in 12/13 AMA-positive SSc cases (a to l), and in one case we had a cytoplasmic staining associated with positive anti-nuclear and nucleolar immunofluorescence staining (m).

proteins identified in this new protein-IP pattern was confirmed using the currently available ELISA, yet not routinely used, i.e. the recombinant pMIT3 antigen (Moteki et al., 1996), and the IP-WB technique. It is possible that AMA positivity we observed by protein-IP may also include the reactivity only of some of the presented bands but this was not observed in our series. Further, we report that 15% of SSc sera are positive for AMA, a significant prevalence as demonstrated also by previous reports with up to 25% AMA positivity in SSc patients (Cavazzana et al., 2011), and ACA are reported in up to 30% of patients with PBC (Rigamonti et al., 2011).

Our data demonstrate that one laboratory technique, protein-IP, could be sufficient to achieve two goals: to screen for autoantibodies in rheumatic patients such as SSc and to identify coexisting autoantibodies that discriminate comorbidities. Moreover, we are currently living in an era characterized by the use of automatic screening method for ANA identification that often do not include the cytoplasmic pattern in AMA-positive patients, thus we could overcome this problem by using IP alone. Nonetheless, we are aware that protein-IP has a limited feasibility in the routine laboratory due to the labor and time-consuming protocols and the significant costs.

Four patients with SSc and the new protein-IP pattern had no sign of PBC nor tested AMA positive by IIF and this is particularly intriguing as the use of pMIT3-based ELISA has been shown to reduce the proportion of AMA-negative PBC cases and was in 100% agreement with protein-IP, confirming the identity of the protein pattern we observed (Bizzaro et al., 2012). Our results have three possible implications. First, we may speculate that AMA detected by protein-IP are more sensitive and possibly appear earlier than IIF-AMA on tissue slides, and they could be recognized in an early phase of autoantibodies screening. Second, previous studies on the coexistence of PBC and SSc may have underestimated the prevalence of the association, as AMA are usually detected when a clinical or laboratory suspect of PBC is present (Cavazzana et al., 2011). We are well aware that AMA positivity in the absence of biochemical cholestasis should be further investigated by liver histology to confirm the presence of PBC but this is currently not advisable for ethical reasons nor a diagnosis at asymptomatic stages would change the natural history of PBC despite the use of ursodeoxycholic acid (Ali et al., 2014). Third and last, we confirm that AMA and PBC are associated with the limited cutaneous form of SSc and positivity of ACA, and liver disease does not seem to worsen the prognosis of our AMA-positive patients with SSc (Rigamonti et al., 2006).

In conclusion, we describe for the first time the protein-IP pattern of AMA and we demonstrate for the first time that protein-IP can be used for AMA detection in our SSc cohort of patients. In 15% of our SSc cases, in fact, a new AMA IP pattern was detected and characterized as a set of 4 proteins corresponding to the subunits of the pyruvate dehydrogenase antigen complex, as described decades ago only by immunoblot (Frazer et al., 1985; Alderuccio et al., 1986; Fussey et al., 1989). The definition of this protein-IP pattern is mandatory in the interpretation of protein-IP, increasingly used for rare autoantibody detection in clinical practice. Ultimately, we could also speculate that IP-AMA are earlier predictors of PBC compared to routine AMA on tissue slides, similar to the predictive role of AMA in patients without cholestasis, but this needs to be verified in a larger prospective cohort.

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References

Alderuccio, F., et al., 1986. Identification and characterization of mitochondria autoantigens in progressive systemic sclerosis: identity with the 72,000 dalton autoantigen in primary biliary cirrhosis. J. Immunol. 137 (6), 1855–1859.
Ali, A.H., Carey, E.J., Lindor, K.D., 2014. Diagnosis and management of primary biliary cirrhosis. Expert. Rev. Clin. Immunol. 10 (12), 1667–1678.
Arbuckle, M.R., et al., 2003. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. N. Engl. J. Med. 349 (16), 1526–1533.
Assassi, S., et al., 2009. Primary biliary cirrhosis (PBC), PBC autoantibodies, and hepatic parameter abnormalities in a large population of systemic sclerosis patients. J. Rheumatol. 36 (10), 2250–2256.
Bizzaro, N., et al., 2012. Overcoming a “probable” diagnosis in antimitochondrial antibody negative primary biliary cirrhosis: study of 100 sera and review of the literature. Clin Rev Allergy Immunol 42 (3), 288–297.
Bowlus, C.L., Gershwin, M.E., 2014. The diagnosis of primary biliary cirrhosis. Autoimmun. Rev. 13 (4–5), 441–444.
Cavazzana, I., et al., 2011. Primary biliary cirrhosis-related autoantibodies in a large cohort of Italian patients with systemic sclerosis. J. Rheumatol. 38 (10), 2180–2185.
Ceribelli, A., et al., 2010. Anti-Th/To are common antinucleolar autoantibodies in Italian patients with scleroderma. J. Rheumatol. 37 (10), 2071–2075.
Ceribelli, A., et al., 2012. Anti-MJ/NXP-2 autoantibody specificity in a cohort of adult Italian patients with polymyositis/dermatomyositis. Arthritis Res. Ther. 14 (2), R97.
Ceribelli, A., et al., 2017. Myositis-specific autoantibodies and their association with malignancy in Italian patients with polymyositis and dermatomyositis. Clin. Rheumatol. 36 (2), 469–475.
Frazer, I.H., et al., 1985. Reactivity of anti-mitochondrial autoantibodies in primary biliary cirrhosis: definition of two novel mitochondrial polypeptide autoantigens. J. Immunol. 135 (3), 1739–1745.
Fregeau, D.R., et al., 1990. Primary biliary cirrhosis. Inhibition of pyruvate dehydrogenase complex activity by autoantibodies specific for E1 alpha, a non-lipoic acid containing mitochondrial enzyme. J. Immunol. 144 (5), 1671–1676.
Fussey, S.P., et al., 1989. The E1 alpha and beta subunits of the pyruvate dehydrogenase complex are M2'd' and M2'e' autoantigens in primary biliary cirrhosis. Clin. Sci. (Lond) 77 (4), 365–368.
Gershwin, M.E., et al., 2000. Primary biliary cirrhosis: an orchestrated immune response against epithelial cells. Immunol. Rev. 174, 210–225.
Gershwin, M.E., et al., 2005. Risk factors and comorbidities in primary biliary cirrhosis: a controlled interview-based study of 1032 patients. Hepatology 42 (5), 1194–1202.
van den Hoogen, F., et al., 2013. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League against Rheumatism collaborative initiative. Arthritis Rheum. 65 (11), 2737–2747.
Kaplan, M.M., Gershwin, M.E., 2005. Primary biliary cirrhosis. N. Engl. J. Med. 353 (12), 1261–1273.
Kayser, C., Fritzler, M.J., 2015. Autoantibodies in systemic sclerosis: unanswered questions. Front. Immunol. 6, 167.
Kikuchi, K., et al., 2009. Ursodeoxycholic acid reduces CpG-induced IgM production in patients with primary biliary cirrhosis. Hepatol. Res. 39 (5), 448–454.
McHugh, N.J., et al., 1990. Autoantibodies to mitochondrial and centromere antigens in primary biliary cirrhosis and systemic sclerosis. Clin. Exp. Immunol. 81 (2), 244–249.
Minier, T., et al., 2014. Preliminary analysis of the very early diagnosis of systemic sclerosis (VEDOSS) EUSTAR multicentre study: evidence for puffy fingers as a pivotal sign for suspicion of systemic sclerosis. Ann. Rheum. Dis. 73 (12), 2087–2093.
Miyachi, K., et al., 1980. Precipitating antibodies to mitochondrial antigens in patients with primary biliary cirrhosis. Clin. Exp. Immunol. 39 (3), 599–606.
Mosca, M., et al., 2014. The diagnosis and classification of undifferentiated connective tissue diseases. J. Autoimmun. 48-49, 50–52.
Moteki, S., et al., 1996. Use of a designer triple expression hybrid clone for three different lipoyl domain for the detection of antimitochondrial autoantibodies. Hepatology 24 (1), 97–103.
Nielen, M.M., et al., 2004. Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. Arthritis Rheum. 50 (2), 380–386.
Norman, G.L., et al., 2009. Is prevalence of PBC underestimated in patients with systemic sclerosis? Dig. Liver Dis. 41 (10), 762–764.
Poormoghim, H., et al., 2000. Systemic sclerosis sine scleroderma: demographic, clinical, and serologic features and survival in forty-eight patients. Arthritis Rheum. 43 (2), 444–451.
Pope, J.E., Johnson, S.R., 2015. New classification criteria for systemic sclerosis (scleroderma). Rheum. Dis. Clin. N. Am. 41 (3), 383–398.
Rigamonti, C., et al., 2006. Clinical features and prognosis of primary biliary cirrhosis associated with systemic sclerosis. Gut 55 (3), 388–394.
Rigamonti, C., et al., 2011. Primary biliary cirrhosis associated with systemic sclerosis: diagnostic and clinical challenges. Int. J. Rheum. Dis. 2011, 976427.
Selmi, C., et al., 2004. Primary biliary cirrhosis in monozygotic and dizygotic twins: genetics, epigenetics, and environment. Gastroenterology 127 (2), 485–492.
Selmi, C., et al., 2014. Genome-wide analysis of DNA methylation, copy number variation, and gene expression in monozygotic twins discordant for primary biliary cirrhosis. Front. Immunol. 5, 128.
Selmi, C., et al., 2016. Serum antinuclear and extractable nuclear antigen antibody prevalence and associated morbidity and mortality in the general population over 15 years. Autoimmun. Rev. 15 (2), 162–166.
Targoff, I.N., et al., 1997. Classification criteria for the idiopathic inflammatory myopathies. Curr. Opin. Rheumatol. 9 (6), 527–535.

Case Report

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Immunotherapy

Immune-mediated thrombocytopenia and hypothyroidism in a lung cancer patient treated with nivolumab

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Patients treated with immune checkpoint inhibitors can develop various immunological complications; however, few cases of immune thrombocytopenia occurring in association with the administration of these agents have so far been reported. We herein report the case of a 62-year-old Japanese man with non-small-cell lung cancer who developed immune thrombocytopenia and hypothyroidism during nivolumab therapy. After the second administration of the drug, his peripheral blood platelet count rapidly decreased to 1.6 × 10^4/μl with a petechial rash and symptoms associated with a low thyroid function. Nivolumab-induced immune thrombocytopenia and hypothyroidism were suspected based on the presence of platelet-associated IgG, an increased level of autoantibodies to thyroglobulin and thyroid peroxidase and an enlarged thyroid gland. The patient eventually made a full recovery after treatment with oral prednisolone and levothyroxine. Further investigations and the accumulation of data are necessary to elucidate the precise mechanisms underlying the autoimmune responses that occur in patients treated with immune checkpoint inhibitors.

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Keywords: hypothyroidism • immune thrombocytopenia • lung cancer • nivolumab

Certain cancer cells express the programmed death ligand (PD-L1/L2), which interacts with programmed death-1 (PD-1) on activated T cells, leading to the suppression of tumor immunity against cancer cells. Thus, blocking this inhibitory pathway to enhance the immune reaction to cancer cells has been considered to be a promising new strategy in cancer immunotherapy. New biologics that target PD-1 in order to enhance tumor immunity, nivolumab and pembrolizumab, have recently become available for the treatment of non-small-cell lung cancer (NSCLC). Since these new drugs exhibit their effects by blocking the inhibitory pathway in tumor immunity, induction of autoimmunity is a potential concern. Various adverse effects, in particular immune-related ones, have been reported in clinical trials of immune checkpoint inhibitors as unique events, which are not usually seen with conventional anticancer therapeutic agents [1]. We herein present a rare case of a NSCLC patient who developed immune thrombocytopenia and hypothyroidism as possible immunological side effects of nivolumab treatment. In the present study, we also review the literature to highlight the association between immune checkpoint inhibitors and immune thrombocytopenia and hypothyroidism.

Case report

The patient was a 62-year-old Japanese man who was diagnosed to have stage IV lung adenocarcinoma with bilateral malignant pleural effusion 2 years prior to this presentation. Immune checkpoint inhibitor treatment with nivolumab (3 mg/kg) every 2 weeks was initiated as the third-line treatment regimen, although he was suspected to have asymptomatic Hashimoto's thyroiditis, based on positive anti-TPO (thyroid peroxidase) (510 U/ml; normal range: <16 U/ml) and anti-TG (thyroglobulin) antibodies (572 U/ml; normal range: <28 U/ml) but normal levels

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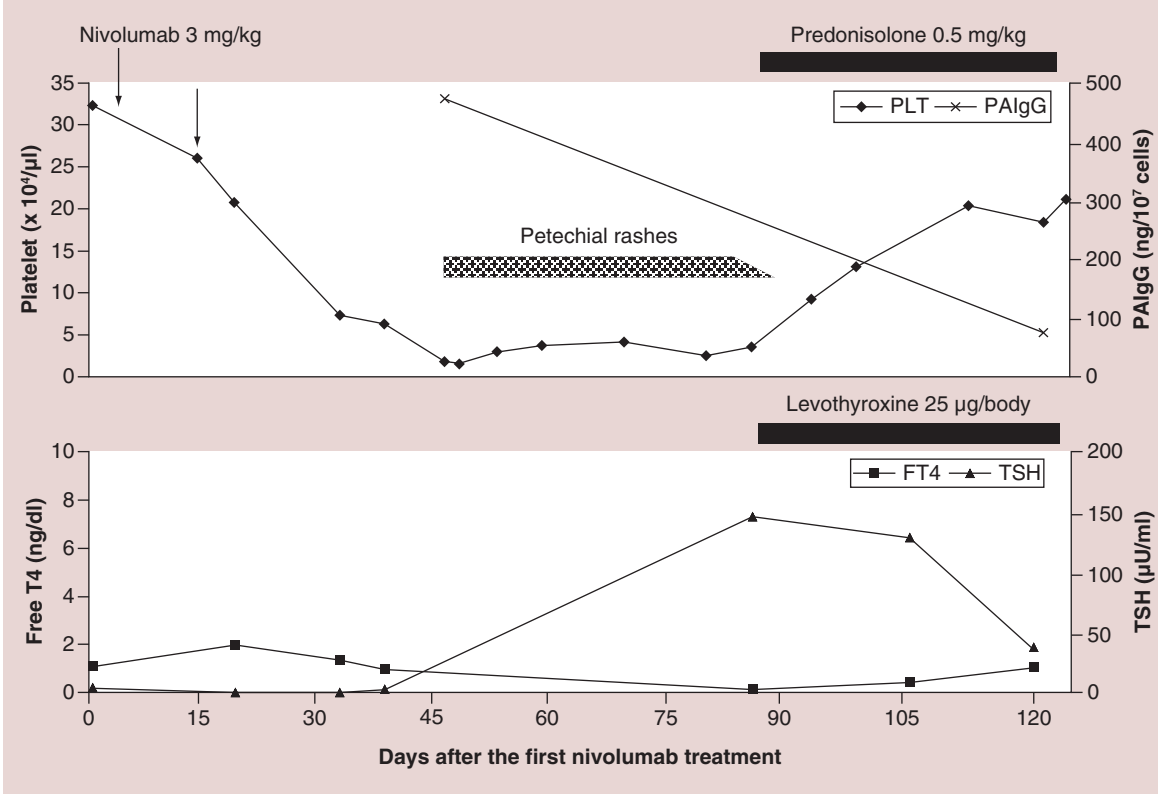

Figure 1. The time course of the laboratory tests related to immune thrombocytopenia and hypothyroidism. The time course of the PLT counts, PAIgG (top) and free T4 and TSH (bottom) during nivolumab treatment, is shown. PAIgG: Platelet-associated immunoglobulin; PLT: Platelet; T4: Thyroxine; TSH: Thyroid-stimulating hormone.

of free T4 (0.97 ng/dl; normal range: 0.97–1.79 ng/dl) and thyroid-stimulating hormone (TSH; 2.28 ng/dl; normal range: 0.34–6.50 μU/ml). The size of the primary lung lesions and the amount of bilateral pleural effusion were reduced after the second administration of nivolumab. However, he visited our clinic because of general fatigue and a loss of appetite. A physical examination revealed a petechial rash on both legs, and the laboratory findings showed a decreased platelet count (1.6×10^4/μl) but a white blood cell count of 9600/μl (54% neutrophils and 42% lymphocytes) and a normal hemoglobin level. The serum level of free T4 (0.13 ng/dl) was decreased while serum TSH (146.0 μU/ml), anti-TPO antibodies (≥600 U/ml) and anti-TG antibodies (≥4000 U/ml) were increased. Anti-TSH receptor antibody and TSH receptor stimulating antibody were negative. Antinuclear antibodies were positive with a titer of 1:80. On ultrasound examination, both lobes of his thyroid gland were enlarged. In addition, his increased levels of platelet-associated IgG (473 ng/10^7cells; normal range: 0–46 ng/10^7cell), a high percentage of immature platelet (9.3%) (normal range: 0.5–2.7%) and an increased number of megakaryocytes without abnormal cells in a bone marrow biopsy, suggested a diagnosis of immune thrombocytopenia. Based on these findings, he was diagnosed with immune thrombocytopenia and hypothyroidism due to acute exacerbation of autoimmune thyroiditis.

Analysis of autoantibodies in his sequential sera revealed an appearance of a new specificity that recognized approximately 34 kD unidentified protein, after starting nivolumab treatment. Prednisolone (0.5 mg/kg) and levothyroxine (25 μg/body) were initiated to treat his immune thrombocytopenia and hypothyroidism, respectively. His platelet count normalized (20.4×10^4/μl), and the symptoms of hypothyroidism disappeared within a month (Figure 1). He did not develop recurrence of thrombocytopenia or hypothyroidism during the tapering period. Paclitaxel protein-bound was initiated as the fourth-line treatment regimen after the patient recovered from thrombocytopenia and hypothyroidism. After six courses of chemotherapy using paclitaxel protein-bound, repeated computed tomography revealed stable disease.

Table 1. The reported cases of nivolumab- and pembrolizumab-associated immune thrombocytopenia.

Study [Ref.] (year)	Age/sex	Diagnosis	Agents	Cycle	PLT lowest count	PAIgG (ng/10^7cell)	Other autoimmune phenomenon	Treatment	Outcome
Solomon *et al.* [7] (2015)	70/M	Melanoma	Ipilimumab and nivolumab	NR	$115 \times 10^3/\mu l$	NR	None	None	Recover
Le Roy *et al.* [4] (2016)	34/M	Melanoma	Pembrolizumab	1	$100/\mu l$	NR	None	S and I	Recover
	51/F	Melanoma	Pembrolizumab	NR	$9000/\mu l$	NR		S	Recover
Kanameishi *et al.* [5] (2016)	79/M	Melanoma	Nivolumab	2	$2000/\mu l$	NR	None	S, I and RO	Recover
Inadomi *et al.* [3] (2016)	73/M	Melanoma	Nivolumab	7	$23 \times 10^3/\mu l$	28.4	None	S	Not recover
Shiuan *et al.* [2] (2017)	47/F	Melanoma	Ipilimumab and nivolumab	1	$<5000/\mu l$	NR	None	S, I, RO and RI	Recover
	45/F	Melanoma	Ipilimumab and nivolumab	NR	$8000/\mu l$	NR	None	S, I and RI	Recover
	52/F	Melanoma	Ipilimumab and nivolumab	NR	$<5 \times 10^3/\mu l$	NR	None	S, I and RI	NR
	80/M	Melanoma	Pembrolizumab	NR	$104 \times 10^3/\mu l$	NR	Neurological	None	NR
	55/F	Melanoma	Ipilimumab and nivolumab	NR	$61 \times 10^3/\mu l$	NR	Endocrine and skin	None	NR
	44/M	Melanoma	Ipilimumab	NR	$18 \times 10^3/\mu l$	NR	Gastrointestinal	S and I	NR
	67/M	Melanoma	Ipilimumab and nivolumab	NR	$86 \times 10^3/\mu l$	NR	Neurological and liver	S	NR
	45/F	Melanoma	Nivolumab	NR	$49 \times 10^3/\mu l$	NR	None	S	NR
	53/M	Melanoma	Pembrolizumab	NR	$53 \times 10^3/\mu l$	NR	Neurological and liver	None	NR
	48/F	Melanoma	Pembrolizumab	NR	$89 \times 10^3/\mu l$	NR	skin	None	NR
	36/F	Melanoma	Pembrolizumab	NR	$58 \times 10^3/\mu l$	NR	None	None	NR
	56/M	Melanoma	Nivolumab	NR	$73 \times 10^3/\mu l$	NR	None	None	NR
	69/M	Melanoma	Nivolumab	NR	$74 \times 10^3/\mu l$	NR	None	None	NR
Present case (2017)	62/M	Lung cancer	Nivolumab	2	$16 \times 10^3/\mu l$	473	Exacerbation of Hashimoto's disease	S	Recover

I: Immunoglobulin; NR: Not reported; PAIgG: Platelet-associated immunoglobulins; PLT: Platelet; RI: Rituximab; RO: Romiplostim; S: Steroid therapy.

Table 2. Reports in the literature of immune thrombocytopenia induced by immune checkpoint inhibitors.

Agents	Study [Ref.] (year)		Diagnosis	Hypothyroidism			Thrombocytopenia	
Pembrolizumab	Garon EB *et al.* [8] (2015)	KEYNOTE-001 (Phase I)	NSCLC	2 mg/kg every 3 weeks	10 mg/kg every 3 weeks	10 mg/kg every 2 weeks	NR	
				1/6 (16.7%)	14/287 (4.9%)	19/202 (9.4%)		
	Ribas A *et al.* [9] (2015)	KEYNOTE-002 (Phase II)	Melanoma	2 mg/kg (every 3 weeks)		10 mg/kg (every 3 weeks)	2 mg/kg (every 3 weeks)	10 mg/kg (every 3 weeks)
				9/178 (5%)		13/179 (7%)	2/178 (2%)	1/179 (<1%)
	Robert C *et al.* [10] (2015)	KEYNOTE-006 (Phase III)	Melanoma	10 mg/kg (every 2 weeks)		10 mg/kg (every 3 weeks)	NR	
				28/278 (10.1%)		24/277 (8.7%)		
	Herbst RS *et al.* [11] (2016)	KEYNOTE-010 (Phase II/III)	NSCLC	2 mg/kg (every 3 weeks)		10 mg/kg (every 3 weeks)	NR	
				28/339 (8%)		28/343 (8%)		
	Langer CJ *et al.* [12] (2016)	KEYNOTE-021 (Phase II)	NSCLC	200 mg/body (every 3 weeks)				
				9/59 (15%)			3/59 (5%)	
	Reck M *et al.* [13] (2016)	KEYNOTE-024 (Phase III)	NSCLC	200 mg/body (every 3 weeks)				
				14/154 (9.1%)			0/154 (0%)	
	Alley EW *et al.* [14] (2017)	KEYNOTE-028 (Phase IB)	MPM	10 mg/kg (every 2 weeks)				
				1/25 (4%)			1/25 (4%)	
	Yamazaki N *et al.* [15] (2017)	KEYNOTE-041 (Phase IB)	Melanoma	2 mg/kg (every 3 weeks)			NR	
				4/42 (9.5%)				
	Bellmunt J *et al.* [16] (2017)	KEYNOTE-045 (Phase III)	Urothelial carcinoma	200 mg/body (every 3 weeks)			NR	
				17/266 (6.4%)				
	Bauml J *et al.* [17] (2017)	KEYNOTE-055 (Phase II)	Head and neck cancer	200 mg/body (every 3 weeks)			NR	
				16/171 (9%)				
Nivolumab	Brahmer J *et al.* [18] (2015)	CheckMate-017 (Phase III)	NSCLC	3 mg/kg (every 2 weeks)				
				5/133 (4%)			NR	
	Motzer RJ *et al.* [19] (2015)	CheckMate-025 (Phase III)	RCC	3 mg/kg (every 2 weeks)				
				24/406 (5.9%)			2/406 (0.5%)	
	Borghaei H *et al.* [20] (2015)	CheckMate-057 (Phase III)	NSCLC	3 mg/kg (every 2 weeks)				
				19/287 (7%)			NR	
	Robert C *et al.* [21] (2015)	CheckMate-066 (Phase III)	Melanoma	3 mg/kg (every 2 weeks)				
				9/206 (4.4%)			0/206 (0%)	
	Ferris RL *et al.* [22] (2016)	CheckMate-141 (Phase III)	Head and neck cancer	3 mg/kg (every 2 weeks)				
				9/236 (3.8%)			2/236 (0.8%)	

HCC: Hepatocellular carcinoma; MPM: Malignant pleural mesothelioma; NR: Not reported; NSCLC: Non-small-cell lung cancer; RCC: Renal cell cancer.

Discussion

Nivolumab and pembrolizumab are immune checkpoint inhibitors that are used in the treatment of advanced NSCLC as antineoplastic drugs with a new mechanism. They are associated with a unique set of side effects that develop through a mechanism markedly different from those of conventional anticancer chemotherapeutic agents. Since these immune checkpoint inhibitors interfere with the immunoinhibitory PD-1–PD-L1 interactions, adverse reactions caused by immunostimulation and autoimmunity, such as endocrinological dysfunction or interstitial pneumonia, may occur [1]. In the present case, a patient who had NSCLC developed immune thrombocytopenia and hypothyroidism soon after the initiation of nivolumab as a third-line treatment. Several previous studies have reported the induction of autoimmune adverse events such as endocrine and pulmonary disorders in patients treated with nivolumab; however, to the best of our knowledge, this is a rare case of a NSCLC patient who developed concomitant immune thrombocytopenia and hypothyroidism accompanied by increased levels of serum autoantibodies associated with each disease during nivolumab treatment.

Table 1 summarizes the reported cases of immune thrombocytopenia during nivolumab and pembrolizumab therapy. Detailed time point of development of immune thrombocytopenia is not available in most cases, however, immune thrombocytopenia developed as early as only 1–2 cycles of treatment in four of five cases with information. Shiuan *et al.* reported that the incidence of immune thrombocytopenia among malignant melanoma patients receiving immune checkpoint inhibitor was <1% [2]. The mainstay treatment for immune thrombocytopenia is corticosteroids, either with or without initial steroid-pulse therapy, and these therapies increase the platelet counts in most cases within the first few weeks. Nevertheless, a case of corticosteroid-resistant immune thrombocytopenia [3] and cases that required a combination of corticosteroids and intravenous immunoglobulin therapy [2,4,5], during nivolumab treatment have been reported.

Although six potential mechanisms of drug-induced immune thrombocytopenia have been suggested [6], the present case did not seem to correspond to any of these. Immune thrombocytopenia during treatment with immune checkpoint inhibitors is thought to have resulted from the specific induction of autoimmunity to platelets, because of the high levels of platelet-associated IgG, the absence of any other factors that were likely to cause thrombocytopenia during the nivolumab treatment, and the platelet count did not increase until the initiation of corticosteroid therapy. Antibody production and cell-mediated immunity can be stimulated by nivolumab *via* the effects of blocking immunoinhibitory PD-1 on T cells, which may cause an autoimmune reaction. Therefore, searching for drug-induced antibodies will be important for clarifying the mechanism underlying this condition.

Hypothyroidism developed along with the enlargement of the thyroid gland as confirmed by ultrasound and increased levels of autoantibodies to thyroglobulin and thyroid peroxidase, at the same time when the patient developed autoimmune thrombocytopenia. Based on these findings, an acute exacerbation of pre-existing subclinical chronic thyroiditis via the inhibition of immune inhibitory pathway by nivolumab is a likely explanation in our case. Development of hypothyroidism is relatively common, reported in 3.8–7.0% in clinical trials of nivolumab monotherapy for patients with NSCLC (Table 2), however, cases of patients with NSCLC who have multiple complications associated with nivolumab are uncommon.

Conclusion

The increasing use of immune checkpoint inhibitors is likely to affect the prevalence of autoimmune clinical conditions that are previously uncommon in patients with lung cancer. In addition, some patients may develop multiple autoimmune complications, as seen in the present case. Although reports of immune checkpoint inhibitor-associated immune thrombocytopenia are rare, immune thrombocytopenia represents a potentially life-threatening complication. We speculate that nivolumab have triggered an autoimmune reaction to platelets and the thyroid gland in the present case; however, the precise pathogenesis remains unknown and further research is therefore needed.

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Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Executive summary

- Patients treated with immune checkpoint inhibitors can develop various immunological complications.
- We herein report a non-small-cell lung cancer patient treated with nivolumab who developed immune-mediated thrombocytopenia and hypothyroidism.
- After starting nivolumab treatment, we detected a new specificity that recognized approximately 34 kD unidentified protein.
- Some patients being treated with nivolumab may be affected by the production of autoantibodies.
- Further investigations and the accumulation of data are necessary to elucidate the precise mechanisms underlying the autoimmune responses that occur in patients treated with immune checkpoint inhibitors.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained approval from the institutional review board of our university. In addition, informed consent was obtained from the patient for inclusion of his medical and clinical course within this case report and for publication.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1 Topalian SL, Hodi FS, Brahmer JR *et al.* Safety, activity and immune correlates of anti-PD-1 antibody in cancer. *N. Engl. J. Med.* 366(26), 2443–2454 (2012).

2 Shiuan E, Beckermann KE, Ozgun A *et al.* Thrombocytopenia in patients with melanoma receiving immune checkpoint inhibitor therapy. *J. Immunother Cancer* 5, 8 (2017).

•• **A case series of drug-induced immune thrombocytopenia associated with the use of immune checkpoint inhibitors.**

3 Inadomi K, Kumagai H, Arita S *et al.* Bi-cytopenia possibly induced by anti-PD-1 antibody for primary malignant melanoma of the esophagus: a case report. *Medicine (Baltimore)* 95(29), e4283 (2016).

4 Le Roy A, Kempf E, Ackermann F *et al.* Two cases of immune thrombocytopenia associated with pembrolizumab. *Eur. J. Cancer* 54, 172–174 (2016).

5 Kanameishi S, Otsuka A, Nonomura Y, Fujisawa A, Endo Y, Kabashima K. Idiopathic thrombocytopenic purpura induced by nivolumab in a metastatic melanoma patient with elevated PD-1 expression on B cells. *Ann. Oncol.* 27(3), 546–547 (2016).

6 Aster RH, Curtis BR, Mcfarland JG, Bougie DW. Drug-induced immune thrombocytopenia: pathogenesis, diagnosis and management. *J. Thromb. Haemost.* 7(6), 911–918 (2009).

•• **A description of the mechanism underlying drug-induced immune thrombocytopenia.**

7 Solomon LR. Thrombocytopenia due to low-dose colchicine therapy: a possible drug interaction with nivolumab and implications for supportive care. *Acta. Oncol.* 54(8), 1235–1237 (2015).

• **The first report of drug-induced immune thrombocytopenia associated with immune checkpoint inhibitors.**

8 Garon EB, Rizvi NA, Hui R *et al.* Pembrolizumab for the treatment of non-small-cell lung cancer. *N. Engl. J. Med.* 372(21), 2018–2028 (2015).

9 Ribas A, Puzanov I, Dummer R *et al.* Pembrolizumab versus investigator-choice chemotherapy for ipilimumab-refractory melanoma (KEYNOTE-002): a randomised, controlled, Phase 2 trial. *Lancet Oncol.* 16(8), 908–918 (2015).

10 Robert C, Schachter J, Long GV *et al.* Pembrolizumab versus ipilimumab in advanced melanoma. *N. Engl. J. Med.* 372(26), 2521–2532 (2015).

11 Herbst RS, Baas P, Kim DW *et al.* Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. *Lancet* 387(10027), 1540–1550 (2016).

12 Langer CJ, Gadgeel SM, Borghaei H *et al.* Carboplatin and pemetrexed with or without pembrolizumab for advanced, non-squamous non-small-cell lung cancer: a randomised, phase 2 cohort of the open-label KEYNOTE-021 study. *Lancet Oncol.* 17(11), 1497–1508 (2016).

13 Reck M, Rodriguez-Abreu D, Robinson AG *et al.* Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. *N. Engl. J. Med.* 375(19), 1823–1833 (2016).

14 Alley EW, Lopez J, Santoro A *et al.* Clinical safety and activity of pembrolizumab in patients with malignant pleural mesothelioma (KEYNOTE-028): preliminary results from a non-randomised, open-label, Phase 1b trial. *Lancet Oncol.* 18(5), 623–630 (2017).

15 Yamazaki N, Takenouchi T, Fujimoto M *et al.* Phase 1b study of pembrolizumab (MK-3475; anti-PD-1 monoclonal antibody) in Japanese patients with advanced melanoma (KEYNOTE-041). *Cancer Chemother. Pharmacol.* 79(4), 651–660 (2017).

16 Bellmunt J, De Wit R, Vaughn DJ *et al.* Pembrolizumab as second-line therapy for advanced urothelial carcinoma. *N. Engl. J. Med.* 376(11), 1015–1026 (2017).

17 Bauml J, Seiwert TY, Pfister DG *et al.* Pembrolizumab for platinum- and cetuximab-refractory head and neck cancer: results from a single-arm, Phase II study. *J. Clin. Oncol.* 35(14), 1542–1549 (2017).

18 Brahmer J, Reckamp KL, Baas P *et al.* Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. *N. Engl. J. Med.* 373(2), 123–135 (2015).

19 Motzer RJ, Escudier B, Mcdermott DF *et al.* Nivolumab versus everolimus in advanced renal-cell carcinoma. *N. Engl. J. Med.* 373(19), 1803–1813 (2015).

20 Borghaei H, Paz-Ares L, Horn L *et al.* Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. *N. Engl. J. Med.* 373(17), 1627–1639 (2015).

21 Robert C, Long GV, Brady B *et al.* Nivolumab in previously untreated melanoma without BRAF mutation. *N. Engl. J. Med.* 372(4), 320–330 (2015).

22 Ferris RL, Blumenschein G Jr, Fayette J *et al.* Nivolumab for recurrent squamous-cell carcinoma of the head and neck. *N. Engl. J. Med.* 375(19), 1856–1867 (2016).

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RESEARCH ARTICLE Open Access

Elevated α-defensin levels in plasma and bronchoalveolar lavage fluid from patients with myositis-associated interstitial lung disease

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Abstract

Background: Interstitial lung disease (ILD) is a prognostic indicator of poor outcome in myositis. Although the pathogenesis of myositis-associated ILD is not well understood, neutrophils are thought to play a pivotal role. Neutrophils store azurophil granules that contain defensins, which are antimicrobial peptides that regulate the inflammatory response. Here, we evaluated levels of the human neutrophil peptides (HNPs) α-defensin 1 through 3 in patients with myositis-associated ILD to determine whether HNPs represent disease markers and play a role in the pathogenesis of myositis-associated ILD.

Methods: HNP levels were measured in the plasma and bronchoalveolar lavage fluid (BALF) of 56 patients with myositis-associated ILD and 24 healthy controls by enzyme-linked immunosorbent assay.

Results: Analysis revealed significantly higher HNP levels in plasma and BALF samples from patients with myositis-associated ILD as compared to those of healthy controls; however, plasma HNPs were significantly correlated with total cell counts in BALF. Additionally, BALF HNP levels were positively correlated with serum surfactant protein-A and the percentage of neutrophils in BALF, and BALF HNP levels correlated with the percentage of reticular opacities in high-resolution computed tomography results for patients with anti-aminoacyl-tRNA synthetase (ARS) antibody positive myositis-associated ILD. Survival did not differ between patients with higher and lower levels of plasma and BALF HNPs.

Conclusions: Plasma and BALF HNPs might reflect the disease activities of myositis-associated ILD, especially in patients with anti-ARS antibody positive myositis-associated ILD. However further studies are necessary to clarify whether HNPs represent disease markers and play roles in disease pathogenesis.

Keywords: Bronchoalveolar lavage fluid, Human neutrophil peptide, Idiopathic inflammatory myopathies, Interstitial lung disease

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Background

Idiopathic inflammatory myopathies (IIMs) are a heterogeneous group of disorders clinically characterized by chronic muscle weakness, low muscle endurance, and the presence of inflammatory cell infiltrates in muscle tissue [1]. Polymyositis (PM), dermatomyositis (DM), and clinically amyopathic dermatomyositis (CADM) are subsets of IIMs that frequently affect the lungs. Interstitial lung disease (ILD) is a common pulmonary manifestation considered a common cause of morbidity and mortality in myositis [2, 3]. Risk factors for ILD in patients with myositis include genetic predisposition and myositis-specific autoantibodies [4, 5]; however, little is known about the clinical course and pathogenesis of myositis-associated ILD. Cellular profiles in bronchoalveolar lavage fluid (BALF) can be used to help diagnose ILD, but only serve to rule out infection in the current clinical differential diagnosis in patients with myositis [6]. However, some reports suggest that the presence of neutrophils in BALF correlates with poor clinical course [2, 7, 8].

Defensins are small, arginine-rich, cationic peptides that exhibit antimicrobial activity [9]. Human cells express α- and β-defensins, and among the six known α-defensins, human neutrophil peptides (HNPs) 1 to 4 are mainly found in neutrophils, whereas human defensins 5 and 6 are primarily expressed in intestinal Paneth cells and the respiratory and female reproductive tracts [10]. In addition to their antimicrobial functions, defensins might also regulate inflammatory responses [11]. We previously identified elevated plasma and BALF HNP levels in patients with various inflammatory lung diseases, including systemic sclerosis-associated ILD, with these levels correlated with neutrophils in BALF [12–19]. These results indicated that plasma and BALF HNP levels play a pivotal role and might serve as biomarkers of other connective-tissue-disease-associated ILD. Here, we evaluated HNP concentrations in BALF and plasma samples from patients with myositis-associated ILD to determine whether HNPs could be used as markers of myositis-associated ILD.

Methods

Study population

The study population comprised 56 patients with myositis-associated ILD and who visited the Department of Respiratory Medicine at Nagasaki University Hospital between 2000 and 2015, as well as 24 healthy volunteers. PM/DM and CADM diagnoses were based on the criteria reported by Bohan and Peter [20] and Sontheimer et al. [21], respectively. ILD was diagnosed by high-resolution computed tomography (HRCT) of the lung, and three of these patients were pathologically diagnosed with fibrotic nonspecific interstitial pneumonia by surgical lung biopsy. BALF and blood samples were collected from each patient during the primary visit and stored at − 20 °C until use. Patients were not under treatment with systemic steroid and/or immunosuppressants at the time of sample collection. All data, including those from pulmonary function tests, arterial blood gas analyses, markers of interstitial pneumonia, such as Krebs von den Lungen 6 (KL-6), surfactant protein (SP)-A, and SP-D expression, as well as survival rates, were obtained from medical records. All healthy controls were asymptomatic, not taking any medication, and had normal chest radiographs. The study protocol was approved by the Human Ethics Review Committee at Nagasaki University School of Medicine, and all participants provided written, informed consent before enrollment.

Evaluation of ILD

All HRCTs and BALF were obtained at the time of diagnosis and at ~ 4-week intervals. HRCT results for all patients were retrospectively and independently assessed by two pulmonologists (N.S. and H.I.). The extent of visual ground glass opacity, consolidation, reticular opacities, and honeycombing were determined by visually estimating the relative area of abnormality in the upper, middle, and lower zones of each lung to the nearest 10%, as previously described [22, 23]. The upper zone was defined as the area above the level of the carina, the lower zone as the area below the level of the inferior pulmonary vein, and the middle zone as the area between the upper and lower zones. The overall involvement percentage was obtained by averaging the six lung zones, with final involvement obtained by averaging the scores of the two observers. BALF was collected with three instillations of sterile physiological saline (50 mL) through a flexible bronchoscope, as previously described [24]. The collected lavage fluid was passed through two sheets of gauze, centrifuged at 400 *g* for 10 min at 4 °C, and the supernatant stored at − 20 °C until analysis.

HNP quantification

HNP concentrations in plasma and BALF samples were measured using sandwich enzyme-linked immunosorbent assay (ELISA) kits according to manufacturer protocol (HNP1–3; HyCult Biotechnology, Uden, Netherlands). Plasma samples were diluted 1000-fold prior to analysis. The lower limit of detection was 156 pg/mL.

Immunoprecipitation (IP)

Sera were analyzed by IP of K562 cell extracts radiolabeled with ^{35}S-methionine, and the specificities of autoantibodies were determined using specific reference sera [25].

Anti-Jo-1 and MDA5 ELISAs

Anti-Jo-1 and −melanoma-differentiation-associated protein 5 (MDA5) antibodies were also tested by ELISA, as previously described [26], using recombinant Jo-1 and

MDA5 proteins (0.5 μg/mL; Diarect, Freiburg, Germany) and 1:250 diluted sera. The optical density was measured and converted into units using a standard curve created with a prototype-positive serum.

Statistical analysis

All values are expressed as the median and inter-quartile range (IQR). Differences between groups were compared using Mann-Whitney *U* tests. Differences among groups were determined using the Kruskal–Wallis test for continuous variables. If a significant difference was found by the Kruskal–Wallis test, multiple comparisons were performed using the Dunn test. Statistical significance was defined as $p < 0.05$. Correlations between parameters were determined by Spearman's rank correlation coefficient. To account for multiple comparisons, we conducted false-discovery rate (FDR) calculations using the Benjamini–Hochberg procedure [27], with FDR q values of 0.1 considered significant.

Results

Patient characteristics

Patient demographics are shown in Table 1 ($n = 56$). Fifteen of the patients were men, and the median age was 60 years. Half of the patients were diagnosed with DM. The myositis-specific autoantibodies anti-ARS (Jo-1, PL-7, PL-12, EJ, OJ, and KS) and anti-MDA5 were detected in 46% and 18% of patients, respectively, as determined by IP and ELISA analysis. Patient laboratory findings are shown in Table 2.

Table 1 Characteristics of patients with myositis-associated ILD

Characteristic	N (%)
Gender (male/female)	15/41
Age (y)	60 (range, 50–66)
Clinical diagnosis	
Polymyositis	7 (13%)
Dermatomyositis	28 (50%)
Clinically amyopathic dermatomyositis	21 (38%)
Myositis-specific autoantibodies	
anti-ARS Ab	26 (46%)
anti-Jo-1 Ab	11 (20%)
anti-PL-7 Ab	6 (11%)
anti-KS Ab	3 (5%)
anti-PL-12 Ab	2 (4%)
anti-EJ Ab	2 (4%)
anti-OJ Ab	2 (4%)
anti-MDA5 Ab	10 (18%)
anti-Ku Ab	2 (4%)
anti-TIF1 gamma Ab	2 (4%)
Unknown	16 (29%)
Total	56

HNP levels in plasma and BALF samples

HNP analysis revealed significantly higher levels in the plasma of patients with myositis-associated ILD [78.5 pg/mL (40.1–171.1 pg/mL)] than in that of healthy controls [60.5 pg/mL (20.5–83.3 pg/mL), $p < 0.05$; Fig. 1a], as well as that in the BALF [250.9 pg/mL (53.7–821.0 pg/mL) vs. 14.3 pg/mL (7.8–30.9 pg/mL), $p < 0.01$; Fig. 1b]. Plasma HNP levels were elevated only in patients with ARS autoantibody production; however, significant elevations were observed in BALF samples from both anti-ARS-positive and ARS/MDA5-double-negative subjects ("others"; Fig. 2).

Association between HNPs and clinical parameters in myositis-associated ILD

We then analyzed the relationships between clinical parameters and HNP levels in patient plasma and BALF samples (Table 3). Interestingly, the plasma and BALF HNP levels showed no significant association. Plasma HNP levels were significantly correlated with total cell count in BALF (Fig. 3 and Table 2). Moreover, BALF

Table 2 Laboratory findings in patients with myositis-associated ILD

Variables	N	Median	IQR
Laboratory data			
CK (IU/L)	55	150	(66–393)
Aldolase (IU/L)	53	7.4	(5.1–19.0)
AST (IU/L)	55	34	(23–59)
ALT (IU/L)	55	28	(20–46)
LDH (IU/L)	55	302	(239–391)
PaO2 (torr)	44	79.4	(69.6–90.2)
KL-6 (U/mL)	53	1030	(440–1793)
SP-D (ng/mL)	49	204	(95–204)
SP-A (ng/mL)	37	72.7	(55.4–105.1)
Pulmonary function test			
%VC (%)	40	83.6	(67.3–94.7)
FEV1/FEV (%)	40	83.7	(78.8–92.8)
%DLco (%)	40	54.8	(39.0–72.0)
BALF cell findings			
TCC ($\times 10^5$/mL)	45	3.5	(2.8–5.8)
Macrophages (%)	45	45.7	(34.4–63.9)
Lymphocytes (%)	45	33.4	(17.4–47.5)
Neutrophils (%)	45	7.8	(2.2–15.4)
Eosinophils (%)	45	2.2	(0.9–7.2)
CD4/CD8	43	0.4	(0.2–1.1)

ALT: alanine aminotransferase; *AST*: aspartate aminotransferase; *CK*: creatine kinase; *DLco*: diffusing capacity of the lungs for carbon monoxide; *FEV*: forced expiratory volume; *TCC*: total cell count; *VC*: vital capacity

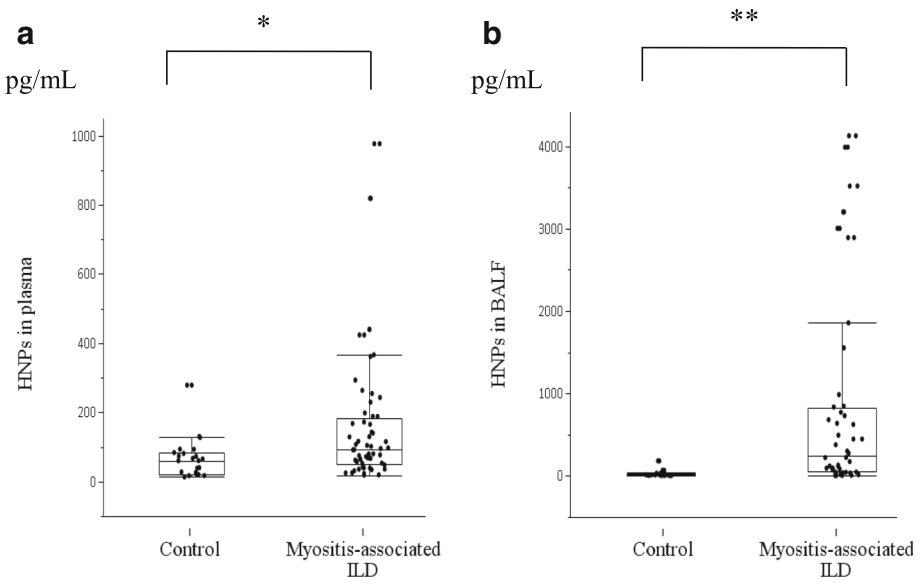

Fig. 1 ELISA analysis of patient samples. HNP levels in plasma (**a**) and BALF (**b**) samples from patients with myositis-associated ILD and healthy controls according to ELISA. Boxes represent the IQR, and the internal line represents the median. Whiskers indicate the lowest and highest values within 1.5 × IQR. *$p < 0.05$; **$p < 0.01$

HNP levels were positively correlated with serum SP-A and neutrophil percentage in BALF (Fig. 4 and Table 2). There were no significant associations between HNP levels and pulmonary function test results or HRCT findings. Furthermore, survival determined by Kaplan–Meier survival analysis showed no association with HNP concentration in the plasma or BALF (data not shown).

Next, we examined the association between HNP levels and clinical parameters in each group according to myositis-related autoantibodies (anti-ARS antibody, anti-MDA5 antibody, others). No other significant correlation was evident in each group according to myositis-related autoantibodies other than plasma HNP levels and total cell count in BALF in patients with anti-ARS-antibody positive myositis-associated ILD. On the other hand, BALF HNP levels in patients with anti-ARS-antibody positive myositis-associated ILD were significantly correlated with the extent of reticular opacities

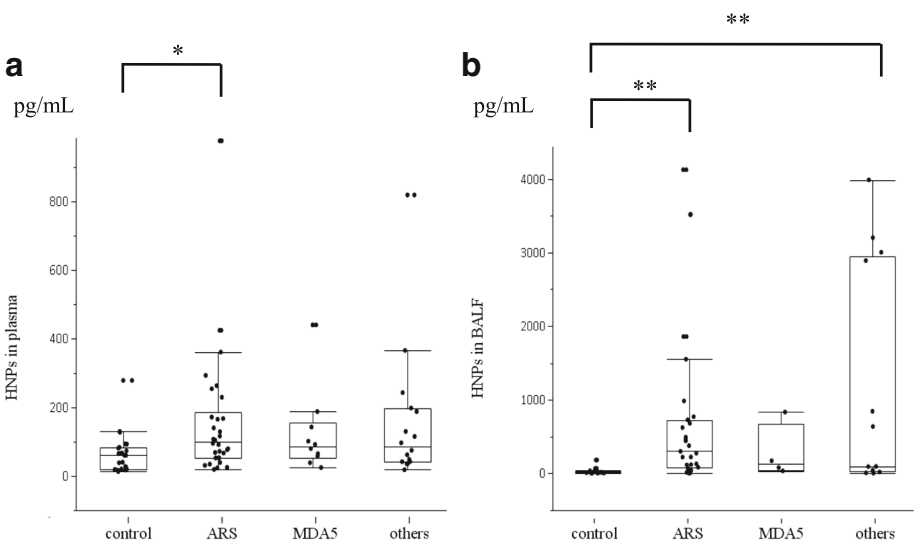

Fig. 2 Associations between plasma and BALF HNP levels and patient autoantibody levels. ELISA analysis of associations between HNP levels in the plasma (**a**) and BALF (**b**) and autoantibody levels in patients with myositis-associated ILD and healthy controls. "Others" indicates patients negative for both ARS and MDA5 autoantibodies. *$p < 0.05$; **$p < 0.01$

Table 3 Correlation between HNPs and clinical parameters in patients with myositis-associated ILD

	HNPs in plasma				HNPs in BALF			
	N	r	95% CI	*q*-value	N	r	95% CI	*q*-value
Laboratory data								
CK (IU/L)	55	0.135	−0.135–0.387	1.00	43	0.029	−0.274–0.327	0.92
PaO_2 (torr)	28	−0.062	−0.425–0.318	1.00	35	0.182	− 0.161–0.486	0.49
KL-6 (U/mL)	53	− 0.122	− 0.380–0.153	1.00	41	0.267	−0.044–0.531	0.23
SP-D (ng/mL)	49	0.029	− 0.254–0.308	1.00	38	0.205	−0.123–0.492	0.41
SP-A (ng/mL)	44	0.010	−0.288–0.306	0.95	29	0.528	0.200–0.750	0.02
Pulmonary function test								
%VC (%)	40	−0.023	−0.332–0.291	1.00	34	−0.048	−0.380–0.295	0.93
%DLco (%)	40	−0.071	−0.374–0.246	1.00	34	−0.017	−0.353–0.323	0.92
BALF cell findings								
TCC ($\times 10^5$/mL)	45	0.409	0.131–0.627	0.07	44	0.147	−0.157–0.425	0.49
Lymphocytes (%)	45	−0.013	−0.305–0.282	1.00	44	−0.305	−0.552–0.009	0.17
Neutrophils (%)	45	0.266	−0.030–0.519	0.52	44	0.693	0.499–0.821	0.01
HRCT findings								
Ground glass opacity (%)	52	0.132	−0.146–0.391	1.00	41	0.102	−0.212–0.397	0.69
Consolidation (%)	52	0.095	−0.183–0.359	1.00	41	−0.237	−0.508–0.076	0.30
Reticular opacities (%)	52	−0.028	−0.299–0.247	1.00	41	0.284	−0.026–0.544	0.23

CI confidence interval, *CK* creatine kinase, *DLco* diffusing capacity of the lungs for carbon monoxide, *TCC* total cell count, *VC* vital capacity

and negatively correlated with consolidation according to HRCT findings (Table 4). No other significant correlation was evident in each group categorized by myositis-related autoantibodies other than BALF HNP levels and neutrophil percentage in BALF from patients with anti-ARS-antibody positive myositis-associated ILD. Additionally, there was no significant correlation between HNP levels and clinical parameters in anti-MDA5-antibody positive myositis-associated ILD and ARS/MDA5-antibody double-negative subjects (data not shown).

Discussion

In this study, we found elevated HNP concentrations in the plasma and BALF from patients with myositis-associated ILD as compared with that observed in those from healthy controls. Notably, plasma HNP levels were associated with total cell counts in the BALF, whereas those in BALF samples were associated with the interstitial pneumonia marker SP-A and the percentage of neutrophils in BALF. Furthermore, BALF HNP levels in patients with anti-ARS-antibody positive myositis-associated ILD were significantly

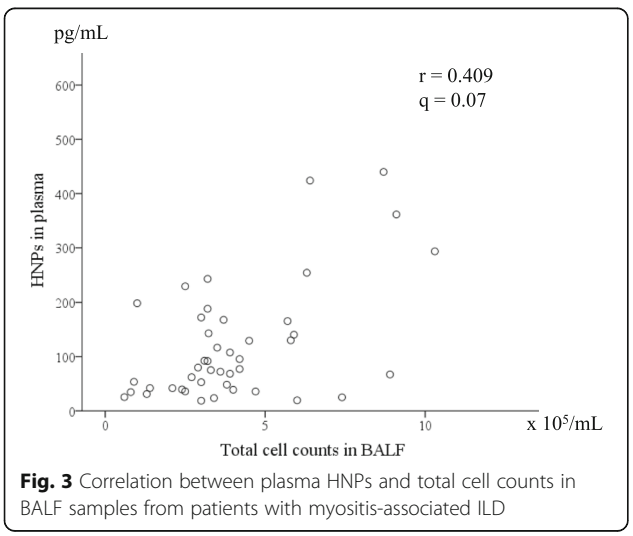

Fig. 3 Correlation between plasma HNPs and total cell counts in BALF samples from patients with myositis-associated ILD

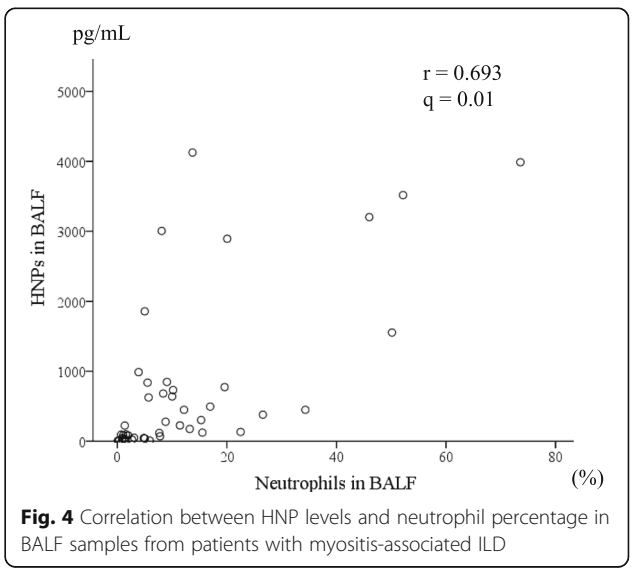

Fig. 4 Correlation between HNP levels and neutrophil percentage in BALF samples from patients with myositis-associated ILD

Table 4 Correlation between HNPs and HRCT findings in patients with anti-ARS antibody positive myositis-associated ILD

	HNPs in plasma				HNPs in BALF			
	N	r	95% CI	*q*-value	N	r	95% CI	*q*-value
HRCT findings								
Ground glass opacity (%)	30	0.328	−0.037–0.616	0.49	27	0.085	−0.305–0.451	0.80
Consolidation (%)	30	0.031	−0.333–0.387	1.00	27	−0.452	−0.710–− 0.087	0.08
Reticular opacities (%)	30	−0.089	−0.435–0.280	1.00	27	0.465	0.103–0.718	0.09

CI confidence interval

correlated with the extent of reticular opacities and negatively correlated with consolidation in HRCT findings.

Previous studies demonstrated that BALF HNP levels are associated with the prevalence of neutrophils in BALF samples and disease activity in patients with various lung diseases, including connective-tissue-disease-associated ILD [14–19]. Moreover, several reports suggest that neutrophils in the BALF correlate with poor clinical course in patients with PM/DM [2, 7, 8]. Consistent with these findings, the present study showed that BALF HNP levels correlated with the amount of neutrophils in the BALF of patients with myositis-associated ILD. Neutrophils release granular and nuclear contents called neutrophil extracellular traps (NETs), including HNPs, in response to different classes of microorganisms, soluble factors, and host molecules [28]. Zang et al. [29] demonstrated that patients with PM/DM have the capacity to form NETs that could not be completely degraded, particularly in patients with PM/DM-ILD. Moreover, they reported that abnormal NET regulation might be involved in PM/DM pathogenesis and could be a factor that initiates and/or aggravates ILD [29]. The authors also reported a higher percentage of low-density granulocytes (LDGs) along with enhanced NET-formation capabilities in patients with DM as compared with healthy controls, and that this percentage was also higher in DM patients with ILD than in those without. Additionally, LDG percentage was positively correlated with lung disease activity scores [30]. In line with these reports, the present results showed that increased HNP levels in the plasma and BALF from patients with myositis-associated ILD suggested that neutrophils are likely to release NETs, including HNPs, which are difficult to degrade in patients with myositis-associated ILD.

Our results also showed that BALF HNP levels correlated with SP-A levels, suggesting the existence of interstitial lung injury [31]; however, this was not observed in HRCT findings for all patients. Additionally, these levels in patients with anti-ARS-antibody positive myositis-associated ILD were significantly correlated with the extent of reticular opacities according to HRCT findings, which we previously reported in patients with systemic sclerosis-associated ILD [19]. Reticular opacities are common HRCT findings in anti-ARS-antibody positive associated ILD with or without myositis [32–34] and reflect fibrosis in anti-ARS-antibody positive ILD [33]. These findings indicate that HNP levels in BALF reflect the fibrotic change in anti-ARS-antibody positive associated ILD. Furthermore, we previously reported that HNPs induce the production of cytokines and growth factors, which act on lung fibroblasts and epithelial cells to induce pulmonary fibrosis and collagen production in vitro [9, 35, 36]. This might suggest that HNPs in the lung both indicate and induce fibrotic change. Further studies are needed to determine whether neutrophil-derived HNPs play a role in the pathogenesis of myositis-associated ILD.

We also observed that plasma HNPs were associated with total cell counts in BALF, which might reflect the lung inflammation observed in patients with myositis-associated ILD. Although the precise mechanism of plasma HNP production is not well understood, these factors are likely derived from neutrophil-precursor cells in the bone marrow following stimulation by inflammatory mediators [18, 37]. Therefore, these results might suggest that lung inflammation elicits increased plasma HNP levels. Nevertheless, further studies are needed to clarify the functional significance of plasma HNPs in patients with myositis-associated ILD.

Although we found that HNP levels were associated with several clinical parameters and suggested to play a role in myositis-associated ILD, it remains unclear whether HNPs can be used as disease markers in myositis-associated ILD. This might be because our definition of myositis-associated ILD included different disease types (PM, DM, or CADM) or the use of myositis-specific antibodies (anti-ARS and anti-MDA5).

The present study has several limitations. First, because the patient population was strictly seen by physicians in the respiratory department, our study only examined HNP levels in patients with myositis-associated ILD, but not those without the ILD component. Therefore, we were unable to confirm that lung pathology was directly responsible for the observed differences in HNP levels. Second, we did not show values for plasma or BALF HNPs, which can discriminate between various types of ILD or infectious processes. We

previously reported elevated HNP levels in several types of ILD, as well as according to infectious status [14–19], indicating that increased levels of HNPs were nonspecific in myositis-associated ILD. Additionally, the small patient cohort limited the clinical application of these findings; therefore, a larger patient population should be examined using a prospective study model in future investigations.

Conclusions

In conclusion, this study demonstrated increased HNP levels in the plasma and BALF of patients with myositis-associated ILD as compared with that observed in those of healthy controls. Although factors were associated with clinical parameters, further studies are necessary to clarify whether HNPs represent a candidate disease marker and to elucidate the role of defensins in myositis-associated ILD.

Abbreviations

ARS: Aminoacyl-tRNA synthetase; BALF: Bronchoalveolar lavage fluid; CADM: Clinically amyopathic dermatomyositis; DM: Dermatomyositis; FDR: False-discovery rate; HNPs: Human neutrophil peptides; HRCT: High-resolution computed tomography; IIMs: Idiopathic inflammatory myopathies; ILD: Interstitial lung disease; IP: Immunoprecipitation; IQR: Inter-quartile range; KL-6: Krebs von den Lungen 6; LDG: Low-density granulocyte; LDH: Lactate dehydrogenase; MDA5: Melanoma-differentiation-associated protein 5; NET: Neutrophil extracellular trap; PM: Polymyositis; SP: Surfactant protein

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Author contributions

NS made substantial contributions to study conception and design. NS, HI, TK, AH, SN, HY, TM, HK, TM, YO, YI, and HM collected clinical samples. NS drafted the article. MS critically revised the article for important intellectual content. MS, TH, and ST performed IP and ELISA assays for autoantibodies. All authors read and approved the final manuscript.

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Availability of data and materials

The data will not be shared due to participant confidentiality.

Ethics approval and consent to participate

The study protocol was approved by the Human Ethics Review Committee at Nagasaki University School of Medicine, and all participants provided written, informed consent before enrollment.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Lundberg IE, Miller FW, Tjarnlund A, Bottai M. Diagnosis and classification of idiopathic inflammatory myopathies. J Intern Med. 2016;280:39–51.
2. Marie I, Hachulla E, Cherin P, Dominique S, Hatron PY, Hellot MF, Devulder B, Herson S, Levesque H, Courtois H. Interstitial lung disease in polymyositis and dermatomyositis. Arthritis Rheum. 2002;47:614–22.
3. Mukae H, Ishimoto H, Sakamoto N, Hara S, Kakugawa T, Nakayama S, Ishimatsu Y, Kawakami A, Eguchi K, Kohno S. Clinical differences between interstitial lung disease associated with clinically amyopathic dermatomyositis and classic dermatomyositis. Chest. 2009;136:1341–7.
4. Chinoy H, Adimulam S, Marriage F, New P, Vincze M, Zilahi E, Kapitany A, Gyetvai A, Ekholm L, Novota P, et al. Interaction of HLA-DRB1*03 and smoking for the development of anti-Jo-1 antibodies in adult idiopathic inflammatory myopathies: a European-wide case study. Ann Rheum Dis. 2012;71:961–5.
5. Hamaguchi Y, Fujimoto M, Matsushita T, Kaji K, Komura K, Hasegawa M, Kodera M, Muroi E, Fujikawa K, Seishima M, et al. Common and distinct clinical features in adult patients with anti-aminoacyl-tRNA synthetase antibodies: heterogeneity within the syndrome. PLoS One. 2013;8:e60442.
6. Lega JC, Reynaud Q, Belot A, Fabien N, Durieu I, Cottin V. Idiopathic inflammatory myopathies and the lung. Eur Respir Rev. 2015;24:216–38.
7. Schnabel A, Reuter M, Biederer J, Richter C, Gross WL. Interstitial lung disease in polymyositis and dermatomyositis: clinical course and response to treatment. Semin Arthritis Rheum. 2003;32:273–84.
8. Fujisawa T, Hozumi H, Kono M, Enomoto N, Hashimoto D, Nakamura Y, Inui N, Yokomura K, Koshimizu N, Toyoshima M, et al. Prognostic factors for myositis-associated interstitial lung disease. PLoS One. 2014;9:e98824.
9. Sakamoto N, Mukae H, Fujii T, Ishii H, Yoshioka S, Kakugawa T, Sugiyama K, Mizuta Y, Kadota J, Nakazato M, et al. Differential effects of alpha- and beta-defensin on cytokine production by cultured human bronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol. 2005;288:L508–13.
10. Aarbiou J, Rabe KF, Hiemstra PS. Role of defensins in inflammatory lung disease. Ann Med. 2002;34:96–101.
11. Mendez-Samperio P. Recent advances in the field of antimicrobial peptides in inflammatory diseases. Adv Biomed Res. 2013;2:50.
12. Mukae H, Matsumoto N, Ashitani J, Mashimoto H, Kadota J, Nakazato M, Kohno S, Matsukura S. Neutrophil-related cytokines and neutrophil products in bronchoalveolar lavage fluid of a patient with ANCA negative Wegener's granulomatosis. Eur Respir J. 1996;9:1950–4.
13. Mukae H, Ishimoto H, Yanagi S, Ishii H, Nakayama S, Ashitani J, Nakazato M, Kohno S. Elevated BALF concentrations of alpha- and beta-defensins in patients with pulmonary alveolar proteinosis. Respir Med. 2007;101:715–21.
14. Sakamoto N, Ishimatsu Y, Kakugawa T, Yura H, Tomonaga M, Harada T, Nakashima S, Hara S, Hara A, Ishimoto H, et al. Elevated plasma alpha-defensins in patients with acute exacerbation of fibrotic interstitial pneumonia. Respir Med. 2015;109:265–71.
15. Mukae H, Iiboshi H, Nakazato M, Hiratsuka T, Tokojima M, Abe K, Ashitani J, Kadota J, Matsukura S, Kohno S. Raised plasma concentrations of alpha-defensins in patients with idiopathic pulmonary fibrosis. Thorax. 2002;57:623–8.
16. Ashitani J, Mukae H, Nakazato M, Ihi T, Mashimoto H, Kadota J, Kohno S, Matsukura S. Elevated concentrations of defensins in bronchoalveolar lavage fluid in diffuse panbronchiolitis. Eur Respir J. 1998;11:104–11.

17. Ashitani J, Mukae H, Hiratsuka T, Nakazato M, Kumamoto K, Matsukura S. Elevated levels of alpha-defensins in plasma and BAL fluid of patients with active pulmonary tuberculosis. Chest. 2002;121:519–26.
18. Ashitani J, Mukae H, Hiratsuka T, Nakazato M, Kumamoto K, Matsukura S. Plasma and BAL fluid concentrations of antimicrobial peptides in patients with Mycobacterium avium-intracellulare infection. Chest. 2001; 119:1131–7.
19. Sakamoto N, Kakugawa T, Hara A, Nakashima S, Yura H, Harada T, Ishimoto H, Yatera K, Kuwatsuka Y, Hara T, et al. Association of elevated alpha-defensin levels with interstitial pneumonia in patients with systemic sclerosis. Respir Res. 2015;16:148.
20. Bohan A, Peter JB. Polymyositis and dermatomyositis (second of two parts). N Engl J Med. 1975;292:403–7.
21. Sontheimer RD. Would a new name hasten the acceptance of amyopathic dermatomyositis (dermatomyositis sine myositis) as a distinctive subset within the idiopathic inflammatory dermatomyopathies spectrum of clinical illness? J Am Acad Dermatol. 2002;46:626–36.
22. Johkoh T, Muller NL, Colby TV, Ichikado K, Taniguchi H, Kondoh Y, Fujimoto K, Kinoshita M, Arakawa H, Yamada H, et al. Nonspecific interstitial pneumonia: correlation between thin-section CT findings and pathologic subgroups in 55 patients. Radiology. 2002;225:199–204.
23. Sumikawa H, Johkoh T, Ichikado K, Taniguchi H, Kondoh Y, Fujimoto K, Tateishi U, Hiramatsu T, Inoue A, Natsag J, et al. Usual interstitial pneumonia and chronic idiopathic interstitial pneumonia: analysis of CT appearance in 92 patients. Radiology. 2006;241:258–66.
24. Sakamoto N, Mukae H, Fujii T, Kakugawa T, Kaida H, Kadota J, Kohno S. Soluble form of Fas and Fas ligand in serum and bronchoalveolar lavage fluid of individuals infected with human T-lymphotropic virus type 1. Respir Med. 2004;98:213–9.
25. Satoh M, Ceribelli A, Hirakata M, Chan E. Immunodiagnosis of autoimmune myopathies. In: Rose N, Hamilton R, Detrick B, Reeves W, editors. Manual of molecular and clinical laboratory immunology. 8th ed. Washington, D.C.: American Society of Microbiology Press; 2016. p. 878–87.
26. Ceribelli A, Fredi M, Taraborelli M, Cavazzana I, Tincani A, Selmi C, Chan JY, Chan EK, Satoh M, Franceschini F. Prevalence and clinical significance of anti-MDA5 antibodies in European patients with polymyositis/dermatomyositis. Clin Exp Rheumatol. 2014;32:891–7.
27. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Series B Stat Methodol. 1995;57:289–300.
28. Porto BN, Stein RT. Neutrophil extracellular traps in pulmonary diseases: too much of a good thing? Front Immunol. 2016;7:311.
29. Zhang S, Shu X, Tian X, Chen F, Lu X, Wang G. Enhanced formation and impaired degradation of neutrophil extracellular traps in dermatomyositis and polymyositis: a potential contributor to interstitial lung disease complications. Clin Exp Immunol. 2014;177:134–41.
30. Zhang S, Shen H, Shu X, Peng Q, Wang G. Abnormally increased low-density granulocytes in peripheral blood mononuclear cells are associated with interstitial lung disease in dermatomyositis. Mod Rheumatol. 2016;27:1–8.
31. Takahashi H, Shiratori M, Kanai A, Chiba H, Kuroki Y, Abe S. Monitoring markers of disease activity for interstitial lung diseases with serum surfactant proteins a and D. Respirology. 2006;11:S51–4.
32. Yura H, Sakamoto N, Satoh M, Ishimoto H, Hanaka T, Ito C, Hasegawa T, Tanaka S, Miyamura T, Nakashima S, et al. Clinical characteristics of patients with anti-aminoacyl-tRNA synthetase antibody positive idiopathic interstitial pneumonia. Respir Med. 2017;132:189–94.
33. Waseda Y, Johkoh T, Egashira R, Sumikawa H, Saeki K, Watanabe S, Matsunuma R, Takato H, Ichikawa Y, Hamaguchi Y, et al. Antisynthetase syndrome: pulmonary computed tomography findings of adult patients with antibodies to aminoacyl-tRNA synthetases. Eur J Radiol. 2016;85:1421–6.
34. Takato H, Waseda Y, Watanabe S, Inuzuka K, Katayama N, Ichikawa Y, Yasui M, Fujimura M. Pulmonary manifestations of anti-ARS antibody positive interstitial pneumonia–with or without PM/DM. Respir Med. 2013;107:128–33.
35. Yoshioka S, Mukae H, Ishii H, Kakugawa T, Ishimoto H, Sakamoto N, Fujii T, Urata Y, Kondo T, Kubota H, et al. Alpha-defensin enhances expression of HSP47 and collagen-1 in human lung fibroblasts. Life Sci. 2007;80:1839–45.
36. Amenomori M, Mukae H, Ishimatsu Y, Sakamoto N, Kakugawa T, Hara A, Hara S, Fujita H, Ishimoto H, Hayashi T, et al. Differential effects of human neutrophil peptide-1 on growth factor and interleukin-8 production by human lung fibroblasts and epithelial cells. Exp Lung Res. 2010;36:411–9.
37. Nakazato M, Shiomi K, Date Y, Matsukura S, Kangawa K, Minamino N, Matsuo H. Isolation and sequence determination of 6- and 8-kDa precursors of human neutrophil peptides from bone marrow, plasma and peripheral blood neutrophils. Biochem Biophys Res Commun. 1995;211:1053–62.

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Reference standards for the detection of anti-mitochondrial and anti-rods/rings autoantibodies

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Abstract

Background: Anti-mitochondrial antibodies (AMA) are found in >90% of primary biliary cholangitis patients. Anti-rods/rings antibodies (anti-RR) are most commonly associated with interferon-α and ribavirin treatment in hepatitis C patients. Clinical laboratories routinely screen for AMA and anti-RR using indirect immunofluorescence on HEp-2 cells (HEp-2-IFA). Therefore, we sought to establish reference materials for use in AMA and anti-RR testing.

Methods: AMA-positive and anti-RR-positive human plasma samples (AMA-REF and RR-REF), identified as potential reference materials based on preliminary data, were further validated by multiple laboratories using HEp-2-IFA, immunoprecipitation (IP), western blotting, IP-western, line immunoassay (LIA), addressable laser bead immunoassay (ALBIA) and enzyme-linked immunosorbent assay (ELISA).

Results: AMA-REF showed a strong positive cytoplasmic reticular/AMA staining pattern by HEp-2-IFA to ≥1:1280 dilution and positive signal on rodent kidney/stomach/liver tissue. AMA-REF reacted with E2/E3, E3BP, E1α and E1β subunits of the pyruvate dehydrogenase complex by IP and western blotting and was positive for AMA antigens by LIA, ALBIA and ELISA. RR-REF showed a strong positive rods and rings staining pattern by HEp-2-IFA to ≥1:1280 dilution. RR-REF reacted with inosine monophosphate dehydrogenase by IP, IP-western and ALBIA. RR-REF also produced a nuclear homogenous staining pattern by HEp-2-IFA, immunoprecipitated proteins associated with anti-U1RNP antibody and reacted weakly with histones, nucleosomes, Sm and nRNP/Sm by LIA.

Conclusions: AMA-REF and RR-REF are useful reference materials for academic or commercial clinical laboratories to calibrate and establish internal reference standards for immunodiagnostic assays. AMA-REF and RR-REF are now available for free distribution to qualified laboratories through Plasma Services Group.

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Keywords: anti-mitochondrial antibody; anti-rods/rings antibody; autoantibody; autoimmunity; hepatitis C; primary biliary cholangitis.

Introduction

Autoantibodies targeting self-antigens are hallmarks of many systemic and organ-specific autoimmune diseases. In a clinical laboratory setting, autoantibodies against nuclear and some cytoplasmic antigens are routinely screened by the indirect immunofluorescence assay (IFA) on HEp-2 cells (HEp-2-IFA). Several multiplex assays, using methodological platforms such as the enzyme-linked immunosorbent assay (ELISA), chemiluminescence immunoassay (CLIA), line immunoassay (LIA), addressable laser bead immunoassay (ALBIA) and new chip-based assays, are becoming more common in diagnostic laboratories. However, HEp-2-IFA remains the "gold standard" method for detection of antinuclear antibodies (ANA) [1]. ANA, which bind components of the nucleus, have been the focus of study for decades, but many autoantibodies that target cytoplasmic antigens are detected by HEp-2-IFA. One example is anti-mitochondrial antibodies (AMA), which target proteins of the inner and outer mitochondrial membranes. AMA are classified into nine distinct categories, referred to as M1 through M9 subtypes [2, 3]. AMA-M2 target the E2 components of the 2-oxo-acid dehydrogenase family of enzyme complexes, including the pyruvate dehydrogenase complex (PDC)-E2, branched-chain 2-oxo-acid dehydrogenase complex (BCOADC-E2) and 2-oxo-glutarate dehydrogenase complex (OGDC-E2), typically referred to as the major AMA antigens [4]. AMA-M2 also target the minor antigens dihydrolipoamide dehydrogenase (E3)-binding protein (E3BP) and the E1α subunit of PDC (PDC-E1α). AMA-M2 are highly specific for primary biliary cholangitis (PBC), formerly known as primary biliary cirrhosis [5, 6], a relatively uncommon chronic autoimmune disease resulting in progressive destruction of the intrahepatic biliary tree that can eventually lead to liver cirrhosis. Approximately 95% of PBC patients are AMA-positive, with PDC-E2 being the main antigen, and circulating AMA-M2 is one of three key diagnostic criteria for this disease [6–8]. As the presence of AMA often precedes the clinical manifestation of PBC, accurate and complete reporting of AMA in AMA-specific assays and HEp-2-IFA is critical to enabling early diagnosis and treatment of PBC [9–11].

Another example of an autoantibody to cytoplasmic components is anti-rods/rings antibody (anti-RR). Unlike AMA, the study of anti-RR has uncovered only one major autoantigen, inosine 5′-monophosphate dehydrogenase 2 (IMPDH2) [12–14]. IMPDH1 might also be recognized by anti-RR, considering the 84% sequence similarity between the two isoforms. Some human sera positive for anti-RR by HEp-2-IFA do not immunoprecipitate IMPDH, suggesting there are other antigens yet to be identified [15]. Despite reports that cytidine triphosphate synthase (CTPS) colocalizes with IMPDH in rods/rings structures under certain conditions [12, 16, 17], no human anti-RR serum has been found to react with CTPS [13, 15]. Several studies showed that anti-RR is strongly associated with hepatitis C virus (HCV) after treatment with interferon-α and ribavirin (IFN/RBV) therapy [13, 18–22]. However, anti-RR has been observed occasionally in individuals without HCV infection [23] and in rare cases of systemic lupus erythematosus [24] and hepatitis B [19]. Low-titer anti-RR has also been reported in the general population of the United States [24]. Although the use of IFN/RBV therapy will likely decrease with the recent development of novel direct-acting antivirals (DAA), many DAA-based treatment regimens still include RBV [25, 26]. IFN/RBV therapy might also continue in parts of the world where the considerable cost of new DAA prevent their rapid adoption [27]. Considering how much is still unknown about anti-RR antibody, it will be useful to continue to accurately monitor and report its presence during routine ANA testing in clinical laboratories.

In this report, we describe the development of reference materials for the detection of AMA and anti-RR autoantibodies, which we refer to here as the AMA reference material (AMA-REF) and RR reference material (RR-REF). AMA-REF and RR-REF can be used to calibrate and establish internal reference standards for daily use by academic or commercial clinical laboratories performing HEp-2-IFA, immunoprecipitation (IP) or other immunodiagnostic assays, including western blot, ELISA, CLIA, LIA and ALBIA.

Materials and methods

Patient information and reference sample preparation

AMA-REF and RR-REF sera were collected by Plasma Services Group (PSG, Huntingdon Valley, PA, USA) from single donors. The AMA-REF donor was a 65-year-old Caucasian woman diagnosed with PBC. The RR-REF donor was a 47-year-old African-American woman diagnosed with systemic lupus erythematosus, but is anti-dsDNA-negative, HCV-negative and has never received RBV or IFN. Both samples were prepared by PSG as defibrinated human plasma, undiluted without preservative.

Ethical approval

Research using only de-identified human samples in this study complies with all relevant national regulations and institutional policies. Informed consent was obtained by PSG in the collection of reference materials and is approved by appropriate institutional review boards.

HEp-2 indirect immunofluorescence assay (HEp-2-IFA)

HEp-2-IFA was performed on AMA-REF, RR-REF and positive control sera using HEp-2 cell substrate from Inova Diagnostics (San Diego, CA, USA), Bio-Rad (Hercules, CA, USA) or AESKU.Diagnostics GmbH (Wendelsheim, Germany) as previously described [28]. AMA-REF and RR-REF were confirmed by expert technicians to produce characteristic staining patterns as designated by the International Consensus on ANA Patterns (ICAP) [29]. AMA-REF and RR-REF were tested by twofold serial dilution from a starting dilution of 1:40 to at least 1:1280 and were positive at 1:1280 even when using different secondary antibodies, which included the following: Alexa Fluor 488-conjugated AffiniPure $F(ab')_2$ fragment goat anti-human IgG, Fcγ fragment specific (109-546-098, Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:400 in phosphate-buffered saline (PBS) (used in Figures 1 and 2); Alexa Fluor 488-conjugated goat anti-human IgG (A11013, Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:400 in PBS; and FITC IgG conjugate with DAPI premixed solution, undiluted (508102, Inova Diagnostics). Fluorescent images were captured with a 40× objective manually or with the NOVA View automated microscopy system (Inova Diagnostics), which produced the same results for AMA-REF and RR-REF up to 1:1280 dilution. Images shown in Figures 1 and 2 were captured on an Olympus BX53 fluorescence microscope.

Radioimmunoprecipitation assay

Antigens recognized by AMA-REF, RR-REF and positive control sera were analyzed by IP of radiolabeled K562 (human erythroleukemia) cell extract and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described [30–32]. Briefly, cells were labeled for 14 h with 4.2 mCi in 45 mL ^{35}S-L-methionine and ^{35}S-L-cysteine (NEG072, PerkinElmer, Waltham, MA, USA) and lysed in 0.5 M NaCl NET/IGEPAL CA-630 buffer (500 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl pH 7.5, 0.3% IGEPAL CA-630) containing 0.5 mM PMSF and 0.3 TIU/mL aprotinin. Cell extract was cleared by centrifugation and immunoprecipitated on Protein A Sepharose beads (17-0780-01, GE Healthcare, Marlborough, MA, USA) coated with antibodies from 8 µL of human serum. Beads were then washed with 0.5 M NaCl NET/IGEPAL CA-630 buffer. Immunoprecipitated proteins were subjected to SDS-PAGE followed by autoradiography.

AMA-REF western blotting

Twenty-five micrograms of mitochondrial extract from bovine heart tissue lysate (ab110338, Abcam, Cambridge, MA, USA) was subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane. After blocking for 1 h in 5% non-fat dry milk, the membrane was incubated with AMA-REF (diluted at 1:3000 or 1:5000) for 1 h at room temperature, followed by washing in PBS with 0.05% Tween 20 before incubation with goat $F(ab')_2$ anti-human IgG conjugated to horseradish peroxidase (HRP) (2042-05, SouthernBiotech, Birmingham, AL, USA) at 1:10,000 dilution for 1 h at room temperature. Reactivity of AMA-REF was detected by SuperSignal West Pico PLUS chemiluminescent substrate (34577, Thermo Fisher).

IP-western analysis of RR-REF

MOLT-4 (human acute lymphoblastic leukemia) cells (1×10^9) were lysed with 2 mL Buffer A (150 mM NaCl, 10 mM Tris-HCl pH 7.5, 1.5 mM $MgCl_2$, 0.5% NP-40) on ice for 15 min and centrifuged for 15 min at 4 °C to obtain cell lysate. IP was performed using 5 µL RR-REF serum, 100 µL cell lysate and 50 µL Dynabeads Protein A (10001D, Thermo Fisher) per manufacturer's instructions. Immunoprecipitated proteins were subjected to 10% SDS-PAGE followed by immunoblotting with affinity-purified rabbit polyclonal anti-IMPDH1 antibody (22092-1-AP, Proteintech, Chicago, IL, USA) at 1:1000 dilution and HRP-conjugated goat anti-rabbit IgG (4050-05, SouthernBiotech) at 1:5000 dilution.

Line immunoassay (LIA), addressable laser bead immunoassay (ALBIA) and enzyme-linked immunosorbent assay (ELISA)

Aliquots of the reference materials were tested for AMA (M2, M2-3E) and other autoimmune liver disease-related autoantibodies (sp100, LKM-1, SLA/LP, gp210, Ro52/TRIM21) by LIA (EUROLINE Autoimmune Liver Diseases Profile, Euroimmun, Lübeck, Germany) and ALBIA (Inova Diagnostics) using kits and protocols provided by the manufacturers. The cutoffs for ALBIA were set at three standard deviations above the mean of control samples (100 median fluorescence intensity, MFI). RR-REF was tested for antibodies to IMPDH2 by utilizing the full-length human protein (Abnova, Taipei City, Taiwan) covalently coupled to addressable laser beads (Luminex Corporation, Austin, TX, USA) analyzed on a Luminex 200 fluorometer as previously described [21]. ELISA for PDC-E2 was performed using an in-house protocol at Fleury Medicine and Health Laboratories.

Results

Validation of AMA-REF and RR-REF as appropriate reference materials was performed by nine laboratories affiliated with the Autoantibody Standardization Committee of the International Union of Immunological Societies (www.AutoAb.org). These nine laboratories acquired consistent results, and a consensus was reached that these are appropriate reference materials for detection of AMA and anti-RR. Data reported here are representative of all data collected by these nine laboratories.

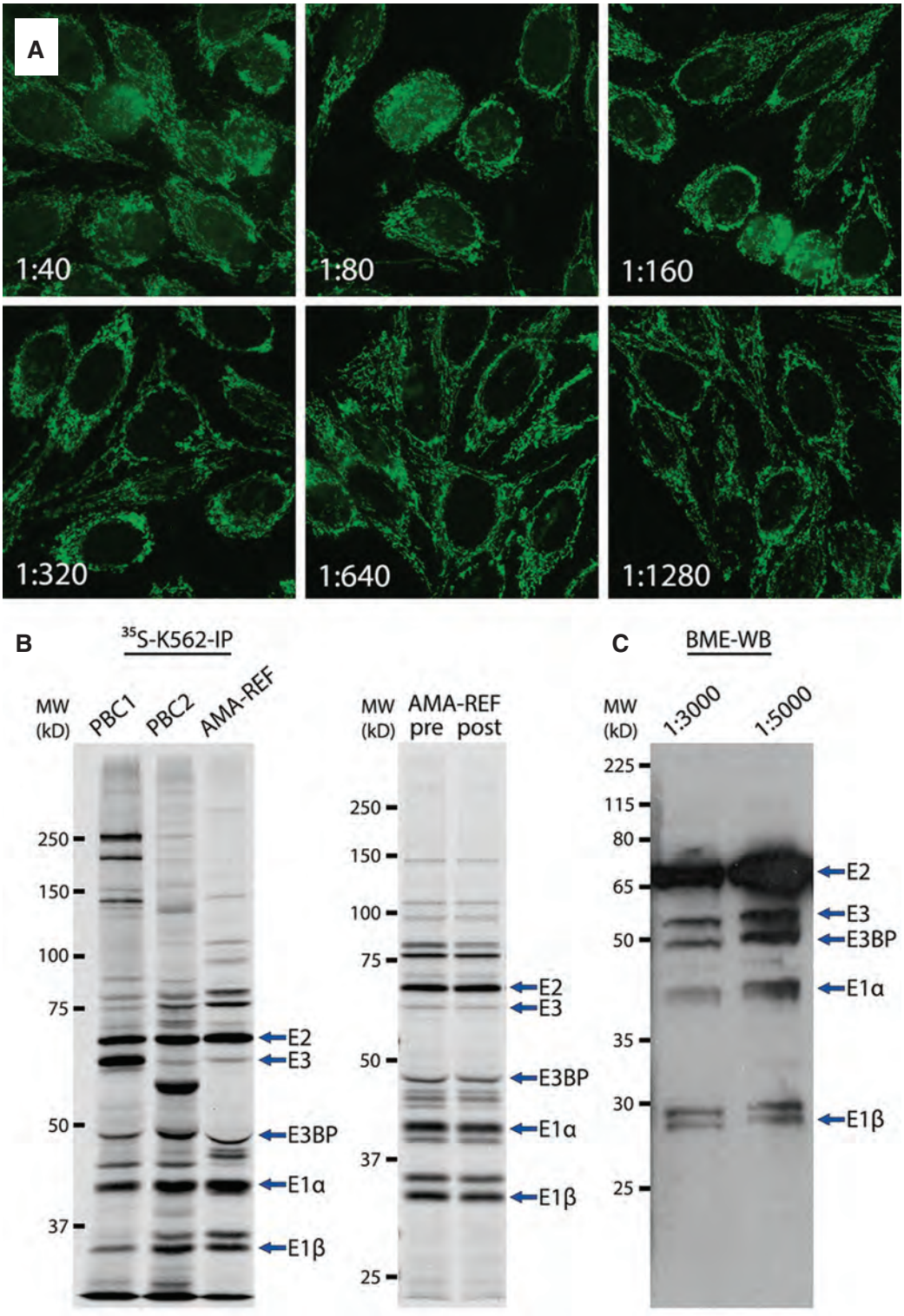

Figure 1: AMA-REF validation by HEp-2-IFA, radioimmunoprecipitation and western blotting.
(A) AMA-REF serum was serially diluted from 1:40 to 1:1280 and analyzed by HEp-2-IFA, which showed a coarse granular filamentous staining pattern in the cytoplasm (ICAP AC-21) typical of known AMA sera. (B) Left panel: IP using ^{35}S-methionine-labeled K562 cell extract (^{35}S-K562-IP) of AMA-REF and AMA-positive control sera PBC1 and PBC2. Right panel: ^{35}S-K562-IP of AMA-REF pre- and post-lyophilization. Immunoprecipitated proteins were subjected to SDS-PAGE in an 8.5% gel followed by autoradiography. Like PBC1 and PBC2, AMA-REF immunoprecipitated four protein subunits E2/E3, E3BP, E1α and E1β of the pyruvate dehydrogenase complex (PDC), typical of AMA sera. (C) Western blotting of bovine mitochondrial extract (BME-WB) with AMA-REF diluted to 1:3000 or 1:5000 demonstrated reactivity with the same E2/E3, E3BP, E1α and E1β PDC subunits.

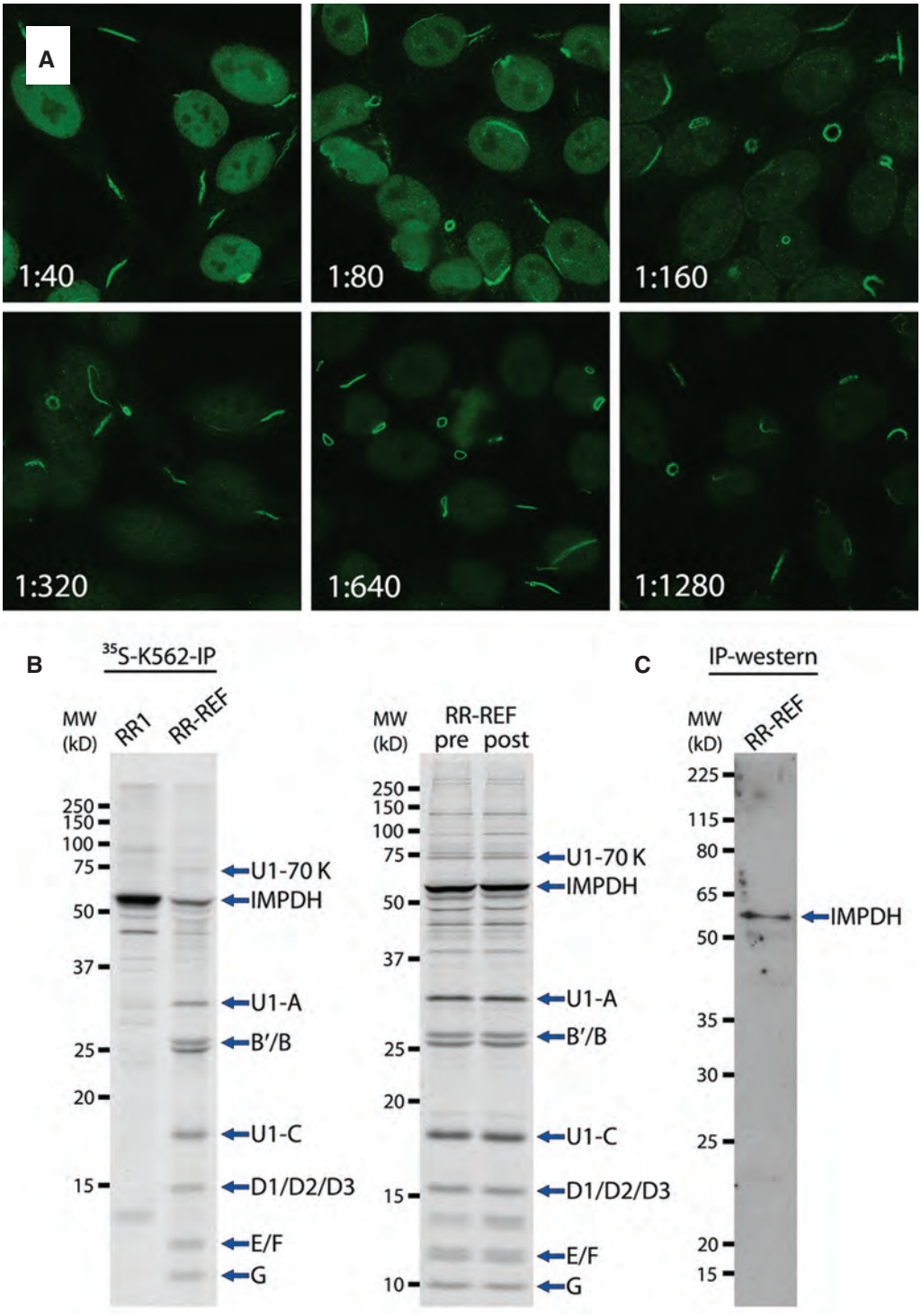

Figure 2: RR-REF validation by HEp-2-IFA, radioimmunoprecipitation and IP-western.
(A) RR-REF serum was serially diluted from 1:40 to 1:1280 and analyzed by HEp-2-IFA, which showed distinct rod- and ring-shaped structures, mainly in the cytoplasm of interphase cells, corresponding to the characteristic rods and rings staining pattern (ICAP AC-23). (B) Left panel: IP using ^{35}S-methionine-labeled K562 cell extract (^{35}S-K562-IP) of RR-REF and anti-RR-positive control serum RR1. Right panel: ^{35}S-K562-IP of RR-REF pre- and post-lyophilization. Immunoprecipitated proteins were subjected to SDS-PAGE in a 13% gel followed by autoradiography. Like RR1, RR-REF immunoprecipitated ~56 kDa inosine 5′-monophosphate dehydrogenase (IMPDH). RR-REF also immunoprecipitated proteins associated with anti-U1RNP antibody, including the U1-70K, U1-A and U1-C proteins, as well as Sm core proteins B′/B, D1/D2/D3, E, F and G. (C) IP of unlabeled MOLT-4 cell extract with RR-REF followed by western blotting (IP-western) with affinity-purified rabbit polyclonal anti-IMPDH1 antibody. A ~56-kDa band corresponding to IMPDH was visible with no other bands detected, confirming reactivity of RR-REF with IMPDH.

AMA-REF validation

AMA-REF was first validated using HEp-2-IFA by seven reference laboratories using HEp-2 cell substrate from Inova Diagnostics (San Diego, CA, USA), and all reported that AMA-REF had a strong positive cytoplasmic reticular/AMA staining pattern from 1:40 to 1:1280 dilution (Figure 1A, representative images). Although five of these laboratories tested the sample only to 1:1280, the other two laboratories reported positive AMA staining at 1:2560 and 1:5120, respectively. Additionally, none of the seven laboratories reported any other staining patterns produced by this sample. One additional reference laboratory that uses HEp-2 cell substrate from AESKU.Diagnostics GmbH (Wendelsheim, Germany) also tested AMA-REF and reported similar results. In the case of AMA, HEp-2-IFA is often performed in addition to IFA on rat (or mouse) kidney, stomach and liver tissue, the traditional method used to detect AMA [33]. In kidney/stomach/liver slides, the typical AMA pattern shows coarse granular staining in the cytoplasm of distal renal tubules, gastric parietal cells and hepatocytes. Aside from HEp-2-IFA, two laboratories also performed the kidney/stomach/liver assay, and both determined that AMA-REF was positive for this characteristic AMA pattern at 1:1280 dilution (data not shown).

In addition to IFA, one reference laboratory utilized IP to determine antigens recognized by AMA-REF. Using ^{35}S-methionine-labeled K562 cell extract, AMA-REF immunoprecipitated protein bands corresponding to the four subunits E2/E3, E3BP, E1α and E1β of PDC recognized by AMA, which were clearly detected in the AMA-positive control sera PBC1 and PBC2 (Figure 1B, left panel) [32, 34, 35]. Ceribelli et al. [32] were recently the first to describe IP band patterns for AMA sera, identical to the bands we observed for AMA-REF. After initial validation that AMA-REF produced the correct IFA pattern and recognized typical AMA antigens, PSG prepared prototype vials of lyophilized AMA-REF and distributed them to eight reference laboratories to demonstrate the lyophilization, packaging and distribution process for these sera. AMA-REF post-lyophilization was retested by IFA, and all results were consistent with our first round of testing (data not shown). Additionally, IP of AMA-REF pre- and post-lyophilization produced identical band patterns (Figure 1B, right panel). Western blotting of bovine mitochondrial extract with AMA-REF was also performed, at various dilutions ranging from 1:200 to 1:10,000. Characteristic bands associated with major PDC antigens were observed from 1:1000 to 1:5000 dilutions, with 1:3000 and 1:5000 showing the best signal-to-noise ratio (Figure 1C).

Table 1: Additional immunoassay data for AMA-REF.

Assay	Positive antigens	Negative antigens
LIA	M2-3E[a]	sp100, PML, gp210, LKM-1, LC-1, SLA/LP, SSA/Ro60, SSA/Ro52, Scl70, CENP-A, CENP-B, PGDH
ALBIA	M2-3E, sp100	LKM-1, SLA/LP, LC-1, HK, KL, gp210, VCP
ELISA	PDC-E2	

[a]3E/M2 includes PDC-E2, BCOADC-E2 and OGDC-E2 antigens.

We then analyzed AMA-REF with additional immunoassays, such as LIA, ALBIA and ELISA. Two laboratories performed LIA to determine reactivity of AMA-REF to highly purified antigens associated with autoimmune liver diseases (EUROLINE Autoimmune Liver Diseases Profile, Euroimmun, Lübeck, Germany). By LIA, AMA-REF recognized AMA-M2 and M2-3E (PDC-E2, BCOADC-E2 and OGDC-E2) antigens, but was negative for other autoantibodies associated with autoimmune liver disease, such as antibodies to sp100, PML, gp210, LKM-1, LC-1 and SLA/LP (Table 1). AMA-REF was also negative on LIA containing common autoantigens, such as Sm, Scl-70, Jo-1, Ro52 and others. When tested by ALBIA, AMA-REF had a strong positive reactivity with M2-3E (MFI: 4569) and weak positive reactivity with sp100 (MFI: 561). AMA-REF did not react with gp210, LKM-1, LC-1, SLA/LP, HK, KL and VCP antigens by ALBIA. Finally, AMA-REF was also positive by ELISA for reactivity with PDC-E2, the most common AMA antigen, with an absorbance value of 6.5 compared to a cutoff of 0.9.

RR-REF validation

RR-REF was validated first with HEp-2-IFA by six laboratories using HEp-2 substrate from Inova Diagnostics. All laboratories reported that RR-REF showed a strong positive rods and rings staining pattern from 1:40 to 1:1280 dilution (Figure 2A, representative images). Although five of these laboratories tested the sample only to 1:1280, one group reported positive rods and rings staining at 1:5120. In addition to the rods and rings pattern, RR-REF also produced the nuclear homogenous pattern (ICAP AC-1) in HEp-2 cells at 1:40 and 1:80 dilutions. The coexisting AC-1 pattern may serve as an internal positive control for detection of anti-RR, as the antigenic rods/rings structures appear in cells only under certain culture conditions [12, 16, 36, 37]. It follows that if rods/rings structures are not present in cells used as substrate, anti-RR antibodies cannot be detected. To date, in our hands, only HEp-2

Table 2: Additional immunoassay data for RR-REF.

Assay	Positive antigens	Negative antigens
LIA	Histones, nucleosomes, Sm, nRNP/Sm	DFS70
ALBIA	IMPDH2	GW182, GE-1, Ago2, LAMP2, EEA1, elastase, Jo-1, ribosome, Sm, Sm/RNP, Ro52, Ro60, SSB, PM/Scl, PCNA, CENP-B

cells from Inova Diagnostics and Euroimmun allow for consistent detection of anti-RR. Two laboratories tested RR-REF on HEp-2 cells from Bio-Rad (Hercules, CA, USA) and AESKU.Diagnostics GmbH (Wendelsheim, Germany). They also reported positive AC-1 pattern at low dilutions but were unable to detect the rods/rings pattern, indicating that these substrates are not suitable for anti-RR testing. Thus, this additional AC-1 pattern is an important aspect of the utility of RR-REF as a reference serum for detection of anti-RR.

As IMPDH is the only known antigen of anti-RR, one reference laboratory also analyzed the reactivity of RR-REF with IMPDH by IP. RR-REF immunoprecipitated a ~56-kDa protein corresponding to IMPDH (Figure 2B, left panel), similar to anti-RR-positive control serum RR1 and known anti-RR-positive sera [38]. RR-REF also immunoprecipitated proteins associated with anti-U1RNP antibody, including the U1-70K, U1-A and U1-C proteins, as well as Sm core proteins B′/B, D1/D2/D3, E, F and G [30, 39]. We then tested RR-REF pre- and post-lyophilization by IFA and IP. Both assays demonstrated that lyophilization and distribution of RR-REF did not affect its reactivity (IP shown in Figure 1B, right panel). To further confirm reactivity of RR-REF with IMPDH, we performed an IP-western blot procedure using unlabeled MOLT-4 cell lysate. Proteins immunoprecipitated by RR-REF were subjected to 10% SDS-PAGE followed by immunoblotting with purified anti-IMPDH antibody, which showed positive reactivity with a 56-kDa protein corresponding to IMPDH (Figure 2C).

RR-REF reactivity was then analyzed by LIA and ALBIA in a similar manner to AMA-REF. Although IMPDH is not an antigen included in commercial LIA assays from Euroimmun, we wanted to determine additional reactivity of this serum, considering its nuclear homogenous pattern by IFA and reactivity with anti-U1RNP proteins by IP. By LIA using the ANA Profile 3 from Euroimmun, RR-REF reacted positively with histones and nucleosomes and had a weak positive signal with Sm and nRNP/Sm antigens, and was negative for all other antigens (Table 2). By ALBIA, RR-REF reacted strongly with IMPDH2 but was negative for other antigens included in an extractable nuclear antigen panel (Jo-1, Sm, nRNP/Sm, Ro52, Ro60, SSB, Pm/Scl, PCNA, CENPB, ribosome) and cytodot panel (GW182, GE-1, Ago2, LAMP2, EEA1, elastase).

Discussion

Detection of serum autoantibodies is a crucial factor in the diagnosis of many autoimmune disorders. Clinicians rely on the accurate and timely detection and quantification of autoantibodies in patients suspected to have an autoimmune disorder. Thus, there have been widespread efforts to either standardize current detection methods or develop novel immunoassays to improve consistency in measurements of autoantibody production. Some high-throughput clinical laboratories have focused on automation to help reduce inter-laboratory variability. Several companies now offer automated HEp-2-IFA reading systems designed to reduce the subjectivity involved in analyzing certain HEp-2-IFA patterns by microscopy. In parallel, groups of experts, such as the Autoantibody Standardization Committee of the International Union of Immunological Societies (www.AutoAb.org), European Autoimmunity Standardization Initiative and ICAP (www.ANApatterns.org), focus on developing guidelines, recommendations and standard reference materials to improve interpretation of immunodiagnostic assays [29, 40–43]. The use of reference sera to properly calibrate assays can allow quantitative measurements to be compared among laboratories.

The presence of circulating AMA-M2 is one of three major criteria for diagnosis of PBC (detectable in ~95% of PBC patients) [6–8] and often precedes clinical manifestations of the disease [9–11], highlighting the importance of accurate detection of AMA. However, significant variability in test results and discrepancies in reporting among different laboratories can affect proper diagnosis. It is therefore critical that we improve the consistency and accuracy in AMA testing across laboratories globally as much as possible. Aside from its high specificity for PBC, AMA-M2 can be detected in a low percentage of autoimmune hepatitis type 1 [44] and systemic sclerosis patients [32]. Although these are often cases of overlapping PBC [45], the measurement of AMA is certainly relevant for patients suspected of having PBC and may be critical in the prediction of PBC development for patients suffering from other autoimmune manifestations. In addition, the characteristic AMA-like AC-21 pattern can be detected by HEp-2-IFA in individuals under diagnosis workout for a variety of reasons [46].

Accordingly, one of our major goals as a group working towards standardization of autoantibody testing was the establishment of a reference serum for AMA that would be readily available to any laboratory seeking to set up internal standards for AMA detection. As our data show, AMA-REF consistently met expectations as a strong positive reference material by IFA, IP, western blotting, LIA, ALBIA and ELISA. IP and western blotting also demonstrated that AMA-REF contains antibodies that recognize antigens of the mitochondrial PDC (E2/E3, E3BP, E1α and E1β) typical of known AMA sera [5, 6, 32]. Further examination by LIA and ALBIA confirmed reactivity with M2-3E antigens, which include PDC-E2, BCOADC-E2 and OGDC-E2, but ALBIA also showed weak positive reactivity with sp100, which was not observed in other assays. The multiple nuclear dots pattern (ICAP AC-6) associated with sp100 was not detected by any laboratory in HEp-2-IFA. Taken together, data from seven different methods show that AMA-REF will be particularly useful for laboratories seeking to establish internal standards for the detection of AMA in a variety of immunoassays.

Although many anti-RR-positive sera are HCV-positive, RR-REF is from an HCV-negative individual, facilitating its safe distribution to laboratories worldwide. In HEp-2-IFA, RR-REF produced the characteristic rods and rings staining pattern (ICAP AC-23), which shows distinct rod- and ring-shaped structures mainly in the cytoplasm of interphase cells [12, 18], with some structures visible in mitotic cells, and smaller structures evident in the nucleus [15]. In addition to the rods and rings pattern, RR-REF showed a nuclear homogenous pattern at 1:40 and 1:80 dilutions. Although HEp-2-IFA is the lone method capable of detecting anti-RR per se, we also evaluated RR-REF for its reactivity with IMPDH, the only known antigen of rods/rings structures. We demonstrated that RR-REF recognizes a ~56-kDa protein corresponding to IMPDH by IP, typical of many known anti-RR-positive sera [15, 38]. We further validated this IMPDH reactivity by IP-western in MOLT-4 cell extract. ALBIA for IMPDH2 also confirmed this positive reactivity, whereas LIA showed additional reactivity to histones, nucleosomes, Sm and nRNP/Sm. Altogether, these data suggest that RR-REF, especially with its internal control nuclear homogenous pattern, will be a useful tool as a reference material for anti-RR detection.

The clinical implications of anti-RR antibodies remain unclear. The rods and rings staining pattern, first described in the context of ANA patterns approximately 10 years ago [47], is considered to be quite rare. However, due to the peculiarity that only HEp-2 substrates from select sources allow for consistent detection of rods/rings structures, it is not clear exactly how rare anti-RR antibodies are. Many patient sera positive for anti-RR may have been missed over the years due to being tested on substrate where immunoreactive rods/rings structures were not present. Additionally, anti-RR positivity does not appear to be useful as a marker for any autoimmune disorder or other types of disease, as the antibody tends to appear in HCV patients only after treatment with IFN/RBV [48–50]. This interpretation is generally correct based on publications focused on anti-RR detection in HCV [13, 18–22] but seems not to be the complete story. For example, low-titer anti-RR were reported in 39 out of 4738 individuals in the National Health and Nutrition Examination Survey, and 38 of these 39 individuals had no history of HCV [24]. Climent et al. [23] also recently reported 14 non-HCV cases of anti-RR in other diseases, mainly of autoimmune origin. A third related point is that due to advancements in HCV treatment, it is likely that fewer patients will be treated with IFN/RBV in the future, thus making anti-RR even more rare. Still, many new treatment regimens continue to include RBV [25, 26] and are extremely costly, potentially necessitating the continued use of IFN/RBV in the developing world [27]. Although the mechanisms behind this autoantibody production remain unclear, we recommend continued monitoring and reporting of anti-RR rather than ignoring the unknown.

In summary, aliquots of AMA-REF and RR-REF are available for free distribution (subjected to shipping charges) to all qualified academic or commercial clinical laboratories through PSG (https://www.plasmaservicesgroup.com) as catalogue numbers IS2724 (AMA) and IS2725 (anti-RR).

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References

1. Meroni PL, Schur PH. ANA screening: an old test with new recommendations. Ann Rheum Dis 2010;69:1420–2.
2. Berg PA, Klein R. Antimitochondrial antibodies in primary biliary cirrhosis and other disorders: definition and clinical relevance. Dig Dis 1992;10:85–101.
3. Berg PA, Klein R. Mitochondrial antigen/antibody systems in primary biliary cirrhosis: revisited. Liver 1995;15:281–92.
4. Oertelt S, Rieger R, Selmi C, Invernizzi P, Ansari AA, Coppel RL, et al. A sensitive bead assay for antimitochondrial antibodies: chipping away at AMA-negative primary biliary cirrhosis. Hepatology 2007;45:659–65.
5. EASL Clinical Practice Guidelines. The diagnosis and management of patients with primary biliary cholangitis. J Hepatol 2017;67:145–72.
6. Shuai Z, Wang J, Badamagunta M, Choi J, Yang G, Zhang W, et al. The fingerprint of antimitochondrial antibodies and the etiology of primary biliary cholangitis. Hepatology 2017;65:1670–82.
7. Bowlus CL, Gershwin ME. The diagnosis of primary biliary cirrhosis. Autoimmun Rev 2014;13:441–4.
8. Kaplan MM, Gershwin ME. Primary biliary cirrhosis. N Engl J Med 2005;353:1261–73.
9. Bogdanos DP, Komorowski L. Disease-specific autoantibodies in primary biliary cirrhosis. Clin Chim Acta 2011;412:502–12.
10. Agmon-Levin N, Shapira Y, Selmi C, Barzilai O, Ram M, Szyper-Kravitz M, et al. A comprehensive evaluation of serum autoantibodies in primary biliary cirrhosis. J Autoimmun 2010;34:55–8.
11. Metcalf JV, Mitchison HC, Palmer JM, Jones DE, Bassendine MF, James OF. Natural history of early primary biliary cirrhosis. Lancet 1996;348:1399–402.
12. Carcamo WC, Satoh M, Kasahara H, Terada N, Hamazaki T, Chan JY, et al. Induction of cytoplasmic rods and rings structures by inhibition of the CTP and GTP synthetic pathway in mammalian cells. PLoS One 2011;6:e29690.
13. Probst C, Radzimski C, Blocker IM, Teegen B, Bogdanos DP, Stocker W, et al. Development of a recombinant cell-based indirect immunofluorescence assay (RC-IFA) for the determination of autoantibodies against "rings and rods"-associated inosine-5'-monophosphate dehydrogenase 2 in viral hepatitis C. Clin Chim Acta 2013;418:91–6.
14. Seelig HP, Appelhans H, Bauer O, Bluthner M, Hartung K, Schranz P, et al. Autoantibodies against inosine-5'-monophosphate dehydrogenase 2--characteristics and prevalence in patients with HCV-infection. Clin Lab 2011;57:753–65.
15. Carcamo WC, Calise SJ, von Mühlen CA, Satoh M, Chan EK. Molecular cell biology and immunobiology of mammalian rod/ring structures. Int Rev Cell Mol Biol 2014;308:35–74.
16. Keppeke GD, Calise SJ, Chan EK, Andrade LE. Assembly of IMPDH2-based, CTPS-based, and mixed rod/ring structures is dependent on cell type and conditions of induction. J Genet Genom 2015;42:287–99.
17. Chang CC, Lin WC, Pai LM, Lee HS, Wu SC, Ding ST, et al. Cytoophidium assembly reflects upregulation of IMPDH activity. J Cell Sci 2015;128:3550–5.
18. Covini G, Carcamo WC, Bredi E, von Mühlen CA, Colombo M, Chan EK. Cytoplasmic rods and rings autoantibodies developed during pegylated interferon and ribavirin therapy in patients with chronic hepatitis C. Antivir Ther 2012;17:805–11.
19. Keppeke GD, Nunes E, Ferraz ML, Silva EA, Granato C, Chan EK, et al. Longitudinal study of a human drug-induced model of autoantibody to cytoplasmic rods/rings following HCV therapy with ribavirin and interferon-alpha. PLoS One 2012;7:e45392.
20. Novembrino C, Aghemo A, Ferraris Fusarini C, Maiavacca R, Matinato C, Lunghi G, et al. Interferon-ribavirin therapy induces serum antibodies determining 'rods and rings' pattern in hepatitis C patients. J Viral Hepat 2014;21:944–9.
21. Stinton LM, Myers RP, Coffin CS, Fritzler MJ. Clinical associations and potential novel antigenic targets of autoantibodies directed against rods and rings in chronic hepatitis C infection. BMC Gastroenterol 2013;13:50.
22. Calise SJ, Bizzaro N, Nguyen T, Bassetti D, Porcelli B, Almi P, et al. Anti-rods/rings autoantibody seropositivity does not affect response to telaprevir treatment for chronic hepatitis C infection. Auto Immun Highlights 2016;7:15.
23. Climent J, Morandeira F, Castellote J, Xiol J, Niubo J, Calatayud L, et al. Clinical correlates of the "rods and rings" antinuclear antibody pattern. Autoimmunity 2016;49:102–8.
24. Calise SJ, Carcamo WC, Ceribelli A, Dominguez Y, Satoh M, Chan EK. Antibodies to rods and rings. In: Meroni PL, Gershwin ME, editors. Autoantibodies, 3rd ed. San Diego, CA: Elsevier, 2014:161–8.

25. Feld JJ, Jacobson IM, Sulkowski MS, Poordad F, Tatsch F, Pawlotsky JM. Ribavirin revisited in the era of direct-acting antiviral therapy for hepatitis C virus infection. Liver Int 2017;37:5–18.
26. Falade-Nwulia O, Suarez-Cuervo C, Nelson DR, Fried MW, Segal JB, Sulkowski MS. Oral direct-acting agent therapy for hepatitis C virus infection: a systematic review. Ann Intern Med 2017;166:637–48.
27. Nelson DR, Peter J. Hepatitis C virus: how to provide the best treatment with what I have. Liver Int 2016;36(Suppl 1):58–61.
28. Buchner C, Bryant C, Eslami A, Lakos G. Anti-nuclear antibody screening using HEp-2 cells. J Vis Exp 2014;88:e51211.
29. Chan EK, Damoiseaux J, Carballo OG, Conrad K, de Melo Cruvinel W, Francescantonio PL, et al. Report of the first international consensus on standardized nomenclature of antinuclear antibody HEp-2 cell patterns 2014–2015. Front Immunol 2015;6:412.
30. Satoh M, Reeves WH. Induction of lupus-associated autoantibodies in BALB/c mice by intraperitoneal injection of pristane. J Exp Med 1994;180:2341–6.
31. Satoh M, Langdon JJ, Hamilton KJ, Richards HB, Panka D, Eisenberg RA, et al. Distinctive immune response patterns of human and murine autoimmune sera to U1 small nuclear ribonucleoprotein C protein. J Clin Invest 1996;97:2619–26.
32. Ceribelli A, Isailovic N, De Santis M, Generali E, Satoh M, Selmi C. Detection of anti-mitochondrial antibodies by immunoprecipitation in patients with systemic sclerosis. J Immunol Methods 2017;452:1–5.
33. Selmi C, Ceribelli A, Gershwin ME. Chapter 57. Antimitochondrial antibodies. In: Meroni PL, Gershwin ME, editors. Autoantibodies, 3rd ed. San Diego, CA: Elsevier, 2014:485–90.
34. Yeaman SJ, Fussey SP, Danner DJ, James OF, Mutimer DJ, Bassendine MF. Primary biliary cirrhosis: identification of two major M2 mitochondrial autoantigens. Lancet 1988;1:1067–70.
35. Fregeau DR, Roche TE, Davis PA, Coppel R, Gershwin ME. Primary biliary cirrhosis. Inhibition of pyruvate dehydrogenase complex activity by autoantibodies specific for E1 alpha, a non-lipoic acid containing mitochondrial enzyme. J Immunol 1990;144:1671–6.
36. Calise SJ, Carcamo WC, Krueger C, Yin JD, Purich DL, Chan EK. Glutamine deprivation initiates reversible assembly of mammalian rods and rings. Cell Mol Life Sci 2014;71:2963–73.
37. Calise SJ, Purich DL, Nguyen T, Saleem DA, Krueger C, Yin JD, et al. 'Rod and ring' formation from IMP dehydrogenase is regulated through the one-carbon metabolic pathway. J Cell Sci 2016;129:3042–52.
38. Carcamo WC, Ceribelli A, Calise SJ, Krueger C, Liu C, Daves M, et al. Differential reactivity to IMPDH2 by anti-rods/rings autoantibodies and unresponsiveness to pegylated interferon-alpha/ribavirin therapy in US and Italian HCV patients. J Clin Immunol 2013;33:420–6.
39. Satoh M, Richards HB, Hamilton KJ, Reeves WH. Human antinuclear ribonucleoprotein antigen autoimmune sera contain a novel subset of autoantibodies that stabilizes the molecular interaction of U1RNP-C protein with the Sm core proteins. J Immunol 1997;158:5017–25.
40. Agmon-Levin N, Damoiseaux J, Kallenberg C, Sack U, Witte T, Herold M, et al. International recommendations for the assessment of autoantibodies to cellular antigens referred to as antinuclear antibodies. Ann Rheum Dis 2014;73:17–23.
41. Meroni PL, Biggioggero M, Pierangeli SS, Sheldon J, Zegers I, Borghi MO. Standardization of autoantibody testing: a paradigm for serology in rheumatic diseases. Nat Rev Rheumatol 2014;10:35–43.
42. Chan EK, Fritzler MJ, Wiik A, Andrade LE, Reeves WH, Tincani A, et al. AutoAbSC.Org – Autoantibody Standardization Committee in 2006. Autoimmun Rev 2007;6:577–80.
43. Shoenfeld Y, Cervera R, Haass M, Kallenberg C, Khamashta M, Meroni P, et al. EASI – The European Autoimmunity Standardisation Initiative: a new initiative that can contribute to agreed diagnostic models of diagnosing autoimmune disorders throughout Europe. Ann N Y Acad Sci 2007;1109:138–44.
44. Muratori P, Efe C, Muratori L, Ozaslan E, Schiano T, Yoshida EM, et al. Clinical implications of antimitochondrial antibody seropositivity in autoimmune hepatitis: a multicentre study. Eur J Gastroenterol Hepatol 2017;29:777–80.
45. Zheng B, Vincent C, Fritzler MJ, Senecal JL, Koenig M, Joyal F. Prevalence of systemic sclerosis in primary biliary cholangitis using the new ACR/EULAR classification criteria. J Rheumatol 2017;44:33–9.
46. Dellavance A, Cancado EL, Abrantes-Lemos CP, Harriz M, Marvulle V, Andrade LE. Humoral autoimmune response heterogeneity in the spectrum of primary biliary cirrhosis. Hepatol Int 2013;7:775–84.
47. Carcamo WC, Yao B, Satoh M, Reeves W, Liu C, Covini G, et al., editors. Cytoplasmic rings/rods as autoimmune targets of emerging human autoantibodies associated with HCV virus infection and interferon therapy. 9th Dresden Symposium on Autoantibodies. Dresden, Germany: Pabst Science Publishers, 2009.
48. Calise SJ, Keppeke GD, Andrade LE, Chan EK. Anti-rods/rings: a human model of drug-induced autoantibody generation. Front Immunol 2015;6:41.
49. Keppeke GD, Calise SJ, Chan EK, Andrade LE. Anti-rods/rings autoantibody generation in hepatitis C patients during interferon-alpha/ribavirin therapy. World J Gastroenterol 2016;22:1966–74.
50. Keppeke GD, Prado MS, Nunes E, Perazzio SF, Rodrigues SH, Ferraz ML, et al. Differential capacity of therapeutic drugs to induce rods/rings structures in vitro and in vivo and generation of anti-rods/rings autoantibodies. Clin Immunol 2016;173:149–56.

Letter to the Editor

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International Consensus on Antinuclear Antibody Patterns: defining negative results and reporting unidentified patterns

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To the Editor,

An international working committee of experts, aiming to establish a consensus on the nomenclature of staining patterns observed in the indirect immunofluorescence assay (IIFA) on HEp-2 cell substrate (HEp-2 IIFA), was initiated at a workshop designated as the International Consensus on Antinuclear Antibody Pattern (ICAP). This was hosted by the 12th International Workshop on Autoantibodies and Autoimmunity (IWAA 2014) held in Sao Paulo, Brazil, in August 2014 [1]. The resulting consensus nomenclature and representative patterns are now available online at the ICAP website (www.ANApatterns.org). Meanwhile, three consecutive productive ICAP workshops have followed, addressing additional challenges and providing responses and advances by means of systematic discussion and debate by the ICAP panel of experts. The 2nd ICAP workshop was held prior to the 12th Dresden Symposium on Autoantibodies in Dresden, Germany, in September 2015 [2, 3], the 3rd within the 13th IWAA in Kyoto, Japan, in September 2016, and the 4th and latest once again in Dresden, Germany, within the 13th Dresden Symposium on Autoantibodies.

In the original ICAP classification algorithm, 28 distinct immunofluorescence patterns recognized by HEp-2 IIFA were defined and summarized into three main categories, comprising 14 nuclear, nine cytoplasmic and five mitotic patterns, respectively. All patterns were assigned an alphanumeric AC code (anti-cellular pattern), allowing for a concise nomenclature that has been promptly adopted by industry and investigators alike. At the first ICAP meeting it was acknowledged that some patterns were yet to be included and that subsequent editions of the ICAP initiative might add new patterns to the consensus classification tree [1]. As such, a novel pattern (Topo I-like) was recently included in the category of nuclear patterns (paper submitted). In addition, we present here two other AC codes that address opposite aspects of the classification tree, i.e. the absence of HEp-2 IIFA reactivity and the occurrence of yet undefined HEp-2 IIFA pattern, respectively.

An important question was whether a negative HEp-2 IIFA sample as well as other undefined autoantibody-specific patterns should also be assigned an AC code. At the 4th ICAP meeting, consensus was reached on categorizing negative HEp-2 IIFA reactivity as AC-0, and patterns not yet defined by any of the current ICAP AC patterns as AC-XX.

The ICAP committee requested and reviewed more than 50 images representing examples of what were considered as a negative HEp-2 IIFA. A set of AC-0 images

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were defined based on consensus among ICAP members. Images rated AC-0 will be provided at the ICAP website as examples of what is considered an autoantibody negative HEp-2 IIFA, as viewed under the microscope. In the decision tree, AC-0 is placed at the same level as the primary nuclear, cytoplasmic and mitotic categories (Figure 1), to reflect that the first important decision in reading HEp-2 IIFA is to decide whether the assay is negative or positive. As a negative HEp-2 IIFA can be represented by a variety of images, it should be clear that images selected for AC-0 should not be regarded as encyclopedic, but used for comparison purposes only. The guiding feature that links these various possibilities is the absence of clear-cut staining of any given subcellular structure (Figure 2), a definition that is both subjective and semi-quantitative at best.

There should be a discussion regarding how the positive/negative cut-off for the HEp-2 IIFA is determined. As was previously suggested [4], there is general consensus that such cut-offs should be determined experimentally and locally with appropriate population controls. The cut-off is highly dependent on the HEp-2 substrate used by individual laboratories, including factors specific to HEp-2 slide manufacturers and lot-to-lot variations, as well as to peculiarities in fluorochrome-conjugated secondary antibody reagents, microscope and camera settings, serum dilutions and other variables.

It is recognized that the 28 AC patterns initially defined cannot cover HEp-2 IIFA patterns elicited by autoantibodies directed against all components of the cell. The typical autoantibody-related disease is systemic lupus erythematosus (SLE), where more than 100 autoantibodies have been described [5], including antibodies against several intracellular components. HEp-2 IIFA patterns that are not yet classified under the ICAP, including certain autoantibodies described in the literature [6–8] and others not yet identified, could be reported as AC-XX with an additional description of the staining pattern observed. This allows reporting of unusual patterns that might hint at an autoimmune disorder, keeping in mind that the pattern description has not yet been discussed or harmonized by the ICAP.

The introduction of the additional codes AC-0 and AC-XX allows for the moment the complete reporting of all possible HEp-2 IIFA AC patterns. For completion, whatever

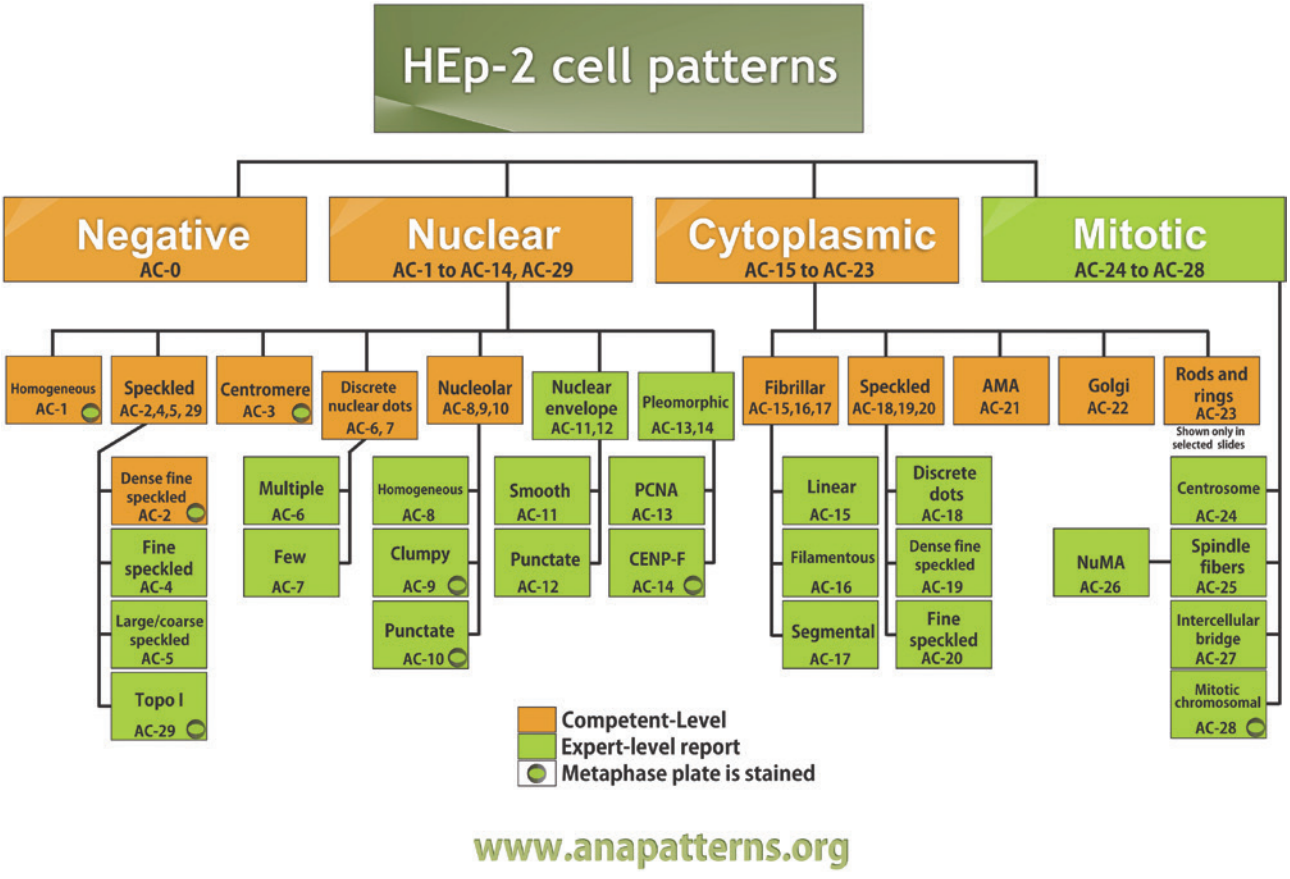

Figure 1: ICAP decision tree for HEp-2 IIFA AC patterns including AC-0.

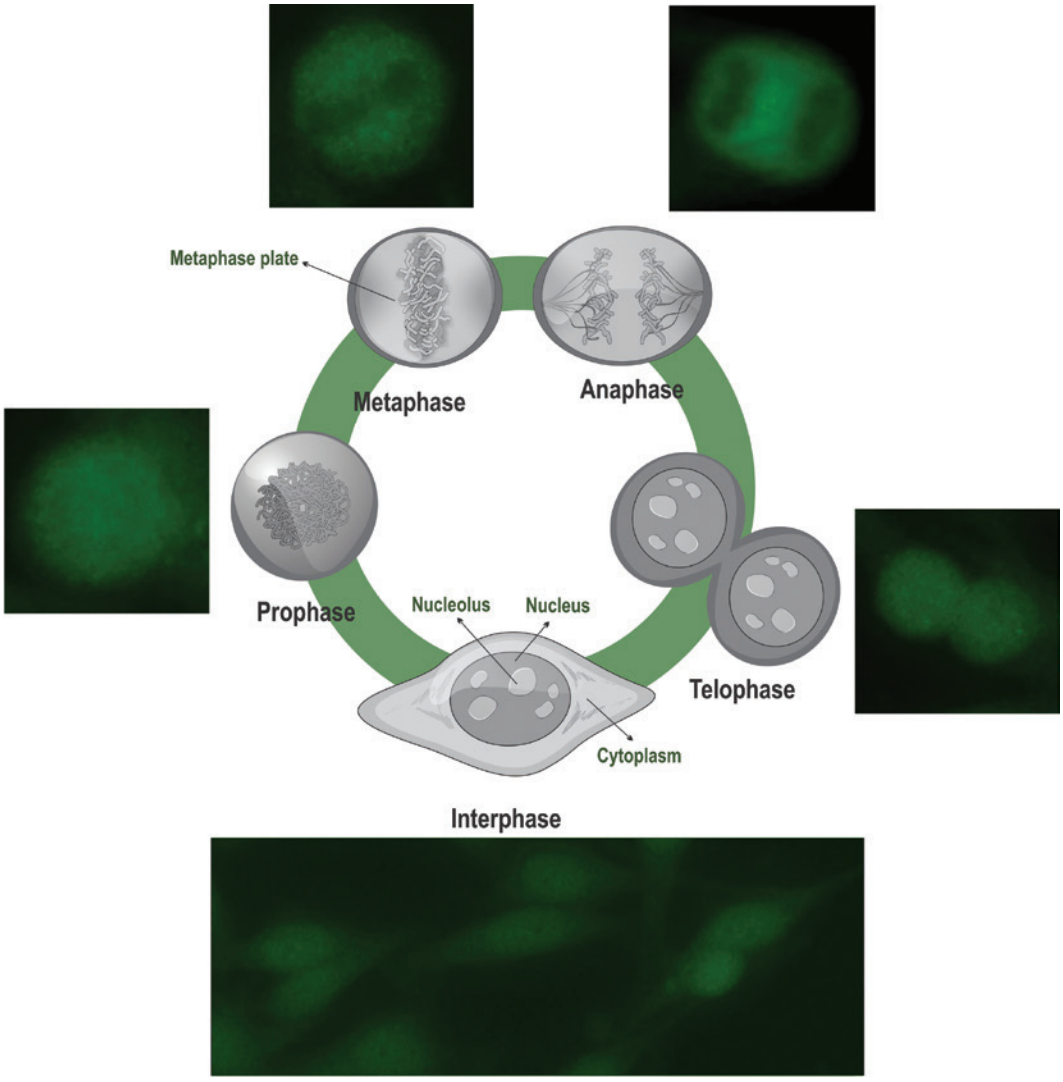

Figure 2: Examples of AC-0 seen in different phases of the cell cycle are planned to be included in the ICAP picture gallery.

is seen by analyzing the slides under a microscope, can be assigned with an AC code. AC-XX is a transitional solution that accommodates yet undefined HEp-2 IIFA patterns and does not prevent new patterns from being defined eventually.

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References

1. Chan EK, Damoiseaux J, Carballo OG, Conrad K, de Melo Cruvinel W, Francescantonio PL, et al. Report of the First International Consensus on Standardized Nomenclature of Antinuclear Antibody HEp-2 Cell Patterns 2014–2015. Front Immunol 2015;6:412.
2. Damoiseaux J, von Mühlen CA, Garcia-De La Torre I, Carballo OG, de Melo Cruvinel W, Francescantonio PL, et al. International Consensus on ANA Patterns (ICAP): the bumpy road towards a

consensus on reporting ANA results. Auto Immun Highlights 2016;7:1.
3. Chan EK, Damoiseaux J, de Melo Cruvinel W, Carballo OG, Conrad K, Francescantonio PL, et al. Report on the Second International Consensus on ANA Pattern (ICAP) Workshop in Dresden 2015. Lupus 2016;25:797–804.
4. Agmon-Levin N, Damoiseaux J, Kallenberg C, Sack U, Witte T, Herold M, et al. International recommendations for the assessment of autoantibodies to cellular antigens referred to as anti-nuclear antibodies. Ann Rheum Dis 2014;73:17–23.
5. Yaniv G, Twig G, Shor DB, Furer A, Sherer Y, Mozes O, et al. A volcanic explosion of autoantibodies in systemic lupus erythematosus: a diversity of 180 different antibodies found in SLE patients. Autoimmun Rev 2015;14:75–9.
6. Muro Y, Chan EK, Landberg G, Tan EM. A cell-cycle nuclear autoantigen containing WD-40 motifs expressed mainly in S and G2 phase cells. Biochem Biophys Res Commun 1995;207:1029–37.
7. Covini G, Chan EK, Nishioka M, Morshed SA, Reed SI, Tan EM. Immune response to cyclin B1 in hepatocellular carcinoma. Hepatology 1997;25:75–80.
8. Fritzler MJ, Kerfoot SM, Feasby TE, Zochodne DW, Westendorf JM, Dalmau JO, et al. Autoantibodies from patients with idiopathic ataxia bind to M-phase phosphoprotein-1 (MPP1). J Investig Med 2000;48:28–39.

Guidelines and Recommendation

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International consensus on antinuclear antibody patterns: definition of the AC-29 pattern associated with antibodies to DNA topoisomerase I

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Abstract: The indirect immunofluorescence assay (IFA) on HEp-2 cells is the reference method for autoantibody screening. The HEp-2 IFA pattern provides useful information on the possible autoantibodies in the sample. The International Consensus on Antinuclear Antibody Patterns (ICAP) initiative seeks to define and harmonize the nomenclature of HEp-2 IFA patterns. The most relevant and usual patterns have been assigned an alphanumeric code from anti-cell (AC)-1 to AC-28 and were organized into a classification algorithm (www.ANApatterns.org). The systemic sclerosis-associated autoantibodies to DNA topoisomerase I (Topo I) produce a peculiar composite 5-element HEp-2 IFA pattern (Topo I-like pattern) comprising the staining of the nucleus, metaphase chromatin plate, nucleolar organizing region, cytoplasm and nucleolus. In a recent assessment of the European Consensus Finding Study Group on autoantibodies, a well-defined anti-Topo I sample was blindly analyzed and classified according to ICAP AC patterns by 43 participant laboratories across Europe. There were wide variations among these laboratories in reporting nuclear, nucleolar and cytoplasmic patterns, indicating the inadequacy of the existing AC patterns to report the Topo I-like pattern. Several ICAP member laboratories independently demonstrated the overall consistency of the HEp-2 IFA Topo I-like pattern using HEp-2 slides from different manufacturers. The ICAP committee reviewed 24 candidate images and selected the four most representative images to be available on the ICAP website. The proper recognition of the AC-29 pattern should trigger suspicion of the presence of anti-Topo I antibodies, which may engender appropriate analyte-specific reflex tests to confirm the autoantibody specificity.

Keywords: antinuclear antibodies; autoantibodies; HEp-2 cell; indirect immunofluorescence.

Autoantibodies are key elements in the diagnosis of systemic autoimmune diseases [1]. The indirect immunofluorescence assay (IFA) on HEp-2 cells (HEp-2 IFA) is a well-established method for the screening of autoantibodies against a wide array of cellular autoantigens [2]. Traditionally known as the antinuclear antibody (ANA) test, HEp-2 IFA has been increasingly recognized as an anti-cell (AC) antibody screening test because in addition to detecting antibodies directed against nuclear targets, it also detects autoantibodies reactive with

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components in the cytoplasm and mitotic apparatus [3]. The semi-quantitative information on the serum level of autoantibodies (represented by the titer in regular microscopy or fluorescence intensity assessed by computer-assisted image analysis) provided by the HEp-2 IFA is augmented by very useful information on the possible molecular targets and the cognate autoantibody specificities, given that the HEp-2 IFA pattern reflects the putative topographic distribution of the target autoantigens across the various cellular domains. Because several autoantigens display peculiar cellular distribution patterns, supervised inferential analysis of HEp-2 IFA patterns allows approximation on the possible autoantibody specificities present in a given serum sample. This property has been recognized in the characterization of a series of AC autoantibodies, including those reacting with the centromere [4], proliferating cell nuclear antigen [5, 6], U3-ribonucleoprotein/fibrillarin [7, 8], CENP-F [9], Cajal bodies [10], NuMA-2/HsEg5 [11, 12], rods and rings [13] and others. Information provided by the HEp-2 IFA pattern is also regularly used in the interpretation of HEp-2 IFA results in the clinical laboratory.

In recognition of the important role of HEp-2 IFA pattern in the interpretation of the test, an international group of specialists has recently promoted an initiative dedicated to define and harmonize the nomenclature of HEp-2 IFA patterns. The International Consensus on ANA Patterns (ICAP) was launched as a workshop in conjunction with the 12th International Workshop on Autoantibodies and Autoimmunity (IWAA) in 2014, São Paulo, Brazil [14]. Three subsequent ICAP workshops were held, respectively, at the 12th Dresden Symposium on Autoantibodies (DSA) in 2015, Dresden, Germany [15, 16]; at the 13th IWAA in 2016, Kyoto, Japan; and at the 13th DSA, in 2017, Dresden, Germany. Through these meetings and related efforts, ICAP established a comprehensive classification of the most relevant and prevalent HEp-2 IFA patterns and harmonized their nomenclature (Figure 1). Each HEp-2 IFA pattern was assigned an alpha-numeric code (AC-#, for Anti-Cell). For example, the nuclear homogeneous pattern is designated AC-1 pattern and the cytoplasmic mitochondrial-like pattern is designated AC-21. ICAP originally classified the HEp-2 IFA patterns into three main groups: the nuclear group of 14 AC patterns, the cytoplasmic group of nine AC patterns and the mitotic apparatus group of five patterns. Each group is further subclassified into subgroups according to the texture of IFA staining or other common features (Figure 1) [14]. The AC pattern algorithm, representative images, detailed descriptions, historically used terminology and autoantigen associations are available in nine languages at www.ANApatterns.org.

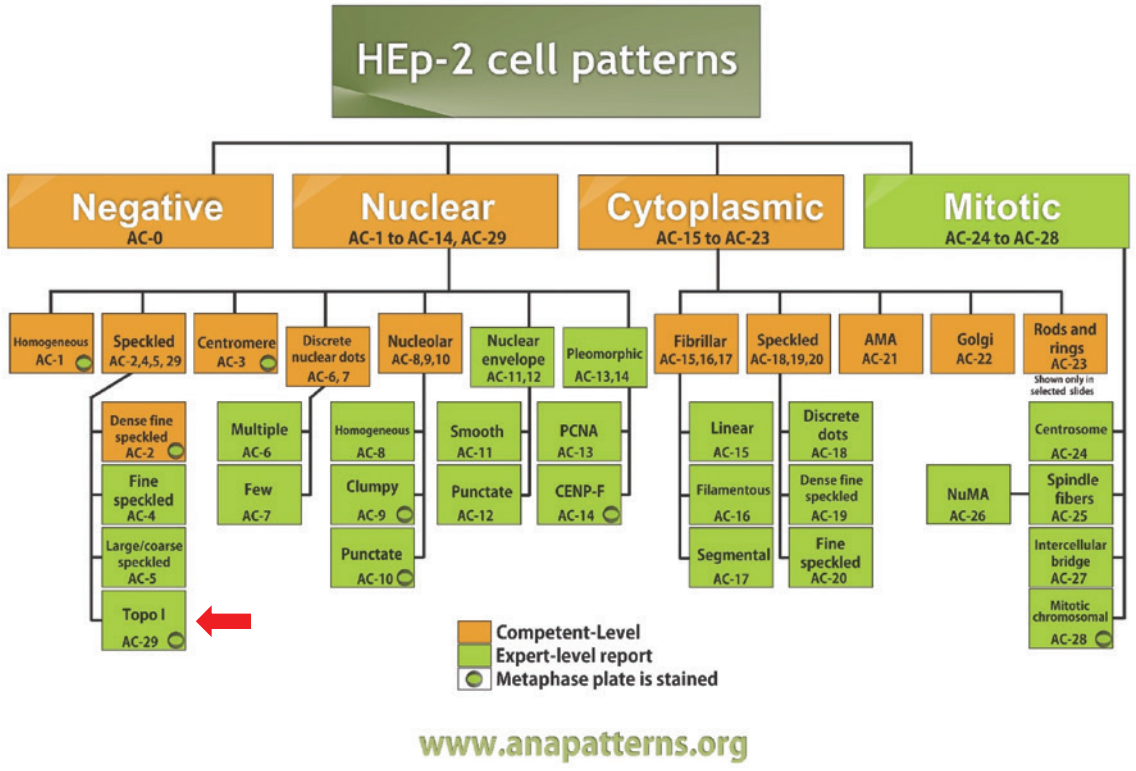

Figure 1: New ICAP decision tree for HEp-2 IFA AC patterns.
The expert-level AC-29 pattern (red arrow) belongs to the speckled pattern group in the nuclear division of the classification tree.

At the first ICAP workshop, it was acknowledged that not all known HEp-2 IFA patterns could be included and that subsequent editions of the ICAP initiative might add new patterns to the consensus classification tree [14]. At the fourth ICAP workshop, three novel AC patterns were approved after extensive exchange of images and discussion among the panel of experts. The AC-0 pattern was defined as a negative HEp-2 IFA test, and the AC-XX pattern was coined as an alternative to facilitate reporting unusual HEp-2 IFA patterns not covered by the current ICAP classification [17]. In this paper, we describe the features of the AC-29 pattern, which was recently defined and achieved consensus at the fourth ICAP as strongly associated with antibodies against the enzyme DNA topoisomerase I (Topo I).

Autoantibodies from systemic sclerosis (SSc) sera that recognized DNA Topo I were first reported as reactive to a 70 kDa "Scl-70" antigen by Dr. Eng M. Tan's laboratory in 1979 [18]. Subsequently, the Scl-70 antigen was demonstrated to be a fragment of the 100-kDa Topo I [19, 20], the enzyme responsible for cleaving, relaxing and re-annealing one of the two strands of double-stranded DNA during transcription and duplication of DNA [21]. Anti-Topo I antibody is a very specific biomarker for SSc, especially for the diffuse cutaneous form with more severe disease [22].

In 2009, Dellavance et al. [23] reported on a composite HEp-2 IFA pattern specifically associated with anti-Topo I antibodies. The so-called anti-Scl-70 (hereafter called Topo I-like) pattern was defined by the presence of five elements (Figure 2): (1) prominent nuclear compact fine speckled pattern in interphase cells; (2) consistent strong fine speckled staining of condensed chromatin in mitotic cells (depending on the serum dilution used, the mitotic chromatin staining may appear homogeneous); (3) strong staining of nucleolar organizing region (NOR) associated on condensed chromosomes in mitotic cells (this NOR staining may be obscured by the bright chromosomal staining as NOR are not always in the same focal plane); (4) delicate and weak cytoplasmic weblike staining radiating from the perinuclear area to the vicinity of plasma membrane (in general, the cytoplasmic staining becomes more prominent during titering the sample to higher dilutions); and (5) inconsistent staining of the nucleoli. One additional and subtle feature of the Topo I-like pattern is the hazy interface between the nuclear fine speckled staining and the cytoplasmic staining. In contrast to most nuclear patterns that show a sharp nuclear border, the Topo I nuclear staining has a rather blurry border in which the nuclear fine speckling overreaches the neighbor cytoplasmic region (Figure 3). The strong association of the HEp-2 IFA Topo I-like pattern with anti-Topo I antibodies was demonstrated by showing that 81 serum samples consecutively selected by presenting the Topo I-like pattern yielded a positive reactivity in specific assays to Topo I [23]. In addition, 16 samples consecutively selected by providing a positive reactivity to Topo I yielded the characteristic Topo I-like pattern [23]. The complete Topo I-like pattern was also obtained with the international anti-Topo I standard provided by the Autoantibody Standardization Committee, affiliated with the International Union of Immunology Societies [24]. Finally, two anti-Topo I affinity-purified antibody preparations reproduced the complete Topo I pattern [23].

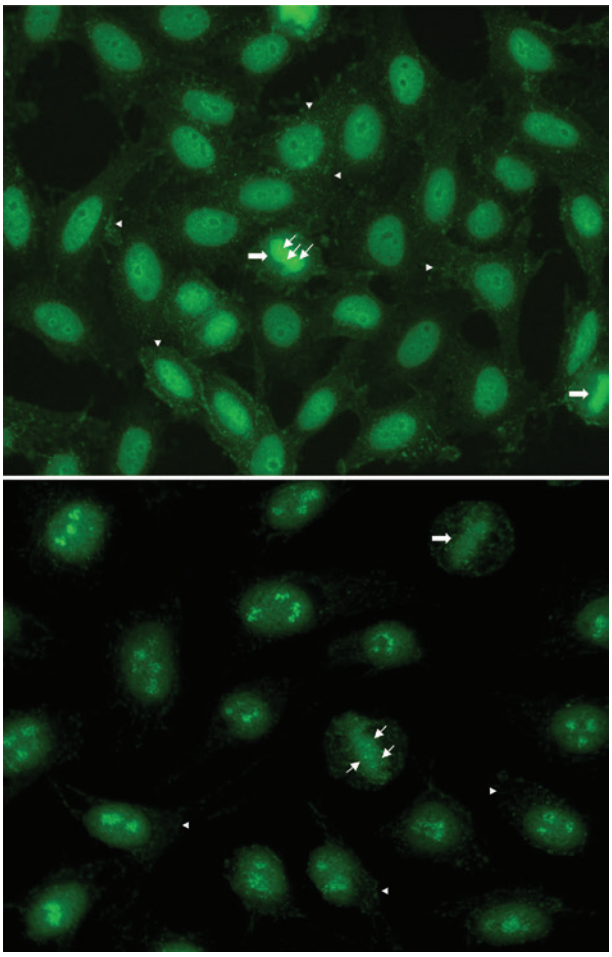

Figure 2: General characteristic features of the Topo I-like HEp-2 IFA pattern (AC-29).
Indirect immunofluorescence on HEp-2 cells with human serum with reactivity to DNA topoisomerase I diluted 1:100. Magnification ×400. Slides from Inova Diagnostics (upper panel) and MBL (lower panel). Interphase nuclei are stained with fine speckled pattern. Metaphase chromatin plate is stained with a compact fine specked pattern (large arrows) as well as with three to eight tiny discrete dots representing the nucleolar organizing regions (small arrows). Upper panel: the cytoplasm depicts a faint weblike staining pattern spanning from the nuclear periphery to the plasma membrane region (arrowheads). Lower panel: the staining of the nucleoli is variable according to the HEp-2 cell preparation.

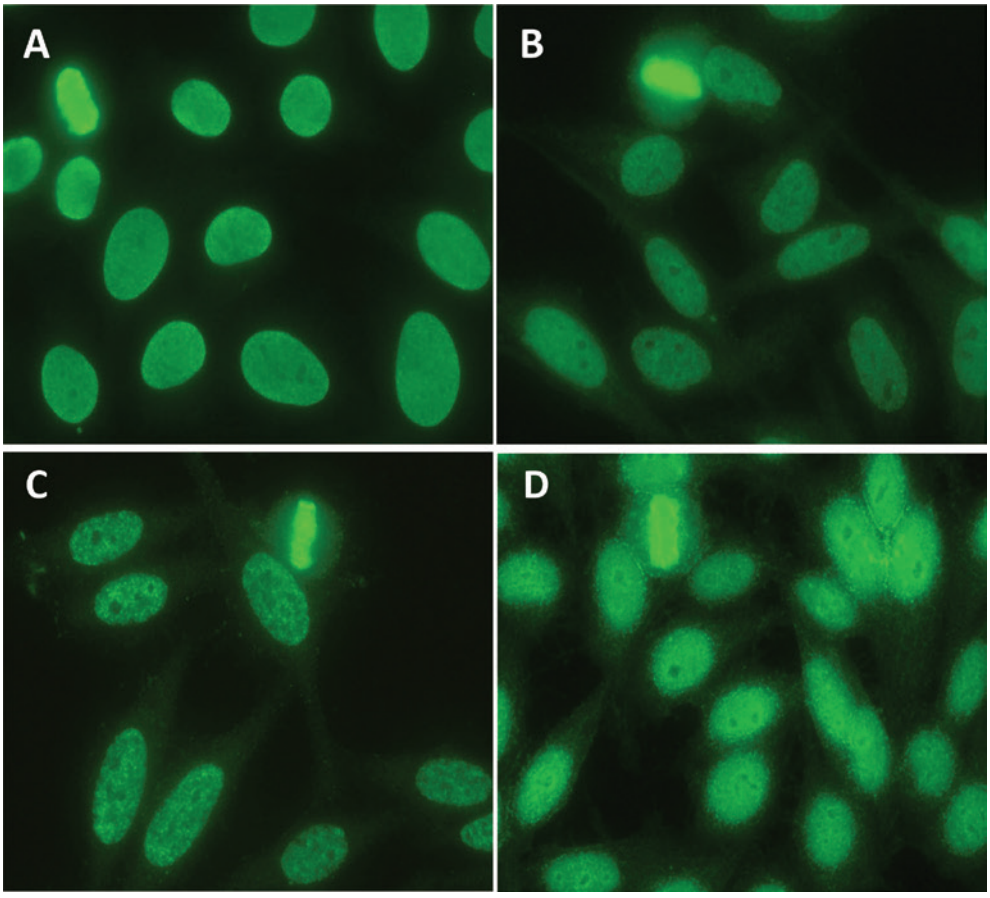

Figure 3: Peculiar aspect of the Topo I-like HEp-2 IFA pattern (AC-29): the fuzzy nuclear border.
Indirect immunofluorescence on HEp-2 cells (Inova Diagnostics) with human serum with reactivity to Topo I diluted 1:100. Magnification ×400. Nuclear borders are sharply defined in (A) nuclear homogeneous pattern (AC-1); (B) mixed nuclear pattern (AC-1+AC-4); and (C) nuclear dense fine speckled pattern (AC-2). By contrast, the AC-29 composite pattern shows ill-defined nuclear borders (D).

The incorporation of the Topo I-like pattern into the ICAP algorithm was initially proposed at the 2nd ICAP edition at the 12th DSA in 2015, Dresden, Germany [15]. The consensus at the time was that more experience with the Topo I-like pattern should be acquired, and in the meantime, experts sought to analyze the reproducibility of this pattern using various HEp-2 slide brands and particular laboratory conditions. Soon it became evident that the main features of the Topo I-like pattern could be observed independently by at least seven ICAP member laboratories who used a variety of HEp-2 slides from different manufacturers (i.e. Bion, Biorad, Bioscientifica, Inova, Euroimmun, Menarini, Immco [including DFS70 knock-out cells], MBL). The need to incorporate an AC pattern specific for the Topo I-like pattern was evidenced by the inadequacy of reports by clinical laboratories across Europe in a recent assessment of the European Consensus Finding Study Group on autoantibodies (ECFSG) (Figure 4). In this ECFSG exercise, a well-defined anti-Topo I serum was blindly analyzed by 43 participant laboratories that were asked to classify the HEp-2 IFA reactivity of the samples according to the existent ICAP classification algorithm. As shown in Figure 4, there was wide variation among the several laboratories, ranging from nuclear (AC-1, AC-2, AC-4), nucleolar (AC-8, AC-9, AC-10) and cytoplasmic (AC-15, AC-17, AC-18, AC-19, AC-23) patterns. Similar findings were obtained in a survey by the Austrian Association for Quality Assurance and Standardization of Medical Diagnostic Tests (ÖQUASTA) (Figure 4). This wide variation clearly demonstrated the inadequacy of the existing AC patterns for classification of the HEp-2 IFA pattern associated with anti-Topo I antibodies.

At the fourth ICAP meeting in 2017, there was a consensus that the Topo I-like pattern be designated as AC-29. The peculiar features that compose the Topo I-like pattern were depicted in great detail and acknowledged by most experts. The ICAP committee reviewed 24 candidate images provided by four ICAP laboratories and achieved consensus on those considered as representative of the proposed AC-29 pattern. Seven images were approved by consensus

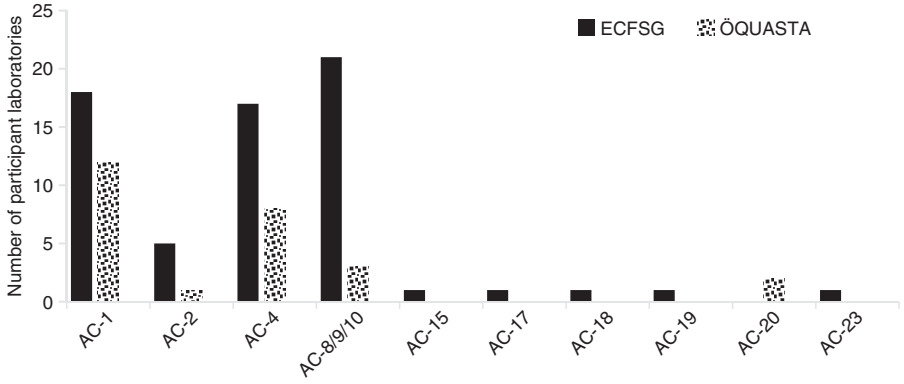

Figure 4: Previous ICAP classification tree was inadequate for reporting on HEp-2 IFA pattern for anti-DNA topoisomerase I antibodies. A blinded reference sample with anti-DNA topoisomerase I antibodies was distributed to 43 participant laboratories in the European Consensus Finding Study Group on autoantibodies (ECFSG) and 25 participant laboratories in the Austrian Association for Quality Assurance and Standardization of Medical Diagnostic Tests (ÖQUASTA). Laboratories should report according to ICAP classification (before establishment of AC-29). Reports from ECFSG laboratories ■ and ÖQUASTA laboratories ▪▪▪ show absence of consensus. AC-1, nuclear homogeneous; AC-2, nuclear dense fine speckled; AC-4, nuclear fine speckled; AC-8/9/10, nucleolar homogenous, clumply and speckled patterns, respectively; AC-15, cytoplasmic fibrillary linear; AC-17, cytoplasmic fibrillary segmental; AC-18, cytoplasmic discrete dots; AC-19, cytoplasmic dense fine speckled; AC-20, cytoplasmic fine speckled; AC-23, cytoplasmic rods and rings.

voting, and the top four are now available at www.ANApatterns.org. Due to the complexity of AC-29 pattern, not all images optimally depict all five of the AC-29 features. Thus, the NOR dots at the metaphase chromatin mass may be in different focal planes and not appear in focus in certain images; the nucleolus is inconsistently stained and may not be apparent on HEp-2 cells subjected to a variety of fixatives and other manufacturing conditions; the delicate weblike cytoplasmic staining may not be readily recognized at lower dilutions (1/80; 1/160), only to become more evident at higher dilutions (≥1/320). In the ICAP classification tree, the AC-29 pattern is arranged together with other nuclear speckled patterns (Figure 1) due to the fact that the nuclear staining is the most prominent of its five features. It is classified as an "expert level" pattern due to its complexity that requires training and expertise in the interpretation of HEp-2 IFA patterns.

Although the five-element compound AC-29 pattern has been observed in most commercial HEp-2 cell slides, there may be some variations in the expression of each element according to the slide brand. The detection of all five elements may be a challenge especially when only a single serum dilution is used (e.g. strong mitotic chromatin staining may obscure NOR) or in semi-automated systems when images are often selected from a single focal plane (e.g. NOR or cytoplasmic staining may not be in same focal plane as interphase nuclei).

There are some practical recommendations on how to routinely screen for the AC-29 pattern. If a compact fine speckled pattern is observed in interphase nuclei and at the mitotic chromatin mass, one should carefully look for positive NOR staining by searching different focal planes at the mitotic chromatin plates. Next, one should look for the presence of the weblike cytoplasmic staining, especially at higher dilutions of the sample. Lastly, the nucleolar staining should be evaluated, although in some HEp-2 slides the nucleolar staining is only visible near the edge of the wells or not visible at all.

In conclusion, the establishment and incorporation of the AC-29 pattern as a reliable representative of anti-Topo I autoantibodies enriches and refines the ICAP classification algorithm and contributes to the nomenclature harmonization and dissemination of this important HEp-2 IFA pattern. The proper recognition of the AC-29 pattern should trigger suspicion on the presence of anti-Topo I antibodies, which should engender appropriate analyte-specific reflex tests to confirm that autoantibody specificity.

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Employment or leadership: MJF was and/or continues to be a consultant to Inova Diagnostics, Werfen International, Alexion Pharmaceuticals and Bio-Rad (Hercules, CA, USA).
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References

1. Tan EM, Chan EK, Sullivan KF, Rubin RL. Antinuclear antibodies: diagnostically specific immune markers and clues to the understanding of systemic autoimmunity. Clin Immunol Immunopathol 1988;47:121–43.
2. Meroni PL, Schur PH. ANA screening: an old test with new recommendations. Ann Rheum Dis 2010;69:1420–2.
3. Agmon-Levin N, Damoiseaux J, Kallenberg C, Sack U, Witte T, Herold M, et al. International recommendations for the assessment of autoantibodies to cellular antigens referred to as antinuclear antibodies. Ann Rheum Dis 2014;73:17–23.
4. Tan EM, Rodnan GP, Garcia I, Moroi Y, Fritzler MJ, Peebles C. Anticentromere antibody and its relationship to CREST syndrome. Arthritis Rheum 1980;23:617–25.
5. Miyachi K, Fritzler MJ, Tan EM. Autoantibody to a nuclear antigen in proliferating cells. J Immunol 1978;121:2228–34.
6. Takasaki Y, Deng JS, Tan EM. A nuclear antigen associated with cell proliferation and blast transformation. J Exp Med 1981;154:1899–909.
7. Reddy R, Tan EM, Henning D, Nohga K, Busch H. Detection of a nucleolar 7-2 ribonucleoprotein and a cytoplasmic 8-2 ribonucleoprotein with autoantibodies from patients with scleroderma. J Biol Chem 1981;258:1383–6.
8. Ochs RL, Lischwe MA, Spohn WE, Busch H. Fibrillarin: a new protein of the nucleolus identified by autoimmune sera. Biol Cell 1985;54:123–33.
9. Casiano CA, Humbel RL, Peebles C, Covini G, Tan EM. Autoimmunity to the cell cycle-dependent centromere protein p330d/CENP-F in disorders associated with cell proliferation. J Autoimmun 1995;8:575–86.
10. Andrade LE, Chan EK, Raska I, Peebles CL, Roos G, Tan EM. Human autoantibody to a novel protein of the nuclear coiled body: immunological characterization and cDNA cloning of p80-coilin. J Exp Med 1991;173:1407–19.
11. Andrade LE, Chan EK, Peebles CL, Tan EM. Two major autoantigen-antibody systems of the mitotic spindle apparatus. Arthritis Rheum 1996;39:1643–53.
12. Whitehead CM, Winkfein RJ, Fritzler MJ, Rattner JB. The spindle kinesin-like protein HsEg5 is an autoantigen in systemic lupus erythematosus. Arthritis Rheum 1996;39:1635–42.
13. Covini G, Carcamo WC, Bredi E, von Mühlen CA, Colombo M, Chan EK. Cytoplasmic rods and rings autoantibodies developed during pegylated interferon and ribavirin therapy in patients with chronic hepatitis C. Antivir Ther 2012;17:805–11.
14. Chan EK, Damoiseaux J, Carballo OG, Conrad K, de Melo Cruvinel W, Francescantonio PL, et al. Report of the First International Consensus on Standardized Nomenclature of Antinuclear Antibody HEp-2 cell patterns 2014–2015. Front Immunol 2015;6:412.
15. Damoiseaux J, von Mühlen CA, Garcia-De La Torre I, Carballo OG, de Melo Cruvinel W, Francescantonio PL, et al. International consensus on ANA patterns (ICAP): the bumpy road towards a consensus on reporting ANA results. Auto Immun Highlights 2016;7:1.
16. Chan EK, Damoiseaux J, de Melo Cruvinel W, Carballo OG, Conrad K, Francescantonio PL, et al. Report on the second International Consensus on ANA Pattern (ICAP) workshop in Dresden 2015. Lupus 2016;25:797–804.
17. Herold M, Klotz W, Andrade LE, Conrad K, Cruvinel WM, Damoiseaux J, et al. International Consensus on Antinuclear Antibody Patterns: defining negative results and reporting unidentified patterns. Clin Chem Lab Med 2018;56:1799–802.
18. Douvas AS, Achten M, Tan EM. Identification of a nuclear protein (Scl-70) as a unique target of human antinuclear antibodies in scleroderma. J Biol Chem 1979;254:10514–22.
19. van Venrooij WJ, Stapel SO, Houben H, Habets WJ, Kallenberg CG, Penner E, et al. Scl-86, a marker antigen for diffuse scleroderma. J Clin Invest 1985;75:1053–60.
20. Guldner HH, Szostecki C, Vosberg HP, Lakomek HJ, Penner E, Bautz FA. Scl 70 autoantibodies from scleroderma patients recognize a 95 kDa protein identified as DNA topoisomerase I. Chromosoma 1986;94:132–8.
21. Wang JC. Cellular roles of DNA topoisomerases: a molecular perspective. Nat Rev Mol Cell Biol 2002;3:430–40.
22. Shero JH, Bordwell B, Rothfield NF, Earnshaw WC. High titers of autoantibodies to topoisomerase I (Scl-70) in sera from scleroderma patients. Science 1986;231:737–40.
23. Dellavance A, Gallindo C, Soares MG, Silva NP, Mortara RA, Andrade LE. Redefining the Scl-70 indirect immunofluorescence pattern: autoantibodies to DNA topoisomerase I yield a specific immunofluorescence pattern. Rheumatology (Oxford) 2009;48:632–8.
24. Chan EK, Fritzler MJ, Wiik A, Andrade LE, Reeves WH, Tincani A, et al. The IUIS/WHO/AF/CDC committee for the standardization of autoantibodies in rheumatic and related diseases – AutoAbSC.Org – Autoantibody Standardization Committee in 2006. Autoimmun Rev 2007;6:577–80.

Arthritis Research & Therapy

RESEARCH ARTICLE Open Access

Relevance of interferon-gamma in pathogenesis of life-threatening rapidly progressive interstitial lung disease in patients with dermatomyositis

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Abstract

Background: Dermatomyositis (DM) with rapidly progressive interstitial lung disease (DM RP-ILD) is a life-threatening condition. Serum cytokine levels are potentially suitable biomarkers for DM RP-ILD. However, the relationships among cytokine levels, lung imaging findings, and lung pathology have not been investigated. The aim of the present retrospective study was to determine the association between hypercytokinemia and lung inflammation in patients with DM RP-ILD.

Methods: The study subjects were nine patients with life-threatening DM RP-ILD and severe hypoxemia (partial arterial oxygen pressure (PaO_2)/fraction of inspired oxygen (FiO_2) ratio ≤ 200) before receiving intensive care management, who were admitted to our hospital between 2006 and 2015. The controls included 10 patients with DM without RP-ILD and 19 healthy subjects. We assessed the association between serum cytokine levels and computed tomography (CT) scores of the lung (ground glass opacity-score, G-score; fibrosis-score, F-score). Lung, hilar lymph nodes, and spleen from two autopsies were examined by hematoxylin-eosin (H&E) staining and immunostaining.

Results: Serum interferon (IFN)-γ, interleukin (IL)-1β and IL-12 levels were significantly higher in patients with DM RP-ILD than in the other two groups, whereas serum IL-6 levels were elevated in the two patient groups but not in the healthy subjects. Serum levels of IL-2, IL-4, IL-8, IL-10, IFN-α, and TNF (tumor necrosis factor)-α were not characteristically elevated in the DM RP-ILD group. Serum IFN-γ levels correlated with G-scores in patients with DM RP-ILD, while IL-1β was negatively correlation with F-scores. Immunohistochemical staining showed infiltration of numerous IFN-γ-positive histiocytes in the lung and hilar lymph nodes; but not in the spleen. Serum IL-6 levels did not correlate with the CT scores. Numerous IL-6-positive plasma cells were found in hilar lymph nodes, but not in the lungs or spleen.

Conclusions: Our results suggest strong IFN-γ-related immune reaction in the lungs and hilar lymph nodes of patients with life-threatening DM RP-ILD, and potential IFN-γ involvement in the pathogenesis of DM, specifically in the pulmonary lesions of RP-ILD.

Keywords: Rapidly progressive interstitial lung disease, Dermatomyositis, IFN-γ

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Background

The rate of interstitial lung disease (ILD) in patients with dermatomyositis (DM) is approximately 30% [1, 2]. While most patients exhibit slow progression of ILD, some exhibit rapidly progressive ILD (RP-ILD), in which the respiratory status deteriorates rapidly within 2–3 months from the onset of ILD [3–5]. In particular, a high incidence of RP-ILD has been reported in patients with clinically amyopathic dermatomyositis (cADM) who are positive for anti-melanoma differentiation-associated gene 5 (MDA5) antibodies (Abs) [6, 7]. RP-ILD in cADM is extremely difficult to treat and associated with a high mortality rate. Kameda et al. [8] reported the efficacy of intensive therapy with high-dose glucocorticoids (GC), intravenous cyclophosphamide (IVCY), and cyclosporine-A (CsA) in patients with DM complicated with RP-ILD (DM RP-ILD). Nakashima et al. [9] also reported marked improvement in prognosis of anti-MDA5 Abs-positive patients with DM using the same regimen, from the early stages of RP-ILD, with 75% survival rate by intensive immunosuppressive regimen versus only about 29% by conventional step-up therapy. Despite these encouraging reports, poor prognosis has been reported even in patients on intensive therapy, such as those with anti-MDA5 Abs-positive cADM, with a mortality rate after 6 months of treatment of as high as 25% [8]. In a retrospective analysis of 56 patients (including 49 patients with RP-ILD) treated in the intensive care unit (ICU) for exacerbation of DM/polymyositis (PM), Peng et al. [10] reported an overall survival rate of 14% (n = 8 out of 56), though the survival rate after 28 days was 0% in patients with cADM. Thus, the prognosis of anti-MDA5 Abs-positive cADM patients with RP-ILD is poor, as is the prognosis of patients with DM who develop RP-ILD during the course of treatment. Although it has been reported that treatment with tacrolimus (TAC), a calcineurin inhibitor, similar to CsA, and rituximab (RTX), is effective for life-threatening DM RP-ILD refractory to the above intensive therapy [11–13], this outcome remains to be confirmed.

Almost all anti-MDA5 Abs-positive patients have cADM with a high incidence of acute or subacute ILD [6, 14]. In a retrospective analysis of 13 patients with anti-MDA5 Abs-positive cADM, Takada et al. [15] reported that mortality was associated with high levels of anti-MDA5 Abs, suggesting that the levels of anti-MDA5 Abs could be useful in predicting prognosis. Since a strong association between DM RP-ILD and anti-MDA5 Abs has been confirmed previously in several studies, research on the pathophysiology of DM RP-ILD has been conducted mainly in anti-MDA5 Abs-positive patients [16]. High serum levels of ferritin and several types of inflammatory cytokines have been described in patients with DM RP-ILD [17–21], suggesting their involvement in the pathogenesis of RP-ILD. The pathophysiology of DM RP-ILD could be similar to that of macrophage activation syndrome (MAS), in which a variety of cytokines (e.g., interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α) are involved [22]. However, despite studies suggesting that serum cytokines levels could be useful biomarkers for monitoring disease activity and to predict the prognosis of DM RP-ILD, the associations among serum cytokine levels, pulmonary image findings (e.g., computed tomography (CT) score) and lung pathology, have not been investigated thoroughly. The present study was designed to determine the relationships among serum cytokine levels, CT scores of the lung, and the histopathologic assessment of lung tissue.

Methods

Study design and patients

This study included nine Japanese patients with DM, aged ≥ 20 years, who had life-threatening RP-ILD and were admitted to our department between 2006 and 2015 and treated at the in-patient intensive care management unit. The term RP-ILD is not well-established and is used mainly by rheumatologists but not by pneumologists. Since we understand that the lack of standardization of the term RP-ILD can cause clinical bias and confusion, we defined RP-ILD with reference to the definition of acute respiratory distress syndrome (ARDS) in this study [23, 24]. Life-threatening RP-ILD was defined based on previous reports [7, 23, 24] as "a critical condition characterized by severe hypoxemia (PaO_2/FiO_2 ratio ≤200) that progressed within 3 months before initiation of treatment or intensification". The control groups included 10 patients with DM with ILD (that did not meet the definition of RP-ILD) who underwent high-dose GC therapy (equivalent to prednisolone (PSL) of > 1 mg/kg/day) and 19 healthy individuals. Age-matched patients with DM without RP-ILD were randomly selected from the cohort of patients with DM/PM who were admitted to our department (n = 38) between 2014 and 2015. Thus, the total number of subjects in this study was 38. With regard to evaluation of serum cytokines, the major cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, interferon (IFN)-γ, IFN-α, TNF-α) involved in DM RP-ILD and MAS were selected based on the literature [17–22]. Cytokine levels were measured in all disease groups before the initiation or intensification of the treatment.

For patients with DM RP-ILD, the CT scores of the lung (ground glass opacity (GGO) score (G-score), fibrosis score (F-score)) and their association with serum cytokine levels were analyzed. This study was approved by the institutional review board of our university (#H28–033).

Diagnostic criteria

The diagnosis of DM was based on the Bohan and Peter criteria for PM/DM while that of cADM was

based on the diagnostic criteria of Euwer and Sontheimer [25–28].

Exclusion criteria

Patients with pulmonary lesions due to bacterial pneumonia, fungal pneumonia, or pneumocystis pneumonia (PCP) and those with sepsis were excluded. Bacterial pneumonia was diagnosed based on positive sputum culture and detection of bacteria phagocytosed by leukocytes. Fungal pneumonia was diagnosed based on positive sputum or bronchoalveolar lavage fluid (BALF) culture for fungi, high serum β-D-glucan levels, positivity for antigens of Candida, Aspergillus or Cryptococcus, and chest CT findings consistent with fungal pneumonia. PCP was diagnosed based on positive polymerase chain reaction (PCR) of the sputum or BALF for *Pneumocystis jirovecii*, and chest CT findings consistent with PCP. Sepsis was diagnosed based on The Third International Consensus Definitions for Sepsis and Septic Shock [29].

RP-ILD assessment by CT scores

Lung CT was evaluated semi-quantitatively using two-types of CT score; the G-score, which reflects changes in the acute and active phases, and the F-score, which reflects changes mainly in the chronic phase. Images were scored by two rheumatologists with at least 15 years of clinical experience, who were blinded to the demographic and clinical information. The left and right lung fields were divided into three regions and total of six lung zones were scored separately: upper (aortic arch zone), middle (tracheal bifurcation zone), and lower (supradiaphragmatic zone). The G-score and F-score of each zone were scored on a scale of 0–3 (maximum score = 3 points). The final CT score used for the analysis was the mean score of the six zones assessed by the two rheumatologists. The criteria used for the G-score were as follows: 1 point for predominantly subpleural partial GGO (Fig. 1A-a), 2 points for more pronounced GGO relative to that of G-score 1 point (Fig. 1A-b), and 3 points for diffuse GGO extending over a wide area (Fig. 1A-c). On the other hand, the criteria used for the F-score were as follows: 1 point for thickening and fibrosis of parts of the interlobular septa, mainly in the subpleural area (Fig. 1B-a), 2 points for more pronounced fibrosis and bronchiectasis compared with that for 1 point (Fig. 1B-b), and 3 points for diffuse and widespread fibrosis, honeycomb lung, and bronchiectasis (Fig. 1B-c) [30–32].

Endpoints and clinical assessment

The primary endpoint was elucidation of the significance of the elevated cytokines in DM RP-ILD. The secondary endpoint was the correlation between serum cytokines and CT scores in DM RP-ILD.

Measurements of serum cytokine levels

We measured the serum concentrations of various cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFN-α, IFN-γ, TNF-α) at the time of admission. Serum samples were isolated and stored at − 80 °C until analysis. The concentrations of these cytokines were measured by cytometric bead array (Becton Dickinson, Franklin Lakes, NJ, USA) using a FACSVerse flow cytometer (Becton Dickinson). Data were analyzed using the FCAP Array software (Becton Dickinson).

Immunohistochemical analysis

Immunohistochemical analysis was performed as described previously [33]. Antigen retrieval was performed by soaking the specimen on slides in 5 M sodium citrate solution in phosphate-buffered saline containing 0.05% (*v*/v) Tween 20 (PBST) (pH 6.0). The slides were subsequently blocked with serum-free protein block (Dako, 2016–08) for 30 min at room temperature and incubated at 4 °C overnight optimally with rabbit polyclonal antibodies to IFN-γ (ab25101, Abcam Inc., Boston, MA, USA) or IL-6 (21865–1-AP, Proteintech Inc., Rosemont, IL, USA) diluted 1:200 in DAKO antibody diluent. After washing three times with PBST, the slides were incubated with anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase-labeled polymer (DakoCytomation, Glostrup, Denmark) and subsequently visualized by treatment with 3,3′ diaminobenzidine (DAB) Chromogen (DakoCytomation, #K3465) according to the instructions provided by the manufacturer. Nuclei were visualized using Mayer's hematoxylin (MERCK, 1:1000 dilution in PBST). For mounting, the sections were rinsed with water, dehydrated in graded ethanol (90% ethanol for 30 s × 3 and 100% ethanol for 30 s × 3), cleared in xylene (for 30 s × 2), and sealed using multi-mount 480 (Matsunami, FM48001). Images were acquired and processed digitally.

Measurement of myositis-specific autoantibodies (MSAs)

We tested all serum samples by immunoprecipitation and enzyme-linked immunosorbent assay (ELISA) using recombinant proteins for anti-MDA5, anti-Jo-1, centromere protein A (CENP-A), CENP-B, Ro-52, and Ro-60 Abs. In addition, in patients positive for anti-aminoacyl-transfer ribonucleic acid synthetase (anti-ARS) Abs, the levels of anti-glycyl-tRNA synthetase (anti-EJ), anti-threonyl-tRNA synthetase (PL-7), anti-PL12, and anti-KS Abs were analyzed by ELISA.

Immunoprecipitation

Myositis-specific autoantibodies in serum were analyzed by immunoprecipitation of K562 cell extracts radiolabeled with ^{35}S-methionine as described previously [34]; the specificities of the autoantibodies were determined

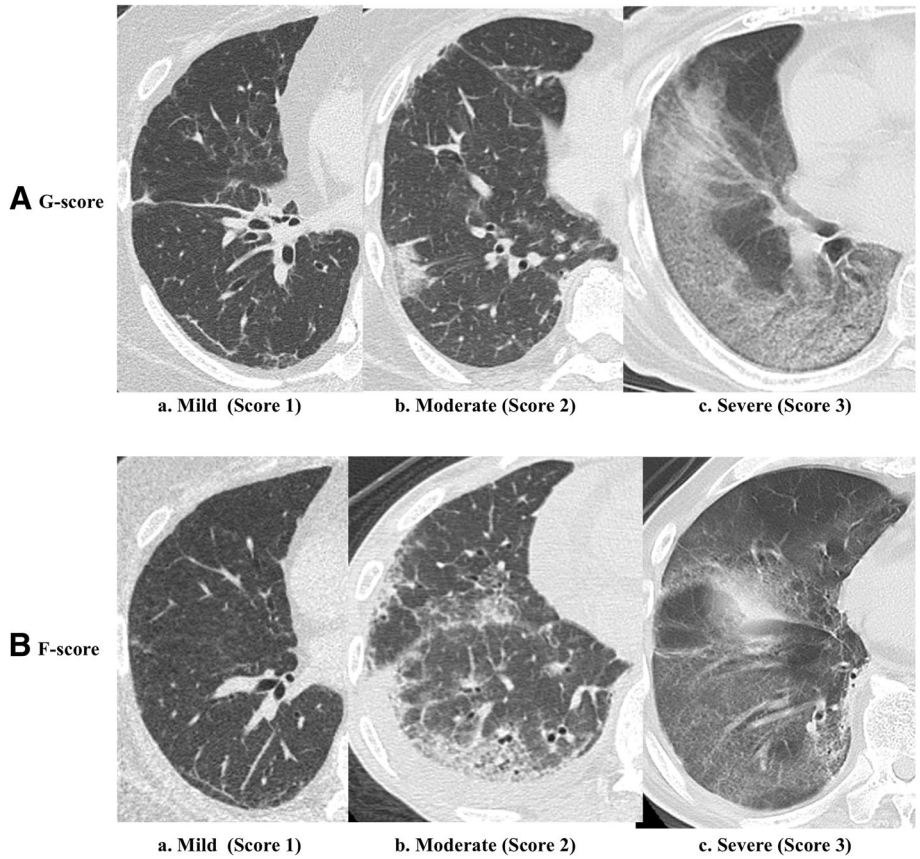

Fig. 1 Assessment of rapidly progressive interstitial lung disease (RP-ILD) by computed tomography (CT) scores. **A** Assessment of RP-ILD by CT ground glass opacity (GGO) scores (G-scores): (**a**) thin-section CT scan shows small areas with GGO compared with normal parenchyma at the right lower lobe (mild GGO = 1); (**b**) CT scan shows extensive GGO that could be easily identified when compared with the normal parenchyma at the right lower lobe (moderate GGO = 2); (**c**) thin-section CT scan shows areas with diffuse GGO at the right lower lobe (severe GGO = 3). **B** Assessment of RP-ILD by CT fibrosis scores (F-scores): (**a**) thin-section CT scan shows areas with thickened interlobular septum or predominant peripheral fibrosis (mild fibrosis = 1). (**b**) CT scan shows extensive fibrosis that could be easily identified when compared with normal parenchyma at the right lower lobe (moderate fibrosis = 2), moderate fibrosis and bronchiolectasis. (**c**) thin-section CT scan shows areas with diffuse fibrosis at the right lower lobe (severe fibrosis = 3). Note honeycombing, bronchiectasis, peribronchovascular thickening, and subpleural cysts

using specific reference serum. Analysis of RNA components of the immunoprecipitates was also performed when necessary.

Anti-MDA5 and Jo-1 ELISA

Anti-MDA5 and Jo-1 Abs were tested by enzyme-linked immunosorbent assay, using recombinant proteins (0.5 μg/ml; Diarect AG, Freiburg, Germany) and 1:250 diluted serum, as described previously [34, 35]. The optical density was measured and converted into units using a standard curve created by a prototype-positive serum. The specificity of ELISA-positive serum was confirmed by immunoprecipitation.

Statistical analysis

Continuous variables were reported as mean plus or minus standard deviation or median (interquartile range). Differences between two groups were compared using the Mann-Whitney U test. Differences among multiple groups were compared using the Kruskal-Wallis test, followed by post-hoc Dunn's multiple comparison test. Multiple group tests using the median test were also performed to determine the median values; the Wilcoxon test was used as the post-hoc test. The correlation between serum cytokine levels and CT scores was calculated using Spearman's correlation coefficient. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using the JMP version 9.0 (SAS Institute Inc., Cary, NC, USA).

Results

Demographic data of patients

The demographic data of patients in the DM RP-ILD groups are summarized in Table 1. The disease duration

Table 1 Clinical characteristics of patients

	DM with RP-ILD	DM without RP-ILD	*p* value
n	9	10	
Age, years	69.3 ± 3.9	63.9 ± 14.2	0.68
Female (*n*, %)	8, 88.9	6, 60.0	0.31
Disease duration (months)	18.1 ± 39.8	7.6 ± 8.7	0.40
Smokers (current and past) (%)	11.1	30.0	0.31
Number of GC pulses	2.2 ± 1.1	N/A	
PaO_2/FiO_2 ratio	160 ± 90	N/A	
Leukocyte count (/μL)	9438 ± 5751	7620 ± 5458	0.35
LDH (U/L)	549 ± 357	376 ± 168	0.27
KL-6 (U/mL)	1087 ± 584	1419 ± 1756	0.46
IgG (mg/dL)	1225 ± 398	1452 ± 454	0.27
Positivity for anti-CADM140/MDA5 Ab (%)	66.7	50.0	0.76
CT score (G)	2.1 ± 0.7	N/A	
CT score (F)	1.2 ± 0.6	N/A	

Data are mean ± SD or number of patients (percentage)
DM dermatomyositis, *RP-ILD* rapidly progressive-interstitial lung disease, *GC* glucocorticoid, PaO_2/FiO_2 partial arterial pressure of oxygen/fraction of inspired oxygen, *KL-6* Kerbs von Lungren 6 antigen, *CT* computed tomograpghy, *G* ground glass opacity, *F* fibrosis

of DM with and without RP-ILD was 18.1 ± 39.8 and 7.6 ± 8.7 months, respectively (Table 1). Among the 19 patients, 11 were considered to have new-onset untreated anti-MDA5 Abs-positive DM. All six anti-MDA5 antibody-positive patients with DM with RP-ILD had hypoxemia (partial arterial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FiO_2) ratio ≤ 200) before starting intensive therapy and their disease duration was 1.2 ± 0.4 months. On the other hand, none of the five anti-MDA5 antibody-positive patients with DM who were free of RP-ILD were hypoxemic before the start of treatment, and their disease duration was 5.5 ± 4.3 months.

The DM RP-ILD group included two patients positive for anti-PL-7 Ab (Additional file 1: Table S1), who developed RP-ILD during the course of remission maintenance therapy and thus, had long disease duration (35 and 120 months). One patient was treated with 7.5 mg/day PSL and the other with 3 mg/day TAC. The disease duration was long in the DM with RP-ILD group because this group not only included anti-MDA5 Abs-positive patients but also two anti-PL-7 antibody-positive patients.

High serum IFN-γ, IL-1β, and IL-12 levels in patients with DM RP-ILD

Figure 2 and Additional file 2: Table S2 compare serum cytokine levels among the DM RP-ILD, DM without RP-ILD, and HD groups, while Additional file 1: Table S1 shows antibody profiles and serum cytokine profiles in the same three groups. Serum levels of IFN-γ, IL-1β, and IL-12 were significantly higher in the DM RP-ILD group compared with the other two groups (IFN-γ, $p < 0.01$ vs DM without RP-ILD, and $p < 0.01$ vs healthy donors (HD); IL-1β, $p = 0.03$ vs DM without RP-ILD, and $p < 0.01$ vs HD; IL-12, $p < 0.01$ vs DM without RP-ILD and $p < 0.01$ vs HD). Furthermore, serum levels of IL-6, IL-10, and IFN-α were significantly higher in the DM RP-ILD group compared with the healthy donors, but were not significantly different from those in the DM without RP-ILD group. Interestingly, the serum levels of IL-2, IL-4, IL-8, and TNF-α levels were within the normal ranges in the DM RP-ILD group (Fig. 2). These results suggest that high serum levels of IFN-γ, IL-1β, and IL-12 are characteristic of DM RP-ILD. Unlike previous studies [17, 20, 21], our results showed no characteristic rises in IL-6, IL-8, IL-10, IFN-α, and TNF-α in DM RP-ILD.

Serum IFN-γ levels correlate significantly with G-score in patients with DM RP-ILD

In addition to the high serum levels of IFN-γ (Fig. 2), in the DM RP-ILD group there was positive correlation between serum IFN-γ levels and the G-scores ($\rho = 0.69$, $p = 0.04$, Table 2). Although serum IL-1β also correlated significantly with the F-scores, the correlation was negative ($\rho = -0.68$, $p = 0.045$, Table 2). None of the other cytokines were significantly correlated with the CT scores. These results demonstrate characteristically high serum IFN-γ in patients with DM RP-ILD, and significant correlation between IFN-γ and the G-score, which is a marker of the acute phase and disease activity in ILD. Moreover, the results suggest that IFN-γ plays a major role in the pathophysiology of DM RP-ILD.

Accumulation of IFN-γ-positive histiocytes in lungs and hilar lymph nodes, and IL-6-positive plasma cells in hilar lymph nodes in patients with DM RP-ILD

Serum IFN-γ was characteristically high and correlated with the G-scores in the DM RP-ILD group, whereas serum IL-6 was not elevated characteristically and did not correlate with the CT scores (Fig. 2 and Table 2), although IL-6 is reported to be important in DM RP-ILD [17, 19, 36, 37]. In the next step, we examined the roles of IFN-γ and IL-6 in the pulmonary pathophysiology of DM RP-ILD by immunostaining and hematoxylin-eosin (H&E) staining of lung tissues, hilar lymph nodes, and spleen tissues from two patients from whom specimens were obtained on autopsy (Fig. 3).

The first patient (Case 1) was a 70-year-old man with anti-MDA5 antibody-positive cADM. The patient was treated with four courses of GC pulse therapy, TAC,

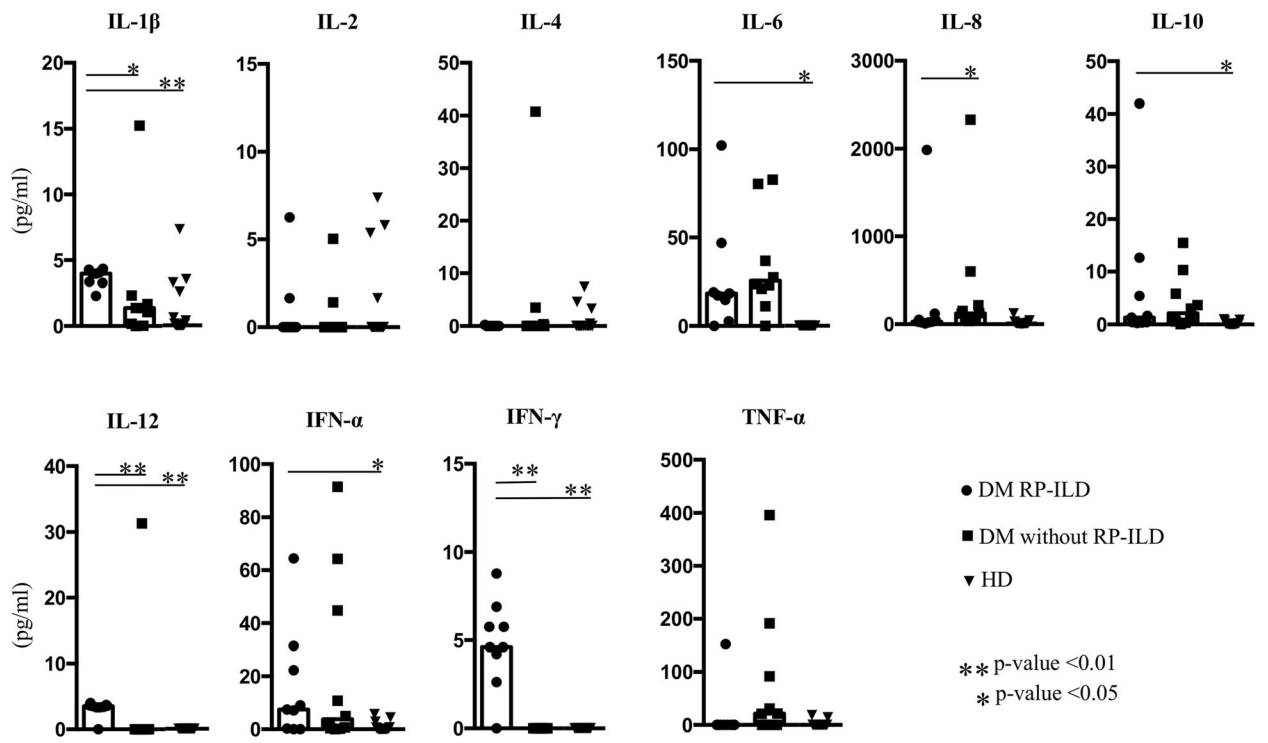

Fig. 2 Serum levels of IL-1β, IL-2, IL-4 IL-6, IL-8, IL-10, IL-12, interferon (IFN)-α, IFN-γ and TNF-α in patients with dermatomyositis (DM), patients with DM complicated with rapidly progressive interstitial lung disease (DM RP-ILD) and healthy donors (HD). Symbols represent data of individual subjects. Statistical analysis is by Kruskal-Wallis followed by Dunn's multiple comparison test

CsA, IVCY, and RTX (Fig. 3a). H&E staining showed diffuse hyaline membrane formation in the alveolar spaces. Fibroblast proliferation and incorporation of this hyaline membrane were observed in some parts, suggesting diffuse alveolar damage (DAD) extending from the exudative phase to the organizing phase (Fig. 3a (1)). Further analysis showed extravasation of erythrocytes and infiltration and aggregation of histiocytes in the alveolar spaces (Fig. 3a (2)). In these same tissues, IFN-γ-stained histiocytes had abundant cytoplasm and eccentrically distributed large nuclei (Fig. 3a (3, 4), red arrows). Marked infiltration of histiocytes was also noted into the lymph sinus of the hilar lymph nodes, with disappearance of nearly all lymphoid follicles (Fig. 3a (5, 6)). In addition to the lung tissue, IFN-γ-stained histiocytes were also found in the hilar lymph nodes (Fig. 3a (7, 8), histiocytes marked by red arrows). However, H&E staining of the spleen showed no marked histopathological changes other than splenic white pulp atrophy (Fig. 3a (9, 10)), with no infiltration of IFN-γ-positive histiocytes (Fig. 3a (11, 12)). The results of IL-6 staining are shown in Fig. 3a (13, 14, 15, 16, 17, 18). In the lung and spleen tissues, a few IL-6-positive histiocytes were observed, but cytoplasmic immunostaining was relatively weak (Fig. 3a (13, 14, 17, 18), histiocytes marked by the red arrow). Numerous IL-6-positive plasma cells were observed in hilar lymph nodes (Fig. 3a (15, 16), plasma cells marked by blue arrows). A small number of IL-6-positive histiocytes and plasma cells were also found in the spleen (Fig. 3a (17, 18), histiocytes and plasma cells are marked by the red and blue arrows).

The other autopsy specimen was from a 65-year-old woman with anti-PL-7 antibody-positive DM treated with one course of GC pulse therapy and IVCY (Fig. 3b). At the onset of RP-ILD, she was treated with 7.5 mg/day PSL, followed by a course of GC pulse therapy, TAC, and IVCY. The histopathological findings were similar to those of the first patient, despite different treatment histories and types of MSA. These results suggest that the pathophysiology of DM RP-ILD seems to be characterized by local appearance of IFN-γ-positive histiocytes in the lung tissues and related lymphoid tissues and the appearance of IL-6-positive plasma cells in hilar lymph nodes, regardless of the treatment history and type of MSA.

Discussion

The present study demonstrated the presence of characteristically high serum IFN-γ in patients with life-threatening

Table 2 Association between CT scores and cytokines in patients with DM RP-ILD

	ρ	*p* value
CT scores (F)		
IFN-γ	0.10	0.80
IL-1β	− 0.68	0.05
IL-6	0.35	0.36
IL-12	− 0.14	0.71
TNF-α	− 0.43	0.25
IL-2	− 0.17	0.67
IL-4	− 0.07	0.85
IL-8	− 0.56	0.12
IL-10	−0.49	0.18
IFN-α	− 0.15	0.70
CT scores (G)		
IFN-γ	0.69	0.04
IL-1β	0.14	0.72
IL-6	0.24	0.53
IL-12	0.10	0.80
TNF-α	− 0.21	0.59
IL-2	− 0.45	0.22
IL-4	0.07	0.86
IL-8	− 0.12	0.76
IL-10	− 0.53	0.14
IFN-α	− 0.35	0.35

DM dermatomyositis, *RP-ILD* rapidly progressive-interstitial lung disease, *CT* computed tomography, *G* ground glass opacity, *F* fibrosis, *IFN* interferon, *IL* interleukin, *TNF* tumor necrosis factor

DM RP-ILD and that such levels correlated significantly with CT scores and histopathologic findings of pulmonary lesions. While high serum IL-6 reported in previous studies was also observed in patients with DM without RP-ILD, this finding might not be a characteristic of DM RP-ILD. Our results also showed significant correlation between serum IFN-γ levels and CT scores/G-scores, and infiltration of IFN-γ-positive histiocytes into the lung and hilar lymph node tissues, but not in the spleen, in patients with high disease activity. Numerous IL-6-positive plasma cells were also observed in the hilar lymph nodes but not in the lung. In the DM RP-ILD group, serum IFN-γ was elevated even in anti-MDA5 antibody-negative cases, whereas in the DM without RP-ILD group, serum IFN-γ was not elevated even in the majority of anti-MDA5 antibody-positive cases (Additional file 1: Table S1 and Additional file 2: Table S2). These results suggest that high serum IFN-γ is associated with the onset of RP-ILD, regardless of the presence of anti-MDA5 antibodies in patients with DM. Gono et al. [19] reported that anti-MDA5 antibody-positive ILD patients with high disease activity and poor prognosis tend to have a low IL-4/IFN-γ ratio, relative to patients with anti-ARS antibody-positive DM complicated with ILD. Considered together, these findings highlight the potential role of IFN-γ in the pathophysiology of anti-MDA5 antibody-positive DM.

No common pathophysiological processes between MAS and DM RP-ILD have previously been described. MAS is a secondary hemophagocytic syndrome (HPS) or hemophagocytic lymphohistiocytosis (i.e., autoimmune-associated HPS), in which various vital organs are damaged due to abnormal production of pro-inflammatory cytokines, such as IFN-γ [38]. It has been reported that hyperferritinemia, which reflects macrophage activation, is observed in 82% of patients with MAS [39, 40]. Moreover, cytopenia and liver dysfunction were often observed in patients with MAS [39, 41]. On the other hand, serum ferritin levels correlated with disease activity in patients with anti-MDA5 antibody-positive DM complicated with RP-ILD [6], who often have liver dysfunction and cytopenia [6, 42, 43]. We identified high serum IFN-γ and the presence of IFN-γ-positive histiocytes in the lung in patients with DM RP-ILD. These results suggest that in addition to its importance in MAS, IFN-γ seems to have a pathological influence in DM RP-ILD by activating macrophages and accelerating inflammation. This is the first study to report the characteristic presence of high serum IFN-γ in DM RP-ILD and that these levels correlate with the severity of pulmonary lesions assessed by CT scores/G-scores and histopathological examination.

In DM, serum IL-6 levels correlate with disease activity [44], and the use of tocilizumab (TCZ) is effective in patients with refractory DM [45]. However, there is no information on whether TCZ is effective against DM complicated with ILD or DM RP-ILD. Our study showed that (1) serum IL-6 was not specifically high only in DM RP-ILD but also in patients with DM without RP-ILD; (2) unlike IFN-γ, high serum IL-6 did not correlate with CT scores; and (3) numerous IL-6-positive plasma cells were found in hilar lymph nodes but not in the lungs. These results suggest that while IL-6 is important in the pathogenesis of DM RP-ILD, it is unlikely to be involved in local lung injury. Although serum IL-1β levels also correlated significantly with F-scores, the correlation was negative. Correlation between serum IL-1β levels and disease activity and pulmonary lesions was examined in previous studies, but no significant correlation was detected [17]. Our results also showed no significant correlation among other cytokines and CT scores in DM RP-ILD.

We expected to find systemic autoimmune inflammation, particularly in secondary lymphoid tissues, such as the spleen, in patients with DM RP-ILD.

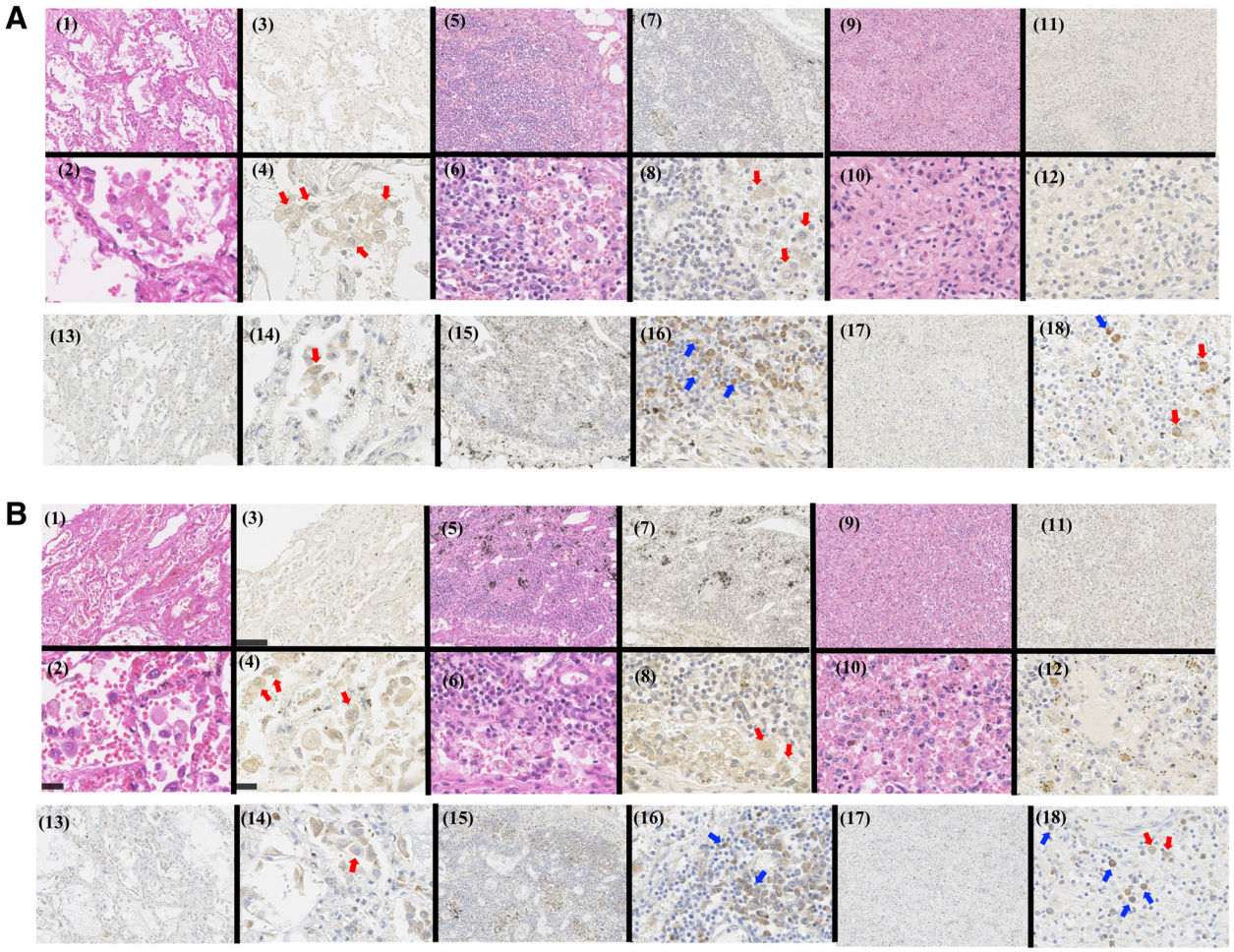

Fig. 3 Histopathological findings from two autopsies. **a** Case 1: (1, 2) lung; hematoxylin-eosin (H&E) staining; (3, 4) lung, immunostaining for interferon (IFN)-γ; (5, 6) hilar lymph nodes, H&E staining; (7, 8) hilar lymph nodes, immunostaining for IFN-γ; (9, 10) spleen, H&E staining; (11, 12) spleen immunostaining for IFN-γ; (13, 14) lung, immunostaining for interleukin (IL)-6; (15, 16) hilar lymph nodes, immunostaining for IL-6; (17, 18) spleen, immunostaining for IL-6. **b** Case 2: (1, 2) lung, H&E staining; (3, 4) lung, immunostaining for IFN-γ; (5, 6) hilar lymph nodes, H&E staining; (7, 8) hilar lymph nodes, immunostaining for IFN-γ; (9, 10) spleen, H&E staining; (11, 12) spleen, immunostaining for IFN-γ; (13, 14) lung, immunostaining for IL-6; (15, 16) hilar lymph nodes, immunostaining for IL-6; (17, 18) spleen, immunostaining for IL-6. Red arrows show histiocytes, blue arrows show plasma cells

However, contrary to our expectation, the presence of IFN-γ-positive histiocytes was limited to local regions of the lungs and pulmonary hilar lymph nodes showing diffuse DAD. Neither infiltration of IFN-γ-positive histiocytes nor histopathological changes suggestive of inflammation were observed in the spleen. Inflammation limited to localized regions of the lungs may be a significant finding that could influence the selection of the drug administration route in the future.

The present study has certain limitations. First, the number of enrolled patients was relatively small because RP-ILD is an uncommon disease. Thus, our findings need to be confirmed in larger cohort studies. Second, it is possible that immunosuppressive therapy itself altered serum cytokine levels in the present study.

Conclusions

IFN-γ was characteristically high in patients with DM RP-ILD after the onset of life-threatening RP-ILD. Furthermore, serum IFN-γ levels correlated with GGO, as evaluated by CT. Our results also suggested that inflammation might occur in localized regions of the lungs. Considered together, the results suggest the high potential of IFN-γ involvement in the pathophysiology of DM, specifically in the formation of pulmonary lesions seen in RP-ILD. Further prospective studies in large numbers of patients are needed.

Additional files

Additional file 1: Table S1 Serum levels of various cytokines, antibody profiles, and treatment at the time of registration. (DOCX 27 kb)

Additional file 2: Table S2 Median values and ranges of the measured cytokines. (DOCX 16 kb)

Abbreviations

Abs: Antibodies; ARDS: Acute respiratory distress syndrome; ARS: Aminoacyl-transfer ribonucleic acid synthetase; BALF: Bronchoalveolar lavage fluid; cADM: Clinically amyopathic dermatomyositis; CsA: Cyclosporine-A; CT: Computed tomography; DAD: Diffuse alveolar damage; DM RP-ILD: Dermatomyositis with rapidly progressive interstitial lung disease; DM: Dermatomyositis; ELISA: Enzyme-linked immunosorbent assay; FiO_2: Fraction of inspired oxygen; F-score: Fibrosis score; GC: Glucocorticoid; GGO: Ground glass opacity; G-score: Ground glass opacity score; H&E: Hematoxylin-eosin; HD: Healthy donors; ICU: Intensive care unit; IFN-α: Interferon alpha; IFN-γ: Interferon gamma; IL: Interleukin; IL-1β: Interleukin-1beta; ILD: Interstitial lung disease; IVCY: Intravenous cyclophosphamide; MAS: Macrophage activation syndrome; MDA5: Melanoma differentiation-associated gene 5; MSA: Myositis-specific antibody; PaO_2: Partial pressure of arterial oxygen; PCP: Pneumocystis pneumonia; PM: Polymyositis; PSL: Prednisolone; RNA: Ribonucleic acid; RP-ILD: Rapidly progressive interstitial lung disease; RTX: Rituximab; TAC: Tacrolimus; TNF-α: Tumor necrosis factor-alpha

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Authors' contributions

YI, SI, KH, SN, KN, and YT conceived the design of the study. YI and YT acquired the clinical data. SI, KH, and SH evaluated the CT scores. KH and SI performed statistical analyses. YI, SI, and KH interpreted the data. KS and MZ measured serum cytokines. MS tested MSA. AN and KY performed histopathological examination. YI drafted the manuscript. All authors revised the manuscript for intellectual content and approved the final version.

Ethics approval and consent to participate

Ethical approval was obtained from the University of Occupational and Environmental Health, Japan Ethics Committee. This retrospective study was approved by the institutional review board, and the requirement to obtain informed consent was waived.

Consent for publication

Not applicable.

Competing interests

Kei Sakata is an employee of Mitsubishi Tanabe Pharma. Shingo Nakayamada has received speaking fees from Bristol-Myers, Sanofi, AbbVie, Eisai, Eli Lilly, Chugai, Pfizer, Takeda (less than US $10,000 each), and research grants from Mitsubishi-Tanabe, Novartis and MSD. Dr Tanaka has received consulting fees, speaking fees, and/or honoraria from Mitsubishi Tanabe Pharma, Eisai, Pfizer, Abbott Immunology, Janssen, Takeda Industrial Pharma, Santen, AstraZeneca, Astellas, Asahi Kasei, UCB, and GlaxoSmithKline (less than US $10,000 each) and from AbbVie and Chugai (more than US $10,000 each) and research grants from Bristol-Myers Squibb, Mitsubishi Tanabe, MSD, Takeda Industrial Pharma, Astellas, Eisai, Chugai, Pfizer, and Daiichi-Sankyo. All other authors declare no conflict of interest.

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References

1. Ikezoe J, Johkoh T, Kohno N, Takeuchi N, Ichikado K, Nakamura H. High-resolution CT findings of lung disease in patients with polymyositis and dermatomyositis. J Thorac Imaging. 1996;11(4):250–9.
2. Fathi M, Vikgren J, Boijsen M, Tylen U, Jorfeldt L, Tornling G, et al. Interstitial lung disease in polymyositis and dermatomyositis: longitudinal evaluation by pulmonary function and radiology. Arthritis Rheum. 2008;59(5):677–85.
3. Fathi M, Lundberg IE. Interstitial lung disease in polymyositis and dermatomyositis. Curr Opin Rheumatol. 2005;17(6):701–6.
4. Bouros D, Nicholson AC, Polychronopoulos V, du Bois RM. Acute interstitial pneumonia. Eur Respir J. 2000;15(2):412–8.
5. Al-Hameed FM, Sharma S. Outcome of patients admitted to the intensive care unit for acute exacerbation of idiopathic pulmonary fibrosis. Can Respir J. 2004;11(2):117–22.
6. Nakashima R, Imura Y, Kobayashi S, Yukawa N, Yoshifuji H, Nojima T, et al. The RIG-I-like receptor IFIH1/MDA5 is a dermatomyositis-specific autoantigen identified by the anti-CADM-140 antibody. Rheumatology (Oxford). 2010;49(3):433–40.
7. Sato S, Hirakata M, Kuwana M, Suwa A, Inada S, Mimori T, et al. Autoantibodies to a 140-kd polypeptide, CADM-140, in Japanese patients with clinically amyopathic dermatomyositis. Arthritis Rheum. 2005;52(5):1571–6.
8. Kameda H, Nagasawa H, Ogawa H, Sekiguchi N, Takei H, Tokuhira M, et al. Combination therapy with corticosteroids, cyclosporin A, and intravenous pulse cyclophosphamide for acute/subacute interstitial pneumonia in patients with dermatomyositis. J Rheumatol. 2005;32(9):1719–26.
9. Nakashima R, Hosono Y, Mimori T. Clinical significance and new detection system of autoantibodies in myositis with interstitial lung disease. Lupus. 2016;25(8):925–33.
10. Peng JM, Du B, Wang Q, Weng L, Hu XY, Wu CY, et al. Dermatomyositis and polymyositis in the intensive care unit: a single-center retrospective cohort study of 102 patients. PLoS One. 2016;11(4):e0154441.
11. Kurita T, Yasuda S, Amengual O, Atsumi T. The efficacy of calcineurin inhibitors for the treatment of interstitial lung disease associated with polymyositis/dermatomyositis. Lupus. 2015;24(1):3–9.
12. Watanabe R, Ishii T, Araki K, Ishizuka M, Kamogawa Y, Fujita Y, et al. Successful multi-target therapy using corticosteroid, tacrolimus, cyclophosphamide, and rituximab for rapidly progressive interstitial lung disease in a patient with clinically amyopathic dermatomyositis. Mod Rheumatol. 2016;26(3):465–6.
13. Kurita T, Yasuda S, Oba K, Odani T, Kono M, Otomo K, et al. The efficacy of tacrolimus in patients with interstitial lung diseases complicated with polymyositis or dermatomyositis. Rheumatology (Oxford). 2015;54(1):39–44.
14. Sato S, Hoshino K, Satoh T, Fujita T, Kawakami Y, Kuwana M. RNA helicase encoded by melanoma differentiation-associated gene 5 is a major autoantigen in patients with clinically amyopathic dermatomyositis: association with rapidly progressive interstitial lung disease. Arthritis Rheum. 2009;60(7):2193–200.
15. Takada T, Aoki A, Asakawa K, Sakagami T, Moriyama H, Narita I, et al. Serum cytokine profiles of patients with interstitial lung disease associated with anti-CADM-140/MDA5 antibody positive amyopathic dermatomyositis. Respir Med. 2015;109(9):1174–80.

16. Abe Y, Matsushita M, Tada K, Yamaji K, Takasaki Y, Tamura N. Clinical characteristics and change in the antibody titres of patients with anti-MDA5 antibody-positive inflammatory myositis. Rheumatology (Oxford). 2017;56(9):1492–7.
17. Kawasumi H, Gono T, Kawaguchi Y, Kaneko H, Katsumata Y, Hanaoka M, et al. IL-6, IL-8, and IL-10 are associated with hyperferritinemia in rapidly progressive interstitial lung disease with polymyositis/dermatomyositis. Biomed Res Int. 2014;2014(8):15245.
18. Gono T, Sato S, Kawaguchi Y, Kuwana M, Hanaoka M, Katsumata Y, et al. Anti-MDA5 antibody, ferritin and IL-18 are useful for the evaluation of response to treatment in interstitial lung disease with anti-MDA5 antibody-positive dermatomyositis. Rheumatology (Oxford). 2012;51(9):1563–70.
19. Gono T, Kawaguchi Y, Hara M, Masuda I, Katsumata Y, Shinozaki M, et al. Increased ferritin predicts development and severity of acute interstitial lung disease as a complication of dermatomyositis. Rheumatology (Oxford). 2010;49(7):1354–60.
20. Gono T, Kaneko H, Kawaguchi Y, Hanaoka M, Kataoka S, Kuwana M, et al. Cytokine profiles in polymyositis and dermatomyositis complicated by rapidly progressive or chronic interstitial lung disease. Rheumatology (Oxford). 2014;53(12):2196–203.
21. Horai Y, Koga T, Fujikawa K, Takatani A, Nishino A, Nakashima Y, et al. Serum interferon-α is a useful biomarker in patients with anti-melanoma differentiation-associated gene 5 (MDA5) antibody-positive dermatomyositis. Mod Rheumatol. 2015;25(1):85–9.
22. Schulert GS, Grom AA. Macrophage activation syndrome and cytokine-directed therapies. Best Pract Res Clin Rheumatol. 2014;28(2):277–92.
23. Ranieri VM, Rubenfeld GD, Thompson BT, Ferguson ND, Caldwell E, Fan E, et al. Acute respiratory distress syndrome: the Berlin Definition. JAMA. 2012; 307(23):2526–33.
24. Fan E, Brodie D, Slutsky AS. Acute respiratory distress syndrome: advances in diagnosis and treatment. JAMA. 2018;319(7):698–710.
25. Bohan A, Peter JB. Polymyositis and dermatomyositis (first of two parts). N Engl J Med. 1975;292(7):344–7.
26. Bohan A, Peter JB. Polymyositis and dermatomyositis (second of two parts). N Engl J Med. 1975;292(8):403–7.
27. Euwer RL, Sontheimer RD. Amyopathic dermatomyositis: a review. J Invest Dermatol. 1993;100(1):124S–7S.
28. Sontheimer RD. Would a new name hasten the acceptance of amyopathic dermatomyositis (dermatomyositis siné myositis) as a distinctive subset within the idiopathic inflammatory dermatomyopathies spectrum of clinical illness? J Am Acad Dermatol. 2002;46(4):626–36.
29. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The third international consensus definitions for sepsis and septic shock (Sepsis-3). JAMA. 2016;315(8):801–10.
30. Brantly M, Avila NA, Shotelersuk V, Lucero C, Huizing M, Gahl WA. Pulmonary function and high-resolution CT findings in patients with an inherited form of pulmonary fibrosis, Hermansky-Pudlak syndrome, due to mutations in HPS-1. Chest. 2000;117(1):129–36.
31. Terriff BA, Kwan SY, Chan-Yeung MM, Müller NL. Fibrosing alveolitis: chest radiography and CT as predictors of clinical and functional impairment at follow-up in 26 patients. Radiology. 1992;184(2):445–9.
32. Ichikado K, Suga M, Muranaka H, Gushima Y, Miyakawa H, Tsubamoto M, et al. Prediction of prognosis for acute respiratory distress syndrome with thin-section CT: validation in 44 cases. Radiology. 2006;238(1):321–9.
33. Yamagata K, Li X, Ikegaki S, Oneyama C, Okada M, Nishita M, et al. Dissection of Wnt5a-Ror2 signaling leading to matrix metalloproteinase (MMP-13) expression. J Biol Chem. 2012;287(2):1588–99.
34. Satoh M, A C MH, EKL C. Immunodiagnosis of autoimmune myopathies. In: B D RGH, JL S, editors. Manual of molecular and clinical laboratory immunology, eighth edition. 8th ed. Washington, D. C: ASM Press; 2016. p. 878–87.
35. Ceribelli A, Fredi M, Taraborelli M, Cavazzana I, Tincani A, Selmi C, et al. Prevalence and clinical significance of anti-MDA5 antibodies in European patients with polymyositis/dermatomyositis. Clin Exp Rheumatol. 2014;32(6):891–7.
36. Nara M, Komatsuda A, Omokawa A, Togashi M, Okuyama S, Sawada K, et al. Serum interleukin 6 levels as a useful prognostic predictor of clinically amyopathic dermatomyositis with rapidly progressive interstitial lung disease. Mod Rheumatol. 2014;24(4):633–6.
37. Yasuda H, Ikeda T, Hamaguchi Y, Furukawa F. Clinically amyopathic dermatomyositis with rapidly progressive interstitial pneumonia: the relation between the disease activity and the serum interleukin-6 level. J Dermatol. 2017;44(10):1164–7.
38. Grom AA, Mellins ED. Macrophage activation syndrome: advances towards understanding pathogenesis. Curr Opin Rheumatol. 2010;22(5):561–6.
39. Kumakura S, Ishikura H, Kondo M, Murakawa Y, Masuda J, Kobayashi S. Autoimmune-associated hemophagocytic syndrome. Mod Rheumatol. 2004; 14(3):205–15.
40. Bracaglia C, de Graaf K, Pires Marafon D, Guilhot F, Ferlin W, Prencipe G, et al. Elevated circulating levels of interferon-γ and interferon-γ-induced chemokines characterise patients with macrophage activation syndrome complicating systemic juvenile idiopathic arthritis. Ann Rheum Dis. 2017; 76(1):166–72.
41. Kumakura S, Murakawa Y. Clinical characteristics and treatment outcomes of autoimmune-associated hemophagocytic syndrome in adults. Arthritis Rheumatol. 2014;66(8):2297–307.
42. Gono T, Kawaguchi Y, Ozeki E, Ota Y, Satoh T, Kuwana M, et al. Serum ferritin correlates with activity of anti-MDA5 antibody-associated acute interstitial lung disease as a complication of dermatomyositis. Mod Rheumatol. 2011;21(2):223–7.
43. Hoshino K, Muro Y, Sugiura K, Tomita Y, Nakashima R, Mimori T. Anti-MDA5 and anti-TIF1-gamma antibodies have clinical significance for patients with dermatomyositis. Rheumatology (Oxford). 2010;49(9):1726–33.
44. Bilgic H, Ytterberg SR, Amin S, McNallan KT, Wilson JC, Koeuth T, et al. Interleukin-6 and type I interferon-regulated genes and chemokines mark disease activity in dermatomyositis. Arthritis Rheum. 2009;60(11):3436–46.
45. Narazaki M, Hagihara K, Shima Y, Ogata A, Kishimoto T, Tanaka T. Therapeutic effect of tocilizumab on two patients with polymyositis. Rheumatology (Oxford). 2011;50(7):1344–6.

ウエアラブル生体センサによる自律神経バランス測定システム：その新しい技術と産業医学への応用

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1．はじめに

私達の体は、気温の変化や精神的ストレス等の刺激に対して体の機能や状態を一定に保とうとする働きがある。この働きを担うのが自律神経で、内臓や血管の収縮・弛緩や内分泌ホルモンの調節など、ほとんどすべての器官の調整を担っている[1)]。自律神経には、２つの神経つまり交感神経と副交感神経があり、私達はこの相反する２つの神経のバランスを調節しながら外界からの刺激に対応して生体の恒常性（ホメオスターシス）を維持している。

ここでは、最近開発されたウエアラブル生体センサ[注)]を用いて心電図Ｒ波のR-R間隔を測定し、その結果を自律神経における交感及び副交感神経の各々３つの合計６項目から成るレーダーチャートとして六角形の図に表示する自律神経バランス測定法を報告する。そして、それを用いた更年期障害患者における自律神経バランス研究を紹介し、さらに自律神経バランスの自動測定・解析アプリケーションソフトの開発とその産業医学への応用について述べる。

2．自律神経とは

自律神経とは、私達の体の隅々にまで神経支配が行きわたり、心臓の動きや胃腸の働き、発汗、体温調節など自分の意思とは無関係に自動（自律）的に働く神経である。この神経は大きく２つに分類され、活動・興奮時に働く交感神経とリラックス時に働く副交感神経がある。ところが、この神経は無意識下で働くため普段は目立たない存在であり、さらにその活動は精神・心理面と深く結びついているためより分かりにくいものとなっている。

2.1　戦うか逃げるか、体の準備をするのが交感神経

アメリカの著名な薬理学教科書[1)]には、「交感神経が興奮すると、心拍数の増加、血圧の上昇、瞳孔や気管支の拡張、皮膚血管の収縮、血流は皮膚や腹部から筋肉へ移動し、血糖値は上昇する。全体として、（敵に遭遇した時）戦うか逃げるか体の準備をしようとするのが交感神経の働きである」と書かれている。すなわち、相手と戦う（又は逃げる）ためには、末梢血管を収縮させて血圧を上げ、心臓を速く動かし血液を筋肉へ送る。瞳孔を拡大し視野を広げ、気管支を拡張し血糖値を上げ酸素とエネルギー源のブドウ糖を筋肉へ送り、相手に力負けしないように戦う。そう考えると私達の体は、交感神経の働きにより活動や闘争する時に非常に上手く対応していることがわかる。

2.2　リラックス・休息時の副交感神経

副交感神経が興奮すると、先程とは全く逆に心拍数の減少、血圧低下、余剰光からの目の保護（瞳孔縮小）、気管支の収縮、皮膚血管の拡張、胃腸

注）手首、耳や胸などの体に装着して生体シグナル（心電図、脈波、体温等）を測定する携帯用小型測定器

運動の亢進、消化腺分泌の亢進、排尿促進、栄養吸収の促進等が見られ全体として休息しエネルギーを貯蔵させる方向に働く[1]。

3．ストレスによる自律神経への影響

昨今の社会を取り巻く急激な変化や職場や生活での様々なストレスが、交感神経の過剰な緊張を招く。適度なストレスは生活のリズムを作るうえで大切であるが、強いストレスが長期にわたると自律神経のバランスが乱れ自律神経機能不全となり、最終的には胃潰瘍等の消化性潰瘍や心筋梗塞や脳卒中のような重大な疾患を引き起こす。

ストレスがなぜ体に悪いかと言うと、一つには強いストレスを感じると私達の体は交感神経を活発に働かせ、交感神経や副腎髄質からカテコールアミン、すなわちノルアドレナリン（NA）やアドレナリン（Ad）を大量に放出する。その結果、血圧が上昇し（高血圧）、動脈硬化や血栓が生じやすくなり心筋梗塞や脳梗塞を引き起こし、さらに免疫系は抑制され最悪の場合死に至る。ストレスや精神的な興奮は大脳皮質が感受し、大脳皮質の直下にある大脳辺縁系（情緒や感情に関与する神経）に影響する（**図1**）[2]。さらに大脳辺縁系の下には視床下部があり、ストレスが大脳皮質からストレートにここに影響する。その視床下部から自律神経は出発しており、交感神経や副腎髄質を支配している腹部交感神経も出ている。従って、強いストレスを長期間受けると、これらの神経が刺激され大量のカテコールアミンを放出し、上記のような病気を引き起こしてしまうと考えらえている。

以前より、ストレスがいろいろな病気や癌を引き起こすことが疫学研究からも強く示唆されていたが、それを科学的に証明した論文が発表された[3]。すなわちストレスを受けると副腎髄質からの大量のアドレナリンが放出され、その結果、２つの経路つまり GTP 結合蛋白質 s（Gs）/cAMP- 依存性蛋白質リン酸化酵素（PKA）系と β-arrestin1（ARRB1）系を介するシグナルを活性化し、最終的に癌抑制因子 p53 レベル等を低下させ、DNA 損傷を引き起こすことが報告された（**図2**）。

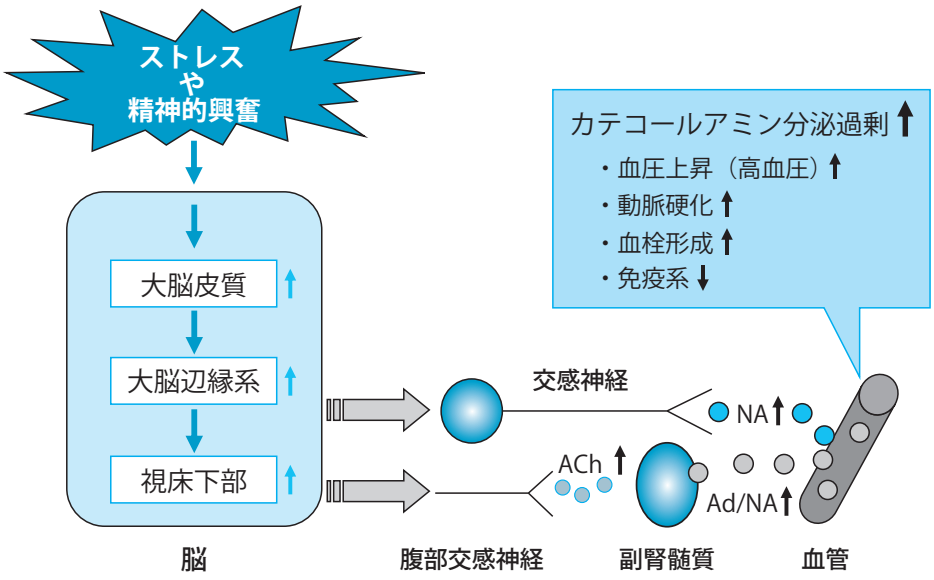

図１　ストレスによる生体カテコールアミン反応[2]（改変）

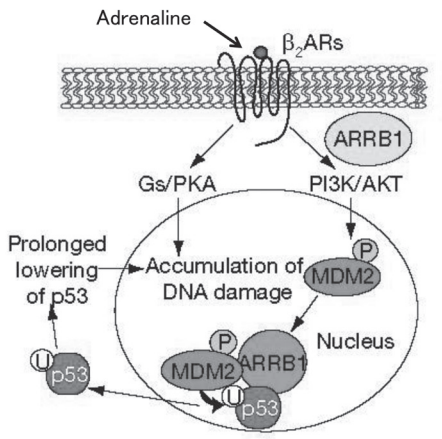

図２　ストレスによる DNA 損傷の機序[3]（改変）

４．自律神経機能の検査

現在までに多くの自律神経機能検査方法が開発され、今日でも利用されている[4]。例えば、①理学的検査として眼球圧迫試験、頸動脈洞圧迫試験、蹲踞（そんきょ）試験、呼吸性不整脈試験、皮膚紋画症検査（腕の内側刺激による皮膚反応検査）、寒冷血圧試験、皮膚毛細血管反応、体位変換試験もしくはシュロング起立試験（安静仰臥位から立位への血圧の変動測定）、②電気的検査として心拍変動検査（脈拍や心電図におけるＲ波とＲ波との間隔を測定）、立位心電図（安静仰臥位から立位への心電図の変化）、皮膚電気抵抗検査、皮膚電気反射検査やミクロビブレーション検査、③薬効的検査としてアドレナリン試験、ピロカルピン試験、アトロピン試験などの自律神経作用薬による試験等、多数ある。

今回紹介する測定法は、上記の電気的検査法の中の心拍変動検査の１つで、後藤幸生氏（福井大学名誉教授）が初めて報告した自律神経機能のレーダーチャート式バランス評価法[5]である。この方法では、まず安静仰臥位状態で 60 秒間心電図を取り、波形からＲ波の間隔（R-R 間隔）を測定する。一見、心電図上では同じようなリズムを打っているように見えるが、R-R 間隔には一定の変動があり、これを心拍変動のゆらぎと言う。健康な人であれば、誰でもこのようなゆらぎがある。安静時測定後、さらに起立して 90 秒間同じように心電図を測定する。従来の多くの自律神経測定法では安静時のみを測定し自律神経バランスを評価するが、後藤式は安静時にさらに起立負荷を加えることによって、自律神経の状態をより複雑化して評価するという方法である。R-R 間隔の経時変化を観察すると、安静時から起立時に一時的に R-R 間隔が短くなり、これを交感神経被刺激度（瞬時反応）と言う（**図３**）[6]。これはすぐに

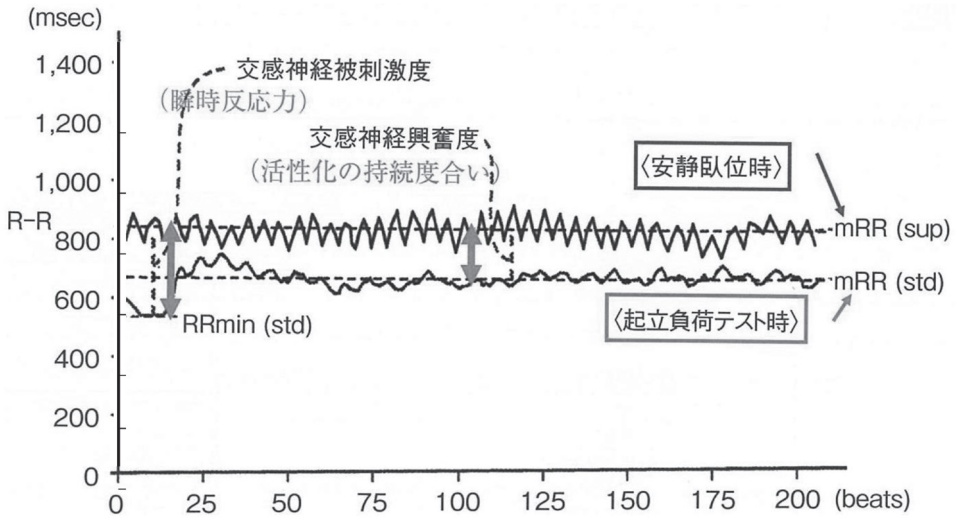

図３　安静仰臥位および立位での心電図 R-R 間隔の変化[6]

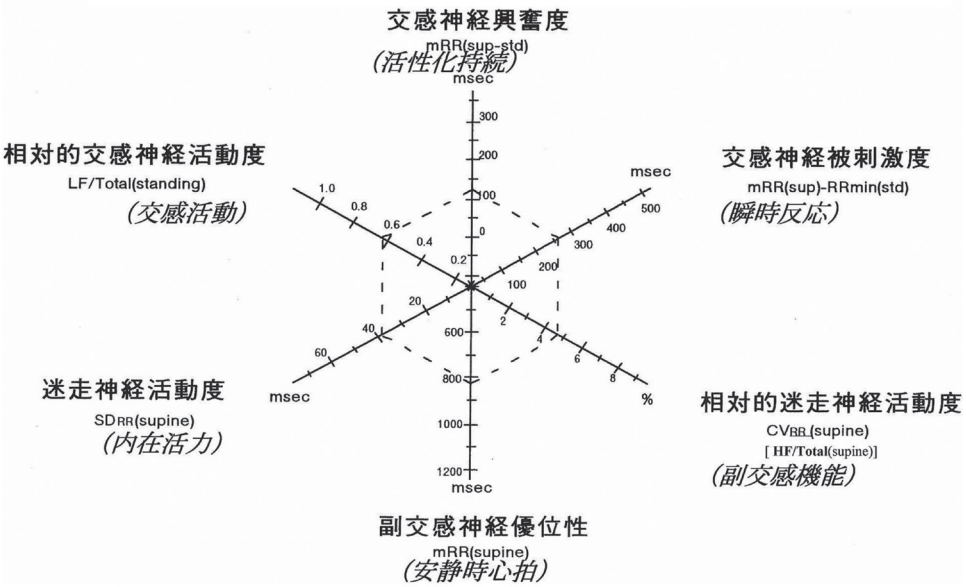

図４　自律神経バランス測定におけるレーダーチャート[6)]

元のレベル近くまで戻るが、起立した状態では安静時レベルまでは戻らない。これを交感神経興奮度（活性化持続）と言う（**図３**）。これらを六角形のレーダーチャートとして表し、上の３つが交感神経のパラメーター（交感神経活動、瞬時反応、活性化持続）と下の３つが副交感神経のパラメーター（副交感神経機能、安静時心拍、内在活力）である（**図４**）[6)]。

５．自律神経バランス測定とその結果の読み方

同じ心拍変動解析法の中でも今回用いた方法は、測定の際に仰臥位時およびそれに続く起立負荷テストとあわせ、真の自律神経活動性を一つの六角形のレーダーチャートで図示するものである。この方法は、単に交感・副交感神経活動の比だけで自律神経バランスの判断するのではなく、自律神経系の機能を６つに分け、その間での活動バランスの良否、および何らかの歪みがないかなどを一目で評価できるように作成された[5, 6)]。すなわちこの図は、６つのパラメーター（３つの交感神経活動指標と３つの副交感神経活動指標）の標準正六角形からなる基準値（約 100 人の成人平均値）のレーダーチャート上に、今測定したばかりの解析データとして自動的に重ね合わせ比較するというもので、即座に自律神経機能のバランスと歪み具合をその場で画面にて確認でき、自律神経症状を客観的なデータとして表示ができるのが特徴である。

その６つの指標（**図４**）[6)] とは、①起立負荷時の低周波 LF 領域パワー／全周波数領域パワーとの比：low frequency/total frequency ratio でもって起立負荷に際しての相対的な交感神経活動レベル、②起立負荷時の交感神経被刺激度：mRR（sup）-RRmin（std）で起立刺激に対する瞬時反応性、③交感神経興奮度：mRR（sup-std）で起立刺激による活性化持続レベル、④安静仰臥位での R-R 間隔平均値：mRR（sup）、すなわち安静時の平均心拍間隔、⑤安静仰臥位時の高周波数領域パワー HF／全周波数領域パワーの比：high frequency/total frequency ratio で相対的な副交感神経活動レベル、そして⑥安静仰臥位の R-R 間隔平均値の標準偏差：SDRR（sup）で被検者が有するその時点での内的活力（疲労、倦怠、楽しめない、不眠、内臓不調などでは低値となる）[6)] が表現され、これら

６つの指標のバランスが基準値の標準図形と比較することで、その歪み具合が評価される。

６．更年期障害患者における自律神経バランス研究

更年期障害患者ではホットフラッシュ（ほてり）、発汗、動悸、頭痛、手足のしびれなどの自律神経失調症や不眠、うつ症状などの更年期特有の症状[7,8]が見られる。しかしながら、その機序についてはまだ詳しいことは不明である。

筆者らの最近の研究[9,10]において、産業医科大学病院の産婦人科外来を受診した 40 ～ 60 歳（平均年齢 =50.1 ± 0.9）で更年期障害と診断された女性（n=40）と、コントロール群として更年期症状が認められない 40 ～ 60 歳（平均年齢 =51.5 ± 0.7）の健常者女性（n=40）の結果を比較した興味ある知見が得られた。なお、この患者群は少なくとも３カ月以上の更年期障害の症状が継続しており、専門医師が診断して甲状腺機能低下症、慢性疲労症候群、もしくはその他の病気に罹患している場合は対象者から除外している。更年期症状のある患者 40 名の簡略更年期指数（SMI：スコア 51 以上が更年期障害と診断）は 72.2 ± 2.2 であった。血中エストラジオール 17 β-E2 は 10.3 ± 1.7 pg/ml、卵胞刺激ホルモン FSH は 52.2 ± 5.4 pg/ml、黄体化ホルモン LH は 26.5 ± 2.6 pg/ml であり、更年期前女性の値（17 β-E2, 83 pg/ml; FSH, 13.5 pg/ml）[11]と比較して減増していた。また、更年期障害患者の中では、エストリオール（22 名）、ベンゾジアゼピン系睡眠薬（12 名）、漢方薬（12 名）、そして抗うつ薬（６名）が処方されていた。

更年期障害患者においては、mRR（sup）-RRmin（std）（瞬時交感神経被刺激度）と mRR（sup-std）（交感神経興奮度の持続）、さらに SDRR（sup）（R-R 間隔平均値の標準偏差）が統計学的に有意に低下していた（各々 $P<0.05$, $P<0.01$, $P<0.001$）（**図５**）[9]。このことは自律神経活動の反応性が鈍っていることを示唆している。次に、SDRR（sup）と更年期障害重症度を示す簡略更年期指数（SMI）[7]の相関性について検討した。その結果、両者には有意に逆相関性があ

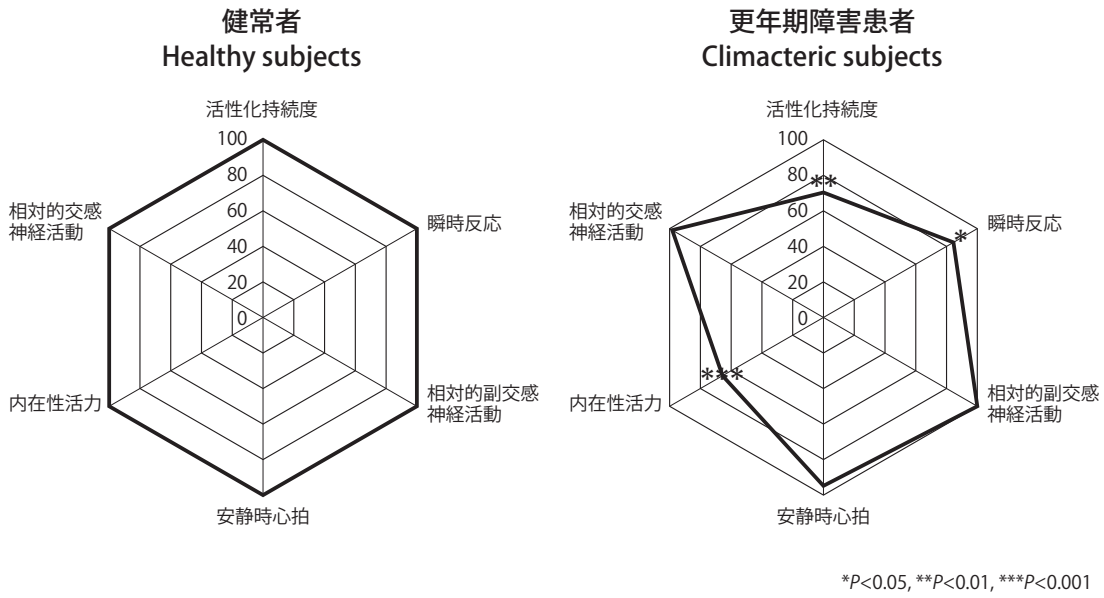

図５　更年期障害患者における自律神経バランス変動[9]

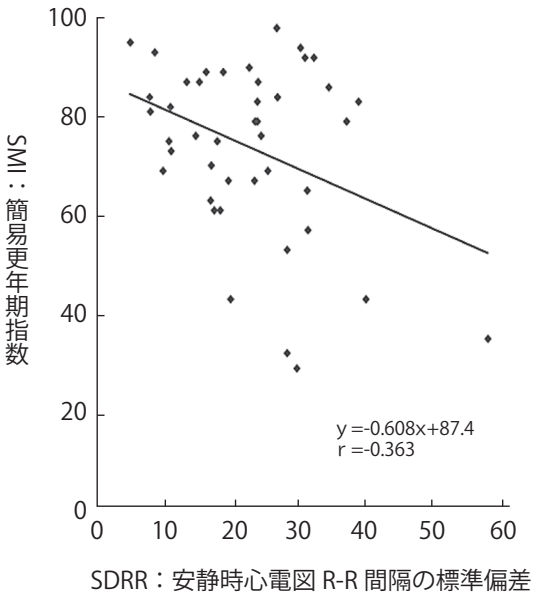

図６　簡略更年期指数（SMI）と SDRR（sup）の逆相関性[9]（改変）

る（相関係数 r = -0.363, *P*=0.0167）との結果を得た（**図６**）[10]。

７．ウエアラブル生体センサを用いた自律神経バランスの自動測定・解析ソフトの開発

本来、自律神経バランス測定を行う場合には、少なくとも測定者が１人別に待機して、被験者の測定を行わなければならない。しかしながら、自分で自分の自律神経バランスをチェックするには、これらの操作をある程度、自動化する必要がある。そこで１人でも測定出来るようにアプリケーションソフトの開発を試みた。ちょうどこの頃（2014 年）に、東芝よりウエアラブル生体センサ Silmee™ Bar type（以下 Silmee）が開発され発売された。Silmee は、約 64mm（幅）×28mm（奥行）×9.6mm（最厚部）、重量は約 14.6g の軽量小型の生体センサである。ゲルパッドで胸―腹部に貼りつけることで、心電位・脈波・体動等の生体情報を同時に連続計測でき、計測したデータを元に、心拍間隔、脈波間隔、体動量、姿勢を算出する。Bluetooth 通信を介して、タブレットにデータが送られ結果が表示・保存される。

その自律神経バランス測定操作法を簡単に説明する。まず、被験者の心窩部に Silmee を貼付し、ベッドに仰臥位にて閉眼させ、滅灯し安静状態を保つ。測定前に４秒毎の呼吸（１秒で吸気、３秒の呼気）でリラックスした状態で行うよう指示し、測定中はタブレットからのアナウンスに合わせ、可能な限り自然にその呼吸を継続させる。約 60 秒間の仰臥位測定後、タブレット音声とともに開眼、速やかに起立させ、起立した状態で引き続き４秒毎の呼吸をする。起立での測定は約 90 秒間行う。測定は２回行い、２回の平均値を測定値とする。測定後はタブレットに表示されるアンケート（疲労度蓄積、ストレスチェック、又は更年期障害のどれか１つ）を被検者自身で行って終了とする。最後に、終了ボタンを押すと、自動的に自律神経バランス測定結果として、六角形のレーダーチャート図とアンケート結果がタブレットの画面に明示される。自律神経バランスのレーダーチャートとその各パラメーターの説明およびその結果を評価する画面を**図７**に表示した[12]。

８．自律神経バランス測定の意義と産業医学への応用

私達が日常生活をスムーズに過ごすには、自律神経の働きは非常に大切である。自律神経は、環境の変化やストレスなどにより神経の存在を意識することなく、それらの変化に自動的に働いて体が上手く対応出来るように調節する。しかし、強いストレスが長期間続いた時に交感神経の過剰な緊張を招き、その結果自律神経系のバランスが乱れて体の変調が生じ、その人の体の弱い場所（部分）において異常や症状、ひいては病気が発生する。以前より多くの研究[3, 13, 14]により、ストレスにさらされると消化器系や循環器系症状、免疫系の抑制や神経精神症状、さらには癌などが生じる事が強く示唆されている。もし何らかのストレスにより自律神経バランスが乱れた場合に、いち早

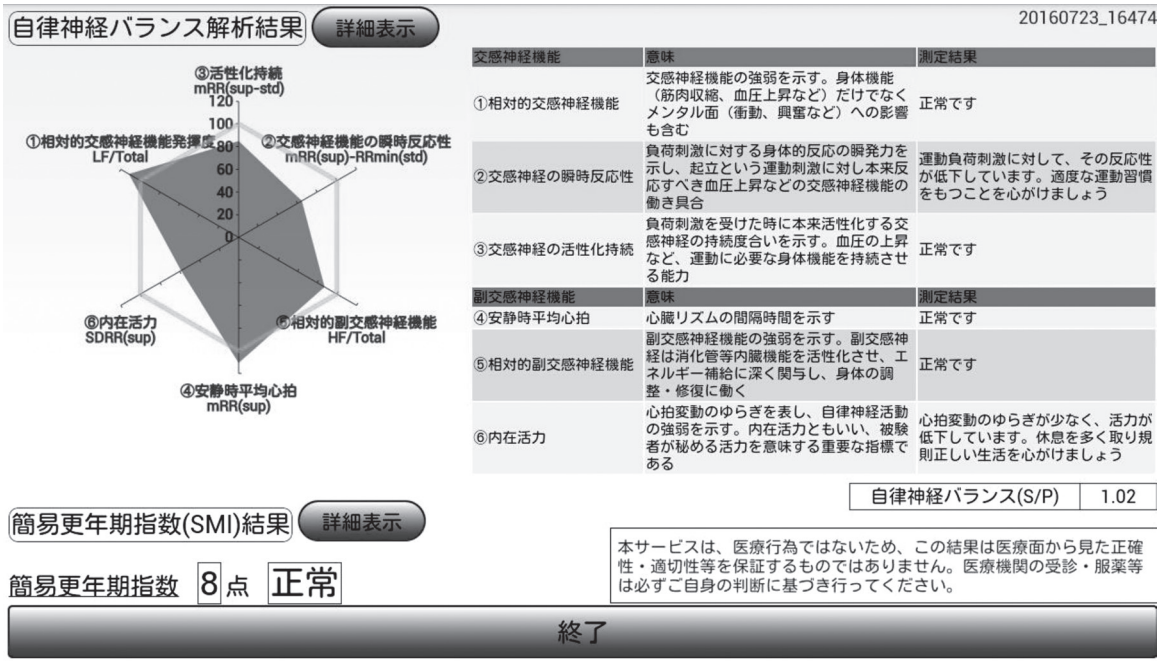

図７　自律神経バランスの自動測定・解析結果 [12)]

くこれをキャッチし、バランスの是正を心がけることは病気にならないためにも重要なことと思われる。

筆者が体験した自律神経バランス変動を１つの症例報告としてここに紹介する。当時、大学で多忙を極めていた時期に、あまりにも体がきつかったので、自分で自分の自律神経バランスを測定した。恐らく、バランスは悪いことが予想されたが、測定してみると思った以上にバランスの悪い結果に驚いた。そこで、意識的に仕事のペースを緩め、その日は大学から出来るだけ早く帰り夕食もすませると早く床に就いた。翌日および翌々日も仕事を軽減して出来るだけ早く帰宅し休息を取った。そしてほぼ同じ時間帯に再度、自律神経バランスを測定してその経過をたどってみた。初日（７月21 日）のバランスの悪い状態から徐々に改善して行く結果が得られた（**図８**）。

現在、産業医科大学のある部署において繁忙期前後での職員の自律神経バランス測定研究を実施しており、近い将来その結果をご報告したい。また、50 人以上の企業においては健康診断時にストレスチェックが義務付けられ実施されている。このストレスチェック（主観的評価）において高スコアを示した従業員に対して、自律神経バランス測定結果（客観的評価）を加えることにより産業医による従業員へのよりきめ細やかな面接指導やその健康管理が出来るのではと期待している。

自律神経バランス測定の利用は、産業医学分野だけにとどまらずいろいろな臨床の場においても共同研究（糖尿病内科、神経内科、精神医学、産婦人科、漢方診療科等）として計画、実施されている。また、鍼灸やマッサージ治療、さらには禅や気功の前後における自律神経バランス変動等についても検討中である。

９．まとめ

今回、ウエアラブル生体センサ Silmee（東芝製、現在は TDK 社の Silmee Lite）による自律神経バランスについて紹介した。従来の自律神経バランスの測定は、最低でも１人の測定者が側にいて測

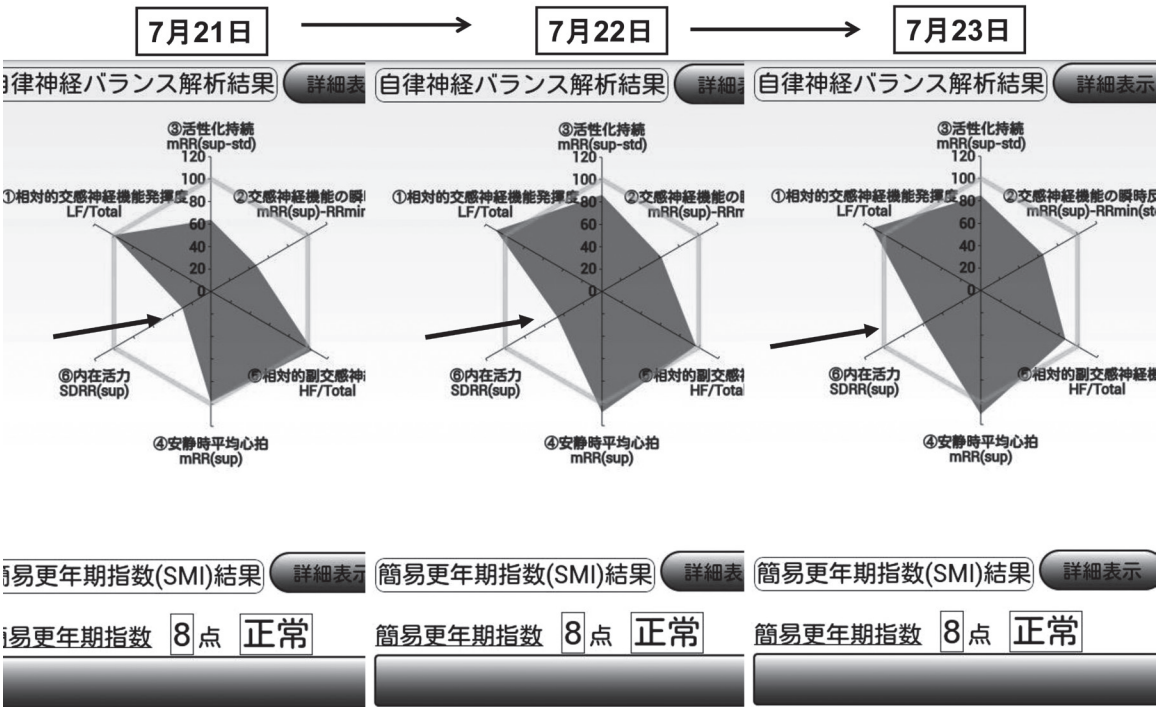

図８　過労後における自律神経バランス経日変化の１例

定する必要があった。今回紹介した方法は、測定操作の大部分を自動化し、被験者自身で１人でも測定可能とした。今後、セルフヘルスケアと言う観点からこの自律神経バランスの自動測定・解析システムは、自分のストレス状態をチェックする有力な手段の一つとして、「いつでもどこでも手軽に」測定可能なウエアラブル生体センサになるのではないかと期待している。

尚、この自律神経バランス測定システムを利用ご希望の方は、筆者までご連絡いただければ幸いである。

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利益相反：本研究における利益相反は無い。

【参考文献】

1）Westfall TC and Westfall DP.: Adrenergic agonists and antagonists, in Goodman & Gilman: the Pharmacological Basis of Therapeutics, 11th ed (Brunton LL, Lazo JS, and Parker KL eds) pp 237-295, McGraw-Hill, NY, 2005

2）柳原延章，佐藤教昭，石明寛．自律神経システムにおける植物フラボノイドと更年期障害の影響について，自律神経，52:13-17, 2015

3）Hara MR, Kovacs JJ, Whalen EJ, et al.: A stress response pathway regulates DNA damage through β2-adrenoreceptors and β-arrestin-1. Nature 477: 349-353, 2011

4）金井正光他，臨床検査法提要 改訂第 34 版 , 2015

5）後藤幸生，柳本政浩，安田善一．レーダーチャー

ト表示による自律神経機能評価法，自律神経，31:660-667, 1994
6）後藤幸生．心身自律神経バランス学，真興交易株式会社医書出版部 , 2011
7）小山嵩夫，麻生武志．更年期婦人における漢方治療；簡略化した更年期指数による評価，産婦人科漢方研究のあゆみ，9:30-34, 1992
8）Freedman RR.: Hot flashes: behavioral treatments, mechanisms, and relation to sleep. Am J Med 118: Suppl 12B, 124-130, 2005
9）Yanagihara N, Seki M, Nakano M, Hachisuga T & Goto Y. Inverse correlation between the standard deviation of R-R intervals in supine position and the simplified menopausal index in women with climacteric symptoms. Menopause 21: 669-672, 2014
10）柳原延章，石明寛，後藤幸生，蜂須賀徹，中野正博．レーダーチャート式自律神経バランス解析の女性更年期障害患者への応用―心拍変動解析値によるバランスと歪み・レーダーチャート式評価法―J UOEH（産業医科大学雑誌），36: 171-177, 2014
11）Burger HG, Cahir N, Robertson DM, et al.: Serum inhibins A and B fall differentially as FSH rises in perimenopausal women. Clin Endocrinol (Oxf). 48: 809-813, 1998
12）柳原延章，佐藤教昭，豊平由美子，李暁佳，石兼真，石明寛，後藤幸生．自律神経調節の基礎と臨床：植物フラボノイドと更年期障害について，日本女性医学会雑誌，24:232-239, 2017
13）Freedman NJ, Lefkowitz RJ.: Anti-β1-adrenergic receptor antibodies and heart failure: causation, not just correlation. J Clin Invest, 113: 1379-1382, 2004
14）Heidt T., et al.: Chronic variable stress activates hematopoietic stem cells. Nature Medicine 20: 754-761, 2014