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Association of Suicidal Ideation with Job Demands and Job Resources: a Large Cross-Sectional Study of Japanese Workers

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Abstract
Purpose This study aimed to identify the association of suicidal ideation with job demands and job resources among Japanese workers.
Methods Valid data from questionnaires that were pre-collected from 42,499 workers (34,882 males and 7617 females) were used for multilevel logistic regression analyses. Job demands and job resources were selected as potential predictors of suicidal ideation. Lifestyle variables, support from family and friends, and suffering from depressive or eating disorders were used as covariates.
Results In the employee-level, most job demands and job resources had significant associations with the risk of suicidal ideation in both sexes. High coworker support had a significant negative association with the risk of suicidal ideation in the organization-level, irrespective of their gender. High physical demands and poor physical environment in the organization-level had significant positive associations with increased risk of suicidal ideation only among females.
Conclusions The risk of suicidal ideation among Japanese workers is associated with job demands and job resources in the employee-level, and coworker support in the organization-level may be important as well.

Keywords Suicidal ideation · Job demands · Job resources · Japanese · Multi-level analysis

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Introduction

Suicide is one of the largest health and behavioral problems since the late twentieth century in most parts of the world, irrespective of the levels of industrialization or wealth. Figures from the World Health Organization (WHO) [1] indicate that one suicide occurs every 40 s, and it is the third leading cause of death in the world. In Japan, around 30,000 people have died from suicide every year since 1998; this equates to 25 deaths from suicide per 100,000 people each year. The Japan Cabinet Office reported that 27,283 people died from suicide in 2013 [2]. Although the total number of suicides has been decreasing continuously since 2010, suicide is still the largest cause of death especially in the younger population in Japan [3]. The number of worker suicides in 2013 reached 9401 [2]; this means that 34.5 % of Japanese people who committed suicide had a job at the time of suicide. Employment-related problems have been the fourth-ranked cause of suicide for several years [2, 4, 5] and have broadly been defined as *karojisatsu* (suicide due to overwork) in Japan [6–8]. Therefore, it is important to identify work-related risk and protective factors associated with suicide among the working population at a more detailed level.

Mann [9] proposed a suicide prevention strategy based on three components: (1) mood or other psychiatric

disorders, (2) suicidal ideation, and (3) factors involved in suicidal behavior. Suicidal ideation, which is a serious thought about killing oneself [10], was deemed to have a direct association with suicidal action. Suicidal ideation has a strong association with completed suicide [11, 12]. It is also a phenomenon that occurs commonly with suicide action; thus, it may be beneficial to identify risk factors for suicidal ideation to prevent consequent suicidal action.

As mentioned earlier, it is important to consider both risk factors and protective factors for suicidal ideation among workers. Within the work stress literature, the Job Demands Resources (JD-R) model [13–15], which considers both health impairment process with job demands and motivation improvement process with job resources, has been one of the most frequently cited theoretical frameworks. In this model, job demands are defined as "physical, social, or organizational aspects of the job that require sustained physical or mental effort and are associated with certain physiological and psychological costs," (p. 501) and job resources are defined as "physical, psychological, social, or organizational aspects of the job that may do any of the following: (a) be functional in achieving work goals; (b) reduce job demands at the associated physiological and psychological costs; (c) stimulate personal growth and development" (p. 501) [13]. For example, high quantitative job demands, numerous or intensive interpersonal conflicts, or long work hours are classified as job demands, while substantial job control or strong support from supervisors or coworkers are classified as job resources. In the JD-R model, specific contents of both job demands and job resources are considered to vary by occupations mainly due to the differences in the nature of their work [13]. Compared to the other prevalent job stress models (e.g., Job Demands Control model [16], NIOSH Generic Job Stress model [17]), the advantage of the JD-R model is its flexibility to include various job demands and job resources in each domain. However, this may also be a downside of the JD-R model, especially when considering the general approach for decreasing suicidal ideation that is applicable to many occupations.

Based on these discussions, this study aimed to identify the association of suicidal ideation with job demands and job resources among Japanese workers in a large sample of working adults. To our knowledge, only a few studies have focused on this topic using a large sample of Japanese workers. Suicidal ideation is one of the common symptoms of the major depression, and thus having such thoughts frequently may reflect one's unhealthy mental status. The study analyses were conducted separately for each gender because, in Japan, males have been shown to have higher suicide rates compared with females [2]. This may reflect social, biological, or psychological differences in the causes of suicidal ideation.

Method

Study Participants

A total of 62,408 Japanese workers representing various industries and occupations were included in this cross-sectional study. Data were drawn from the Mental Health and Life Style Inventory survey conducted in Tokyo, Japan (April 2008 to December 2010), which was distributed to 61 organizations in nine industry sectors. Overall, 55,242 workers completed and returned the questionnaire (response rate = 88.5 %). Informed consent was obtained from all study participants. After excluding 12,743 participants who left certain questionnaire items incomplete, the responses of the remaining 42,499 workers (34,882 males and 7617 females) in 61 organizations were selected for the present analysis (valid response rate = 76.9 %). The number of workers within each organization ranged from 11 to 24,156 (median = 158). The study protocol was approved by the Ethical Committee of the University of Occupational and Environmental Health (H26-029).

Measures

Suicidal Ideation

Suicidal ideation was measured by a single item "I seriously consider that I would feel better if I were to die," during the past 2 weeks, followed by four choices: "1 = never or almost never," "2 = a little or occasionally," "3 = often or fairly often," and "4 = very often or always." In this study, we classified responses into two categories: little or no suicidal ideation ("1 = never or almost never" or "2 = a little or occasionally") and having suicidal ideation ("3 = often or fairly often" or "4 = very often or always"). Kessler et al. [10] defined the suicidal ideation as a serious thought about killing oneself; thus, we set a cutoff point between the answering choices "2 = a little or occasionally" and "3 = often or fairly often."

Potential Predictors

Job demands and job resources were chosen as potential predictors of suicidal ideation. Job demands and job resources were measured with the Brief Job Stress Questionnaire (BJSQ) developed by The Japanese Ministry of Health, Labour and Welfare [18]. In the BJSQ, job demands consisted of five subscales, namely "quantitative job demands" (three items, $\alpha = 0.75$; e.g., I have an extremely large amount of work to do.), "qualitative job demands" (three items, $\alpha = 0.67$; e.g., I have to pay very careful attention.), "physical demands" (one item; My job requires a lot of physical work.), "interpersonal conflict" (three items, $\alpha = 0.64$; e,g., There are differences of opinion within my department.), and "poor

physical environment" (one item; My working environment is poor.). Job resources consisted of five subscales, namely "job control" (three items, $\alpha = 0.68$; e.g., I can work at my own pace.), "suitable jobs" (one item; This job suits me well.), "meaningfulness of work" (one item; My job is worth doing.), "supervisor support" (three items, $\alpha = 0.79$; e.g., How freely can you talk with your supervisor?), and "coworker support" (three items, $\alpha = 0.78$; e.g., How freely can you talk with your coworker?). Each item for the job demands and job resources was answered by participants using a four-point Likert-type scale: "1 = strongly disagree," "2 = disagree," "3 = agree," and "4 = strongly agree." Overtime work, which was considered to be a one component of job demands, was measured with a single item asking, "How long did you work overtime, including work done during holidays, in the last month?" followed by six choices: "less than 15 h," "15 to <30 h," "30 to <45 h," "45 to <60 h," "60 to <80 h," and "80 h or more."

Covariates

Lifestyle variables (hours slept per day, smoking status, and alcohol consumption), support from family and friends, and suffering from depressive or eating disorders were considered as covariates in the analyses. Support from family and friends were measured with three items ($\alpha = 0.84$; e.g., How freely can you talk with family or friends?), which were included in the BJSQ [18]. Participants answered each item with a 4-point Likert-type scale: "1 = strongly disagree," "2 = disagree," "3 = agree," and "4 = strongly agree". In this study, the presence of depressive or eating disorders were not defined clearly by asking the participants whether they are currently suffering from each of the disorder or not. Thus, the response categories were "yes" or "no" for each disorder.

Statistical Analyses

Participants were divided by each mean subscale score for job demands or job resources. The lower score for each scale was considered as reference group. Overtime work of less than 80 h per month was also considered as reference group. The criteria of 80 overtime hours a month were adopted from the Comprehensive Program for the Prevention of Health Impairment Due to Overwork [19]. Under the Japanese law, if the number of overtime hours exceeds 80 h per month and an employee requests administrative guidance or the number of overtime hours exceeds 80 h per month for two to six consecutive months, the employer should make an effort to provide the employee with administrative guidance through an occupational physician.

The risks of suicidal ideation associated with job demands and job resources were estimated using multilevel logistic regression analysis, controlling for lifestyle variables (number of hours slept per day, smoking status, and alcohol consumption), support from family and friends, and suffering from depressive or eating disorders. We considered the workers as a level 1 variable (employee-level) and the organization as a level 2 variable (organization-level) because each worker can be nested within only one organization. Organization-level job demand and job resource scores were calculated by averaging employee-level scores for each organization. All independent variables in the employee-level were centered by group mean and those in the organization-level were centered by grand mean.

IBM SPSS Statistics version 22 (IBM Corp., NY) and Mplus version 7.11 (Muthén & Muthén, LA) were used for statistical analyses. We calculated only employee-level odds ratios (ORs) because Mplus version 7.11 cannot calculate OR in the organization-level, nor calculate the 95 % confidence intervals for each ORs in the employee-level. All analyses were carried out separately for males and females.

Results

Characteristics of the participants stratified by gender are shown in Table 1. To check whether the distributions of each variable differed by gender, χ^2 tests were conducted. The prevalence of suicidal ideation was 4.6 % in males and 4.7 % in females; there was no significant gender difference. These percentages were almost identical to a study with 5238 US adults (5.6 % for males and females) [20]. Overall, most males were older (approximately 40 % of males were more than 40 years old) and worked as full-time workers (96.3 %). The percentage of night shift workers was significantly higher in males (21.2 %) than that in females (2.0 %). Most females were lifetime non-smokers (80.3 %). Approximately 24 % of males drank alcohol almost everyday. The prevalence of eating disorder was significantly higher in females; however, no significant gender difference was found in the prevalence of depressive disorder. Job demands scores were generally higher in males than those in females, but job resources scores were generally lower in males than those in females. Compared to the study samples, samples with missing data ($n = 12{,}743$) were more often females ($n = 2{,}793$, 21.9 %), slightly older (more than 50 years old: $n = 3519$, 27.6 %), working the night shift ($n = 2835$, 22.2 %), and belonging in public affairs ($n = 6135$, 48.1 %). Fifty-eight percent of the participants who had some missing data did not answer their current employment status.

To check the hierarchy of our data, intra-class correlation coefficients (ICC(1)) were calculated for each variable. Among subscales of job demands and job resources, all ICC(1) excluding ICC(1) for overtime work in females were significant in both sexes. Internal consistencies of all variables within each organization (ICC(2)) ranged from 0.90 to 0.98 in males and 0.70 to 0.98 in females (Table 2). Thus, we

Table 1 Characteristics of the study participants and the results of the χ^2 tests for each variable by gender

		Male		Female		Chi-square
		n	%	*n*	%	
Suicidal ideation	No	33,279	(95.4)	7262	(95.3)	0.1
	Yes	1603	(4.6)	355	(4.7)	
Demographic variables						
Age groups						2574.3***
Less than 20 years		358	(1.0)	118	(1.5)	
20 to 29 years		7062	(20.2)	3410	(44.8)	
30 to 39 years		12,826	(36.8)	2775	(36.4)	
40 to 49 years		9262	(26.6)	933	(12.2)	
50 to 59 years		4638	(13.3)	332	(4.4)	
60 years or more		736	(2.1)	49	(0.6)	
Employment status						448.6***
Full-time		33,601	(96.3)	6906	(90.7)	
Part-time		1281	(3.7)	711	(9.3)	
Work shift						1576.9***
Day shift		27,493	(78.8)	7465	(98.0)	
Night shift		7389	(21.2)	152	(2.0)	
Industry sector						1834.4***
Agriculture		778	(2.2)	303	(4.0)	
Manufacturing		22,843	(65.5)	3478	(45.7)	
Wholesale/retail		708	(2.0)	332	(4.4)	
Information technology		2982	(8.5)	1092	(14.3)	
Finance/insurance		2656	(7.6)	1324	(17.4)	
Real estate		1750	(5.0)	411	(5.4)	
Human health/social work		15	(0.0)	93	(1.2)	
Media/publication		264	(0.8)	77	(1.0)	
Public affairs		2886	(8.3)	507	(6.7)	
Lifestyle variables						
Sleep hours per day						12.4**
Less than 5 h		2870	(8.2)	613	(8.0)	
5 to <7 h		22,047	(63.2)	4972	(65.3)	
7 h or more		9965	(28.6)	2032	(26.7)	
Smoking status						4607.4***
Current smoker		15,401	(44.2)	924	(12.1)	
Nonsmoker		13,148	(37.7)	6118	(80.3)	
Past smoker		6333	(18.2)	575	(7.5)	
Alcohol consumption (days)						1996.9***
0		5361	(15.4)	1794	(23.6)	
1 to 3/month		8284	(23.7)	3000	(39.4)	
1 to 2/week		6314	(18.1)	1554	(20.4)	
3 to 4/week		3833	(11.0)	463	(6.1)	
5 to 6/week		2651	(7.6)	207	(2.7)	
Almost everyday		8439	(24.2)	599	(7.9)	
Health-related variables						
Depressive disorder	No	34,392	(98.6)	7520	(98.7)	0.8
	Yes	490	(1.4)	97	(1.3)	
Eating disorder	No	34,866	(100.0)	7576	(99.5)	113.2***
	Yes	16	(0.0)	41	(0.5)	
Work-related variables						
Job demands						

Table 1 (continued)

		Male		Female		Chi-square
		n	%	*n*	%	
Quantitative job demands	Low	13,909	(39.9)	4301	(56.5)	702.8***
	High	20,973	(60.1)	3316	(43.5)	
Qualitative job demands	Low	14,710	(42.2)	4598	(60.4)	834.8***
	High	20,172	(57.8)	3019	(39.6)	
Physical demands	Low	23,696	(67.9)	6345	(83.3)	712.6***
	High	11,186	(32.1)	1272	(16.7)	
Interpersonal conflict	Low	19,632	(56.3)	4810	(63.1)	120.6***
	High	15,250	(43.7)	2807	(36.9)	
Poor physical environment	Low	21,145	(60.6)	4331	(56.9)	36.8***
	High	13,737	(39.4)	3286	(43.1)	
Overtime work per month						111.2***
Less than 80 h		33,702	(96.6)	7532	(98.9)	
80 h or more		1180	(3.4)	85	(1.1)	
Job resources						
Job control	Low	15,903	(45.6)	3047	(40.0)	79.0***
	High	18,979	(54.4)	4570	(60.0)	
Suitable jobs	Low	11,750	(33.7)	2443	(32.1)	7.3**
	High	23,132	(66.3)	5174	(67.9)	
Meaningfulness of work	Low	12,179	(34.9)	2819	(37.0)	12.0***
	High	22,703	(65.1)	4798	(63.0)	
Supervisor support	Low	17,890	(51.3)	4343	(57.0)	82.3***
	High	16,992	(48.7)	3274	(43.0)	
Coworker support	Low	20,513	(58.8)	4139	(54.3)	51.2***
	High	14,369	(41.2)	3478	(45.7)	

**$p < 0.01$; ***$p < 0.001$

Table 2 Intra-class correlation coefficients and internal consistencies of job demands and job resources

	ICC(1)		ICC(2)	
	Male	Female	Male	Female
Job demands				
Quantitative job demands	0.04***	0.08***	0.96	0.92
Qualitative job demands	0.02***	0.07**	0.93	0.90
Physical demands	0.09***	0.26**	0.98	0.98
Interpersonal conflict	0.03**	0.06***	0.94	0.88
Physical environment	0.04***	0.04**	0.96	0.83
Overtime work	0.05**	0.06	0.97	0.89
Job resources				
Job control	0.02***	0.03*	0.93	0.81
Suitable jobs	0.02***	0.02**	0.92	0.70
Meaningfulness of work	0.03***	0.05**	0.95	0.86
Supervisor support	0.02**	0.02*	0.91	0.75
Coworker support	0.02*	0.03***	0.90	0.76

ICC(1) intra-class correlation coefficient, *ICC(2)* internal consistency within the organization

*$p < 0.05$;**$p < 0.01$; ***$p < 0.001$

considered that all job demands and job resources excluding overtime work among females had both employee-level and organization-level information.

Table 3 shows the standardized estimates and odds ratios of multilevel logistic regression analyses controlling for lifestyle variables, support from family and friends, and suffering from depressive or eating disorders, stratified by gender. Among males, all job demands had positive associations with the risk of suicidal ideation in the employee-level. No significant associations were found between any job demands and suicidal ideation in the organization-level. All job resources excluding coworker support were negatively associated with the risk of suicidal ideation in the employee-level. Among job resources, high coworker support had significant negative association with the risk of suicidal ideation in the organization-level.

Among females, all job demands excluding high quantitative job demands and overtime work had positive association with the risk of suicidal ideation in the employee-level. High quantitative job demands had a negative association with the risk of suicidal ideation, and overtime work had no significant association with the risk of suicidal ideation in the employee-level. For the organization-level, high physical demands and

Table 3 Standardized estimates and odds ratios of suicidal ideation among male and female workers

	Male					Female				
	Employee - level			Organization - level		Employee - level			Organization - level	
	Estimate[c]	SE	OR[a,b]	Estimate[c]	SE	Estimate[c]	SE	OR[a,b]	Estimate[c]	SE
Job demands										
High quantitative job demands	0.05**	0.02	1.23	−0.36	0.28	−0.05**	0.02	0.80	0.14	0.26
High qualitative job demands	0.09***	0.01	1.48	−0.15	0.25	0.12***	0.02	1.68	−0.34	0.32
High physical demands	0.07***	0.01	1.40	0.50	0.26	0.05**	0.02	1.31	0.46*	0.23
High interpersonal conflict	0.13***	0.01	1.70	0.00	0.25	0.10***	0.02	1.56	−0.17	0.24
Poor physical environment	0.06***	0.01	1.30	−0.18	0.26	0.06*	0.02	1.27	0.61*	0.29
Overtime work (80 h or more/month)	0.03**	0.01	1.39	0.22	0.15	−0.02	0.03	0.66-	–	–
Job resources										
High job control	−0.06***	0.01	0.77	−0.19	0.29	−0.05	0.04	0.82	−0.14	0.15
Suitable jobs (yes vs. no)	−0.12***	0.01	0.59	0.07	0.46	−0.08**	0.03	0.71	−0.14	0.21
Meaningfulness of work (yes vs. no)	−0.14***	0.01	0.55	−0.31	0.37	−0.08***	0.02	0.69	−0.22	0.28
High supervisor support	−0.04***	0.01	0.85	0.07	0.39	−0.07**	0.03	0.74	0.34	0.21
High coworker support	−0.01	0.01	0.96	−0.61*	0.26	−0.08***	0.02	0.71	−0.78**	0.23

SE standard error

OR odds ratio

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

[a] Low score for each scale as reference group

[b] Controlling for lifestyle variables (sleep hours per day, smoking status, and alcohol consumption), support from family and friends, and suffering from depressive or eating disorders

[c] Standardized

poor physical environment had positive association with the risk of suicidal ideation. Among job resources, all job resources excluding job control had negative association with the risk of suicidal ideation in the employee-level. High coworker support had negative association with the risk of suicidal ideation in the organization-level.

Discussion

This study examined the associations between job demands and job resources and the risk of suicidal ideation among a large cross-sectional cohort of Japanese workers. Multilevel logistic regression analyses revealed that at the employee-level, job demands and job resources had significant associations with the risk of suicidal ideation in both genders. At the organization-level, high physical demands and poor physical environment had positive associations with the risk of suicidal ideation only among females, and high coworker support had negative association with the risk of suicidal ideation in both genders. These results, in particular those at the organization-level, may be the first reported evidence published in work stress literature with regard to the risk of workers' suicidal ideation.

At the organization-level, high coworker support was negatively associated with the risk of suicidal ideation in both genders. Compared to the supervisors, coworkers generally pose as lesser risk and most workers may easily talk about their negative feelings associated with suicidal ideation and thus lessen these feelings through a "cathartic effect". In fact, Fridner et al. [21] revealed that supportive environments when facing difficulties have protective effects on the risk of suicidal ideation among Swedish doctors. Other evidence from cross-sectional studies revealed that organizing a supportive meeting in the workplace had a favorable effect on reducing the risk of suicidal ideation [22, 23]. Although suicidal ideation can be considered as the mentally unhealthy status of workers, organization-level support such as creating a supportive atmosphere, especially from their coworkers, may contribute to reduce workers' suicidal ideation regardless of their gender. Notably, among males, coworker support had no significant association with the risk of suicidal ideation at the employee-level. This may imply that nurturing the coworker support at the organization-level is more important to prevent suicidal ideation, especially for male workers.

With regard to job demands, high physical demands and poor physical environment were positively associated with the risk of suicidal ideation at the organization-level, but only

among females. One potential interpretation of these results may be attributed to the specific biological characteristics of females, which is a regular menstrual period. Within a regular menstrual period, many females suffer from severe body pain such as abdominal pain, low back pain, or headaches [24, 25]. Females who are menstruating are also sensitive to fatigue [25]. Therefore, due in part to menstruation, most females may be more easily be affected by high physical demands and poor physical environment such as moving heavy objects or exposure to heat environment and suffer more from negative feelings, and this may finally lead to suicidal ideation in some cases. However, physical demands and physical environment can easily be improved through organizational change, for example, providing protective equipment for employees, setting up air conditioning equipment, or increasing air ventilation, usually accomplished by adopting some kind of action checklists [26–28]. Based on our study results, ameliorating the physical demands and work environment may be important to counter workers' suicidal ideation, especially for female workers.

At the employee-level, most job demands had a positive association with the risk of suicidal ideation. In a cross-sectional study, Chin et al. [29] revealed a significant association between work-related stress and suicidal ideation in a sample of 6969 Korean male and female workers. Takada et al. [30] also showed a significant association between job stressors and suicidal ideation with 2834 Japanese male workers in a cross-sectional study. However, this association was not found in 1284 female workers. Although a further repeat study should be undertaken, differences between our results and Takada et al. [30] might partly be attributed to the different measurement process of job demands; Takada et al. [30] summed up each scores of job demands and reversed scores of job resources as an indicator of "job stressors." Other proceeding studies revealed a significant association between interpersonal conflict and suicidal ideation. Wall et al. [22] demonstrated a cross-sectional association between interpersonal conflicts such as "recent degrading experiences" or "harassment at work" and the risk of suicidal ideation among a total of 421 Italian and Swedish doctors. In Japan, Wada et al. [31] conducted a cross-sectional study with 3862 doctors and found a significant association between interpersonal conflicts (unreasonable patient demands and complaints) reported in the previous 6 months and their suicidal ideation. Our results also presented that interpersonal conflict had higher levels of OR with the risk of suicidal ideation in both genders. Thus, interpersonal conflict may be one of the crucial factors associated with Japanese workers' suicidal ideation.

Moreover, our results showed a significant association between overtime work and suicidal ideation in males. We found that when overtime work exceeded 80 h per month, the risk of suicidal ideation increased. These results are consistent with the cross-sectional evidence shown by 67,471 Korean workers [32]. Although the prevalence of suicide attempts is generally lower in males than that in females, the ratio of suicide completion is higher in males [33]. This evidence suggests that males might enhance hidden suicidal ideation when levels of job demands including overtime work become excessive. It is well known that job demands lead to adverse psychological, physiological, and behavioral stress reactions [17, 34–37]. Thus, we should pay more attention to workers' psychological functioning to prevent workplace suicide, especially for males who are determined to accomplish suicide without any consultation with others.

Contrary to the findings from job demands-suicidal ideation relationship, most job resources had a favorable effect on the risk of suicidal ideation. On the JD-R model [13–15], job resources are established at the beginning of the motivation process, enhancing work engagement and diminishing burnout. Our recent study [38] revealed that high levels of job resources such as job control and supervisor or coworker support enhanced work engagement, and these associations were mediated by positive affect and meaningfulness of work. These positive aspects of human emotions may have the potential to prevent the risk of suicidal ideation through cultivating psychological strength against stressful situations, although no significant association was found between job control and suicidal ideation in females. In a cross-sectional study, Fridner et al. [21] revealed a significant negative association between job control such as controllability for their own work hours or amount of work assigned and the risk of suicidal ideation among 241 male physicians. Although our study measured a "general" controllability of their work, controllability for their work hours or amount of tasks may be important to consider male workers' risk of suicidal ideation.

With regard to females, there was an unexpected finding of an inverse association between quantitative job demands and the risk of suicidal ideation at the employee-level. This observation is difficult to interpret but we speculate that females who are extremely competent but have a reduced job quantity may feel frustrated with their work which can cause reduced self-esteem leading to increase suicidal ideation. However, this is just one possible explanation and our study design is cross-sectional with limited variables; further longitudinal study design is needed to confirm this hypothesis.

Several limitations of our study should be noted. First, our data were derived from self-reports; thus, we cannot disregard recall reporting and social desirability bias. Overtime work usually can be measured objectively with registered data from each company. However, we did not collect these types of registered data; the methods mentioned above should be adopted in future research to confirm our results. Second, because our study design was cross-sectional, we could not establish causal relations between independent variables and suicidal ideation. Although we found significant associations between several potential predictors and suicidal ideation,

reverse causation is possible. A longitudinal cohort study is required to determine causality of the relationships. Third, although the response rate was relatively high (88.5 %), the non-participants in this population may have had a higher prevalence of suicidal ideation than those who participated. These workers might not have been able to answer the questionnaire; thus, we cannot neglect the healthy worker effect. Fourth, although the prevalence of suicidal ideation was almost identical between males and females presented in past studies [20, 30], it is known that males generally commit suicide more frequently than females [39]. One possible explanation for this difference is that, in general, females may easily share their suicidal ideation with others compared to males [40], but may not take suicide actions. Further study should be undertaken to confirm this assumption. Fifth, although the response rate was relatively high, the representativeness of employees in each organization may not be guaranteed. In this study, we calculated the organization-level job demand and job resource scores based on the individual-level scores, but it was speculated that we can successfully obtained the representative sample in every organization. Because most scores of ICC(1) were significant but small, it also be speculated whether all job demands and job resources had enough information in organization-level. Other organization-derived work-related characteristics should be measured to assess their effects on the associations of the individual-level job demands and job resources with suicidal ideation in the future.

Notwithstanding these limitations, we conclude the following for Japanese workers. In both genders, job demands such as qualitative and physical demands, interpersonal conflict, and physical environment and job resources such as suitable jobs, meaningfulness of work, and supervisor support had significant association with the risk of suicidal ideation at the employee-level. At the organization-level, high physical demands and poor physical environment had positive associations with the risk of suicidal ideation among females, and high coworker support had a negative association with the risk of suicidal in both genders. Organizational campaigns to improve coworker support may have the potential to decrease Japanese workers' suicidal ideation in practice.

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Compliance with Ethical Standards

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

Informed Consent All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all participants for being included in the studies presented.

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Effort–reward imbalance, cortisol secretion, and inflammatory activity in police officers with 24-h work shifts

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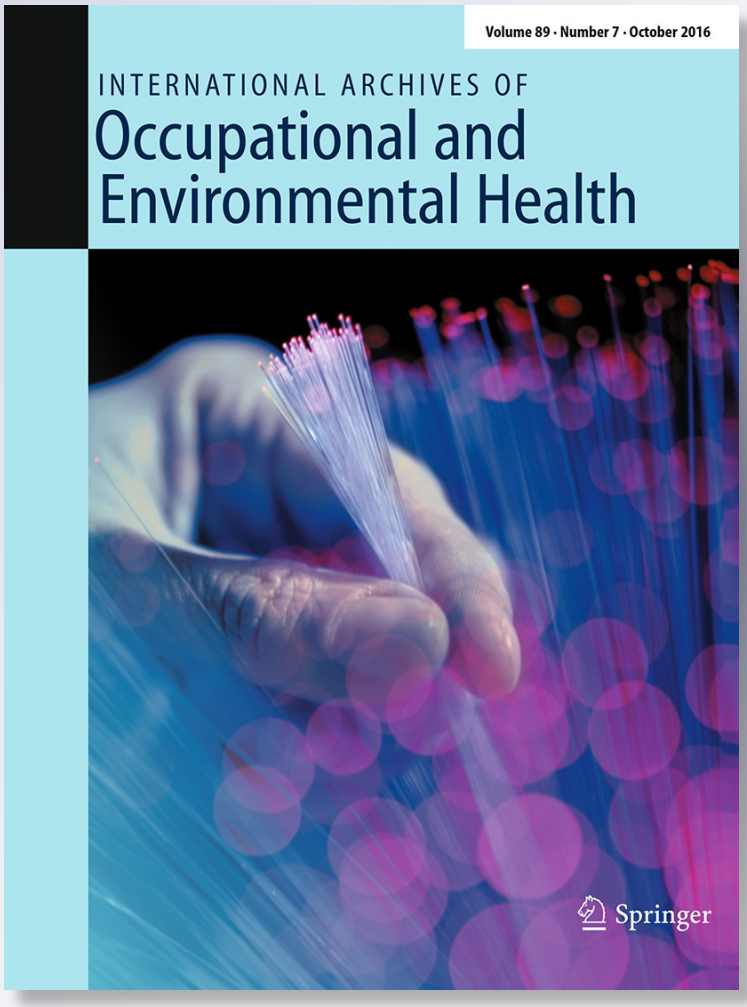

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ORIGINAL ARTICLE

Effort–reward imbalance, cortisol secretion, and inflammatory activity in police officers with 24-h work shifts

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Abstract
Purpose Accumulating evidence shows that effort–reward imbalance (ERI) at work can cause various health problems. However, few studies have investigated the biological pathways linking ERI and health outcomes, and their findings have been inconsistent. In this study, we investigated the associations between ERI, the hypothalamic–pituitary–adrenocortical axis, and inflammation in a sample of police officers.
Methods One hundred forty-two male police officers that were engaged in a working system of 24-h shifts were followed up during the work shift as well as during the two subsequent work-free days. Throughout this period, the participants provided two saliva samples each day for the 3-day period, and we measured the concentrations of cortisol and C-reactive protein (CRP) in the saliva. The police officers also completed the Japanese short version of the Effort–Reward Imbalance Questionnaire.
Results The results of linear mixed model analyses controlled for possible confounding variables indicated that higher effort scores ($p = 0.031$) as well as effort–reward ratio ($p = 0.080$) were associated with lower cortisol levels, and the effect of effort was strengthened in the younger police officers ($p = 0.017$). Furthermore, higher effort scores were associated with higher CRP levels in younger police officers ($p = 0.037$).
Conclusions Our results indicate that effort, a component of ERI, has physiological effects in younger police officers, which possibly contribute to the development of stress-related diseases.

Keywords Effort–reward imbalance · Cortisol · C-reactive protein · Police officers · Job stress

Introduction

The effort–reward imbalance (ERI) model posits that work conditions of high-effort (e.g., work demands, obligation) coupled with low-reward (e.g., money, esteem, job security) are considered particularly stressful and could have adverse health effects (Siegrist 1996). Epidemiological studies have indicated that ERI in the work environment contributes to health problems such as coronary heart disease and depression (Kuper et al. 2002; Siegrist 1996; Siegrist et al. 1992; Tsutsumi et al. 2001a, b).

The physiological mechanism underlying the association between ERI and health problems remains unclear. A potential biological pathway linking ERI to health outcomes is the hypothalamic–pituitary–adrenocortical (HPA) axis. Acute psychosocial stress is known to activate the HPA axis, leading to elevated cortisol levels in the blood and saliva (Dickerson and Kemeny 2004). Moreover, HPA axis dysfunction is associated with various health problems such as depression and cardiovascular disease (McEwen 2000). Previous studies investigating the associations between ERI and cortisol levels (awakening and diurnal levels) yielded inconsistent results. Positive associations (Eller et al. 2006), inverse associations (Eller et al. 2012;

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Maina et al. 2009), and no association (Hanson et al. 2000; Harris et al. 2007; Irie et al. 2004; Marchand et al. 2016; Ota et al. 2014) between ERI and cortisol levels were reported. Characteristics of the participants (e.g., age, sex, and the type of occupation), sample size, and frequencies and timing of cortisol assessment differed between studies, which could affect their findings.

Another possible process linking ERI and health outcomes is inflammatory activity. It is well recognized that psychosocial stress could alter inflammatory activity (Segerstrom and Miller 2004). Further, inflammatory activity could contribute to the development of stress-related diseases, such as cardiovascular disease, by accelerating the process of arteriosclerosis (Steptoe and Brydon 2007). In previous studies, job stress was reported to be associated with higher levels of inflammatory markers including C-reactive protein (CRP), interleukin-6, and tumor necrosis factor (Nakata 2012). However, few studies have investigated the associations between ERI and inflammatory activity. Hamer et al. (2006) reported that higher ERI scores were associated with elevated CRP in response to acute experimental stress, and Almadi et al. (2012) reported a positive association between ERI and CRP in obese male workers. Finally, inflammatory activity is closely connected with HPA axis activity. Specifically, glucocorticoids inhibit the synthesis, release, and/or efficacy of cytokines and other mediators of immune and inflammatory responses (Sapolsky et al. 2000).

The present study focused on the job stress experienced by police officers working at police boxes in 24-h shifts. These officers are required to prepare for the possibility of accidents and emergencies by wearing stab jackets and carrying guns for a prolonged period, including nighttime, which could cause high stress and a chronic burden to their health. Previous studies indicate that the prevalence of cardiovascular diseases and cardiovascular risks in police offers is higher than that of the general population (Franke et al. 1998, 2002; Joseph et al. 2009). However, to our knowledge, no studies have investigated the biological consequences of job stress, such as that caused by ERI, in police officers.

In the present study, we investigated the associations between ERI, cortisol, and CRP in a sample of police officers during their 24-h work shift and the two subsequent work-free days. We took saliva samples and assessed their cortisol and CRP levels. Recent studies have demonstrated the biological validity of salivary CRP, including moderate correlations between circulating and salivary CRP (Ouellet-Morin et al. 2011; Punyadeera et al. 2011). We hypothesize that a stressful work environment, evaluated by ERI, alters the diurnal pattern or basal secretion level of cortisol, and contributes to higher CRP levels in police officers. In the sequence of statistical analyses, we investigated whether ERI components (effort and reward) altered the HPA and inflammatory activities, because previous studies reported biological correlates of only effort or reward (Eller et al. 2006; Maina et al. 2009). We also investigated the effects of ERI on biological parameters in younger and older police officers, considering a possible modification effect of age, because in the past the relationship between job stress and cardiovascular disease was stronger in younger workers (Chandola et al. 2008; Kuper and Marmot 2003; Theorell et al. 1998). Furthermore, correlations between cortisol and CRP levels were calculated considering the known anti-inflammatory effects of cortisol.

Methods

Participants

In this study, male police officers engaged in a working system of 24-h shifts were repeatedly measured for 3 days. The police officers were recruited from police offices located in Chiba Prefecture, Japan in August 2013. The sample initially consisted of 200 male police officers aged 31–59 years, randomly selected by their identification number. The sample size was determined by availability of financial resource. Fifty-eight subjects were excluded for various reasons: 21 for a history of possible inflammation- or adrenal-related diseases (cardiovascular diseases, liver disease, diabetes, or depression), 19 for current use of medications (steroidal, lipid-lowering, or anti-hypertensive medications, or analgesics), 15 for obesity (body mass index (BMI) $\geq$ 30), and 3 for missing data on the ERI questionnaire. Therefore, the final sample consisted of 142 male police officers. The participants' mean (standard deviation [SD]) age was 43.0 (9.1) years. Their mean (SD) body mass index (BMI) was 24.3 (2.6) kg/m^2, and 54 participants were current smokers. Written informed consent was obtained from participants, and the institute's ethical committee approved the study (Kitasato University Medical Ethics Committee, B13-92).

Measurements

Saliva samples for cortisol and CRP measurements were obtained using a Salivette (Sarstedt Ltd.) with a polypropylene and polyethylene polymer swab. The participants were asked to place the swab under their tongue for 3 min to obtain the sample.

ERI was assessed using the 10-item Japanese short version of the Effort–Reward Imbalance Questionnaire (ERIQ), which includes three items for effort (e.g., "I have constant time pressure due to a heavy work load") and seven items for reward assessment (e.g., "My job security

is poor"). The 10-item Japanese short version of the ERIQ was reported to have acceptable internal consistency, reliability, and construct validity (Tsutsumi et al. 2002, 2008). In this sample, Cronbach's alpha coefficients for effort and reward scales were 0.81 and 0.70, respectively. The effort–reward ratio was calculated by the formula effort/reward*c, where 'c' is a correction factor weighting the different numbers of items in numerator and denominator (3/7).

Procedure

The police officers engaged in 3-day rotating shifts with a 24-h work shift (0900–0900 h) followed by two work-free days. In this study, samples were collected during the 3 days, including the 24-h work shift and the 2 work-free days. Saliva samplings were conducted twice a day in the morning (0900 h) and evening (1900 h). Thus, the participants provided six samples in total: before the start of the 24-h shift (0900 h), during the shift (1900 h), after the end of the shift (0900 h), and 10-h (1900 h), 24-h (0900 h), and 34-h (1900 h) after the end of the shift. Previously, cortisol awakening response was frequently investigated in the context of work stress (Karlson et al. 2012). However, in this study, sleep patterns could be largely different between the participants after the 24-h work shift (e.g., time of sleep and awakening, deep sleep or short nap). Therefore, we assessed diurnal pattern of cortisol but not cortisol awakening response.

Before the study started, the participants were verbally instructed on saliva-sampling protocols and were requested to refrain from eating, drinking, smoking, heavy exercise, or brushing their teeth for 1 h before the saliva collections. Participants were also asked to record their bedtime and awakening time during the 3-day study period. Written instructions on the saliva collection protocol were also handed out. We further provided 1-h time windows for morning- and evening-saliva collections (0830–0930 h and 1830–1930 h, respectively) because a stricter schedule was expected to be too difficult for the police officers during their work. Saliva samples were frozen in the freezers of police offices or participants' homes and transported frozen to the laboratory at the end of the experimental period.

Salivary assays

The samples were thawed and centrifuged at 3000 rpm for 15 min. The concentration of cortisol in saliva was determined by an enzyme-linked immunosorbent assay (ELISA) Kit (IBL International, Hamburg, Germany). The lower limit of detection was 0.14 nmol/L, and inter- and intra-assay variations were below 7.3 and 9.3 %, respectively. The concentration of CRP in saliva was determined using an ELISA kit (Salivary C-Reactive Protein ELISA Kit, Salimetrics LLC, PA, USA). The lower limit of detection was 0 pmol/L, and inter- and intra-assay variations were below 11.2 and 5.9 %, respectively.

Statistical analyses

Cortisol data were measured from all six saliva samples, and CRP data were measured from two saliva samples (the evening sample of the first day and the morning sample of the second day); salivary CRP levels were expected to be relatively stable over the course of 3 days (Izawa et al. 2013). Cortisol and CRP concentrations were logarithmically transformed (common logarithm) because the Kolmogorov–Smirnov test indicated a violation of the assumption of normality (skewed distribution).

The differences in the salivary cortisol levels were analyzed using linear mixed models with an unstructured error covariance matrix. We first examined variations in cortisol levels across 3 days, treating day (the first, second, and third days) and time (0900 and 1900 h) as fixed effects and individual intercept as a random effect. Possible confounding variables (age, BMI, and smoking status) were also included in the model as fixed effects, as these variables have previously been reported to be correlated with cortisol and CRP (Ouellet-Morin et al. 2011; Maina et al. 2012). Further, whether or not the sample was collected during the 1-h period after awakening was included in the model as a time-varying factor, because it is well known that cortisol levels rapidly increase during the first hour after awakening (Pruessner et al. 1997). Secondly, we examined the effects of effort, reward, and effort–reward ratio on the cortisol levels; effort score, reward score, or effort–reward ratio were additionally included in the model as fixed effects.

Differences in salivary CRP levels were analyzed by linear mixed models in the same manner as cortisol. We first examined variation in CRP levels during shifts, treating time (evening and morning) as fixed effects and individual intercept as a random effect. Possible confounding variables (age, BMI, and smoking status) were also included in the model as fixed effects. The effort score, reward score, or effort–reward ratio were additionally included in the model as fixed effects.

In preliminary analyses of cortisol and CRP, we found no significant interactions of ERI components with day and time, and thus we did not include these interaction terms in the models. We further examined the effects of ERI components on cortisol and CRP levels in younger (age <40, $N = 72$) and older (age ≥40, $N = 70$) participants, in order to consider the possible modification effect of age.

Pearson and Spearman correlation analyses and partial correlation analyses were conducted to investigate the association between cortisol and CRP. Statistical calculations

Table 1 Characteristics of the participants

	Total ($N = 142$)	Age <40 ($N = 72$)	Age ≥40 ($N = 70$)	p^a
Age (years)	43.0 ± 9.1	35.0 ± 2.9	51.1 ± 5.3	<0.001
Body mass index (kg/m^2)	24.3 ± 2.6	24.2 ± 2.7	24.3 ± 2.6	0.852
Smoking status (%)	38.0	36.1	40.0	0.633
ERIQ				
Effort	7.9 ± 1.9	7.8 ± 1.9	8.0 ± 2.0	0.737
Reward	19.5 ± 2.8	20.3 ± 2.4	18.6 ± 2.9	<0.001
Effort–reward ratio	1.0 ± 0.4	0.9 ± 0.3	1.0 ± 0.4	0.040

Data are presented as mean ± standard deviation

ERIQ Japanese short version of the Effort–Reward Imbalance Questionnaire

[a] Independent *t* tests or Chi-square tests were conducted between younger and older groups

Table 2 Average salivary cortisol and CRP levels adjusted for possible confounders

	24-h work shift			Work-free day		
	1st day 0900	1st day 1900	2nd day 0900	2nd day 1900	3rd day 0900	3rd day 1900
Cortisol (log nmol/l)	1.30 (0.02)	0.85 (0.03)	1.24 (0.02)	0.81 (0.03)	1.19 (0.02)	0.83 (0.02)
CRP (log pmol/l)	–	2.10 (0.03)	2.09 (0.03)	–	–	–

Standard errors of the mean appear in parentheses

were performed with the SPSS statistical package 18.0 for Windows (SPSS Software Inc., Tokyo, Japan).

Results

Characteristics of the participants are shown in Table 1. Younger participants exhibited higher reward scores ($t_{[140]} = 3.7$, $p < 0.001$) and lower effort–reward ratios ($t_{[140]} = 2.1$, $p = 0.040$) than older participants. BMI and smoking status did not differ between groups. Geometric means of cortisol levels (95 % CI) for morning and evening were 17.7 (16.6–18.9) nmol/L and 6.7 (6.0–7.4) nmol/L, respectively. Geometric means of CRP levels (95 % CI) for evening and morning were 126.7 (77.1–176.4) pmol/L and 125.9 (80.7–169.4) pmol/L, respectively. Logarithmically transformed cortisol levels across the 3 days are given in Table 2. In the first model, linear mixed model analyses detected significant differences in morning cortisol levels across the 3 days ($\beta = 0.091$, $p = 0.014$), and post hoc tests indicated that the cortisol levels on the morning of the third day were lower than those on the first and second days. Logarithmically transformed CRP levels during the shift are also shown in Table 2. Linear mixed model analysis of CRP detected no significant differences between the evening and morning levels.

In the second model, linear mixed model analyses for cortisol detected significant effects of effort ($\beta = -0.014$, $p = 0.031$) as well as marginally significant effects of effort–reward ratio ($\beta = -0.063$, $p = 0.080$). The effects of reward were not significant. Further, the effects of effort on cortisol were strengthened in the younger group ($\beta = -0.020$, $p = 0.017$) but weakened in the older group ($\beta = -0.009$, $p = 0.337$, Fig. 1). The effects of the effort–reward ratio on cortisol were not significant in either group. Linear mixed model analyses for CRP detected marginally significant effects of effort ($\beta = 0.026$, $p = 0.081$) but not reward or effort–reward ratio. The effects of effort on CRP were significant in the younger group ($\beta = 0.054$, $p = 0.037$) but not in the older group ($\beta = -0.008$, $p = 0.547$, Fig. 1).

Pearson and Spearman correlation analyses revealed no significant associations between cortisol and CRP levels on either the first evening or the second morning (correlations ranged from −0.02 to 0.04). Partial correlation analyses controlling for age, BMI, smoking status, and timing of saliva collection (within 1 h of awakening or not) revealed no significant associations between cortisol and CRP levels. The correlation analyses were further divided into younger and older groups, but no significant associations were detected.

Discussion

In this study, we investigated the association between ERI and the HPA axis and inflammatory activities in a sample of police officers during a 24-h work shift and the 2 subsequent work-free days. We found that ERI, especially effort, had some physiological effects: higher effort scores were

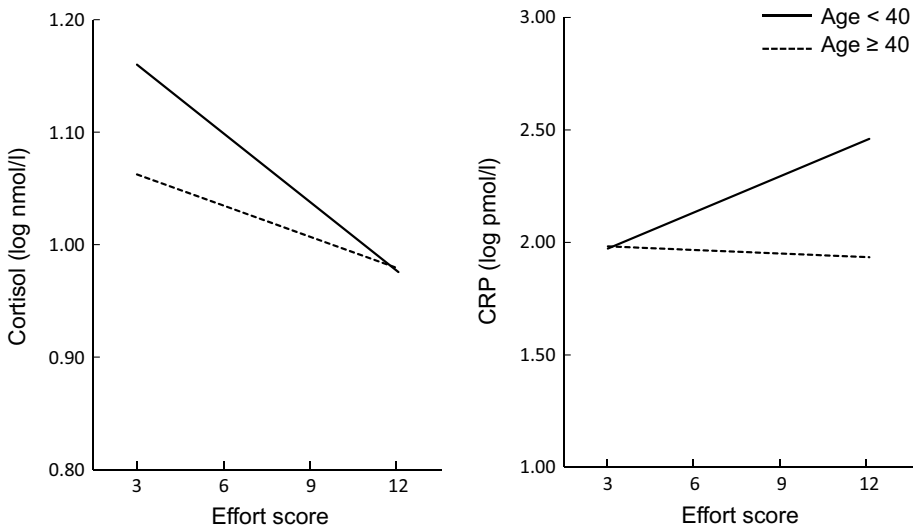

Fig. 1 Effort and averaged levels of salivary cortisol (*left*) and CRP (*right*) in younger (<40) and older (≥40) police officers, adjusted for possible confounders. Regression *lines* for cortisol and CRP are significant in only the younger group

associated with lower salivary cortisol levels and higher salivary CRP levels in younger police officers. We did not find any association between cortisol and CRP levels. This study employed two measures, one endocrine and one inflammatory, to investigate the biological associations of ERI. We believe this to be valuable data because most previous studies employed only one biological parameter (e.g., cortisol or CRP). Further, to our knowledge, this is the first study to investigate the biological effects of ERI in a sample of police officers who are engaged in severe shiftwork (24-h shifts) with both a mentally and physically strenuous work load.

We found that police officers with higher ERI scores, especially effort scores, exhibited lower cortisol levels, consistent with previous findings. Maina et al. (2009) reported lower cortisol secretion after awakening and during the diurnal period in call-center workers with higher ERI or effort scores. Similarly, Eller et al. (2012) reported that higher ERI or effort scores were associated with lower cortisol levels after awakening in female participants. Furthermore, in the previous studies, high ERI or its component was associated with dysregulation of the HPA axis in that there was an attenuated cortisol response to acute psychosocial stress (Siegrist et al. 1997) and lower cortisol awakening response to the dexamethasone test, implying higher negative feedback sensitivity (Bellingrath et al. 2008). Taken together, it can be speculated that workers with high ERI are exposed to stress-induced cortisol for prolonged periods, which causes higher negative feedback sensitivity of the HPA axis, as well as lower cortisol levels or attenuated cortisol responses. This biological speculation was also supported by a recent study in burnout patients, which found that patients with severe burnout exhibited lower cortisol and ACTH response to acute psychosocial stress compared to patients with low burnout and healthy controls (Lennartsson et al. 2015).

Other studies failed to observe an association between ERI and cortisol (Hanson et al. 2000; Harris et al. 2007; Irie et al. 2004; Marchand et al. 2016; Ota et al. 2014). The reason for the discrepant findings remains unclear; however, characteristics of the participants, intensity of the stressor, sample size, and the frequency and timing of cortisol assessment could affect the results, as suggested in a recent review of work stress and cortisol (Karlson et al. 2012). Age could be an important factor, because we found pronounced effects of effort on cortisol in younger participants. No previous studies had investigated the modifying effect of age on the relationship between ERI and cortisol. Furthermore, our study assessed cortisol levels during the work shift as well as the work-free days and appropriately excluded any participants having factors that might affect HPA and inflammatory activities (e.g., history of diseases, medications, and obesity). This could help to increase the statistical power to detect the significance of the effect of ERI on cortisol levels.

The possible effects of a 24-h work shift including night shifts should also be mentioned. In previous studies, working night shifts could reduce cortisol secretion: Lower cortisol levels were observed in the morning after the night shift, compared with levels on a morning not following a night shift (Costa et al. 1994; Leese et al. 1996; Munakata et al. 2001). However, in this study, the cortisol levels after the 24-h shift were comparable to those before the shift (the morning of the first day). We speculate that this was because the police officers involved in this study were allowed to take a short nap during the shift. We found lower cortisol levels on the morning of the third day, compared with those of the first and second days. The previous studies reported that cortisol responses after awakening on the work-free day were lower than that during the work day (Kunz-Ebrecht et al. 2004; Schlotz et al. 2004), which was

speculated to be linked to stress-related anticipation of the upcoming day (Fries et al. 2009). The lower morning cortisol levels on the third day might result from this phenomenon, and not from night shift working per se.

We also found that police officers with higher effort scores exhibited higher CRP levels during the shift. In the past, only two studies had investigated the association between ERI and CRP (Hamer et al. 2006; Almadi et al. 2012), and their findings were consistent with the finding of the current study. Further, we found pronounced effects of effort on CRP in younger participants, similar to the observed association between cortisol and effort. In a previous study on long-haul bus drivers (Tsai et al. 2014), a relationship between job stress (job demand) and CRP was observed only in younger participants, consistent with the finding of this study. In the past, it has been repeatedly reported that the relationship between job stress and incidence of coronary heart diseases was stronger among younger workers (Chandola et al. 2008; Kuper and Marmot 2003; Theorell et al. 1998). Biological factors associated with aging could contribute to the diminished effect of job stress on biological consequence. Furthermore, a survivor effect could be considered; Police officers who are highly vulnerable to the effect of job stress are less likely to work into old age, which could also diminish the biological effects.

In addition, we did not find negative correlations between cortisol and CRP, although we expected reduced cortisol secretion to contribute to increased inflammation. Previously, disturbances in the HPA axis were shown to be associated with higher inflammatory activity (e.g., Theorell et al. 2000). The reason for this finding remains unclear; however, a previous study (Bellingrath et al. 2013) demonstrated that high ERI is also associated with lower glucocorticoid sensitivity of interleukin-6 in vitro indicating the possibility that cortisol unsuccessfully regulated the inflammatory activity under the condition of high ERI. Therefore, a less effective anti-inflammatory regulation by cortisol, rather than reduced cortisol secretion, could largely contribute to increased inflammation in the high-ERI group.

This study has certain limitations that may affect the interpretation of the findings. Firstly, the study population was limited to male police officers because the majority of police officers in Japan are male. Secondly, our findings cannot be generalized to the general working population. The police officers involved in this study were in government employ, implying fairly provided chances of job promotion and salary and good employment security. Such an occupational status could contribute to the results we observed regarding the biological effect of effort, but not reward or effort–reward ratio. Therefore, caution should be exercised in over-generalizing the current findings to a wider population. Furthermore, we conducted many significance tests (effort/reward/ERI, young/old, cortisol, CRP), which should be exercised when interpreting the results.

In conclusion, we investigated the biological consequences of ERI using well-designed protocols. Randomly sampled police officers, selected using appropriate exclusion criteria, were investigated during their work shift as well as the following work-free days by employing endocrine and inflammatory measures. We provided evidence that younger police officers with higher effort scores exhibited lower salivary cortisol levels as well as higher salivary CRP levels. These biological features could possibly contribute to the future development of stress-related diseases such as cardiovascular diseases in the police officers.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in this study.

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Interrelationships Between Job Resources, Vigor, Exercise Habit, and Serum Lipids in Japanese Employees: a Multiple Group Path Analysis Using Medical Checkup Data

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Interrelationships Between Job Resources, Vigor, Exercise Habit, and Serum Lipids in Japanese Employees: a Multiple Group Path Analysis Using Medical Checkup Data

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Abstract
Background Physical inactivity is one of the major risk factors for dyslipidemia and coronary heart disease. Job resources have been identified as determinants of employees' vigor and physical activity habits.
Purpose Our first purpose was to comprehensively analyze the series of relationships of job resources, through vigor and exercise habit (i.e., one aspect of physical activity), to serum lipid levels in a sample of Japanese employees in a manufacturing company. Our second purpose was to investigate sex differences in these relationships using a multiple-group path analysis.
Methods Data were collected from 4543 employees (men = 4018, women = 525) during a medical checkup conducted in February and March 2012. Job resources (job control, skill utilization, suitable jobs, and meaningfulness of work), vigor, exercise habit, triglyceride, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured cross-sectionally.
Results Job resources and vigor were positively associated with exercise habit in both sexes. Exercise habit was inversely associated with triglyceride (−0.03 in men and −0.01 in women, ps < 0.05) and LDL-C (−0.07 in both sexes, ps < 0.05). HDL-C was positively associated with exercise habit (0.03 in both sexes, ps < 0.05). There was no significant difference by sex in path coefficients, except for the covariance between suitable jobs and meaningfulness of work.
Conclusion Higher levels of job resources were associated with greater vigor, leading to exercise habit, which in turn, improved serum lipid levels. Longitudinal studies are required to demonstrate causality.

Keywords Job resources · Vigor · Physical activity · Exercise habit · Serum lipids

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Introduction

Physical inactivity is considered one of the risk factors for mortality [1] and coronary heart disease [2] in the modern society. The Japan Ministry of Health, Labour and Welfare [3] has set improvement of physical activity and exercise as one of the national goals for Japanese health promotion. Several meta-analyses have indicated negative relationships between physical activity and dyslipidemias such as abnormalities in levels of triglyceride, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C), which are considered to be main risk factors for coronary heart disease [4–7]. These studies suggest that regular physical activity may improve serum lipid levels. Hence, identifying the critical factors that increase physical activity is important.

Preceding studies have revealed that biological, psychosocial, or environmental factors are effective in increasing physical activity. According to a review by Bauman et al. [8], physical activity is determined in various dimensions. Sallis et al. [9] suggested an ecological model that defined many multi-level factors as determinants of physical activity and emphasized the interactions among these factors. In Japan, ecological models stressing self-efficacy, social support, and neighborhood environment, to increase physical activity, were proposed by Ishii et al. and Oka et al. [10, 11]. In addition, health behaviors of employees could be influenced by job-related factors. In occupational research, employees' positive feelings, such as vigor, were also found to be associated with physical activity [12–14]. In these studies, greater levels of vigor which was one of the positive feelings or which was a subordinate factor of work engagement [15] were related to higher levels of physical activity. A 20-year follow-up study conducted by Shirom et al. [16] suggested that vigor for work at baseline influenced physical activity and mortality 20 years later. Employees who are vigorous at work not only are likely to have vigorous lifestyles but also engage in physical activities in their personal lives.

The "job demands-resources model" [17] was proposed to enhance these positive aspects at the worksite and has drawn attention in the past decades. In this model, the "health impairment process" is considered important to promote the positive aspects, preventing burnout and negative health outcomes by reducing job demands [18]. The "motivational process," which promotes mental health by enriching job resources, has also gained significance. Several preceding studies have revealed that job resources such as job control, skill utilization, suitable jobs, and meaningfulness of work could promote employees' positive outcomes [19–21]. Job-related factors could promote or inhibit employees' health behaviors through work-home spillover effects [22, 23]. The workplace can be considered a suitable place to promote healthy behaviors in employees because they spend a considerable amount of time there [24, 25]. In addition, the ecological model [9] suggests that multi-level interventions, including not only the domestic environment but also the occupational environment, are effective in promoting physical activity. Therefore, it is important to investigate the association between job-related factors and physical activity. However, to the best of our knowledge, no study to date has assessed these relationships.

In addition, sex differences in these relationships have not been fully investigated. Because sex is an important biological risk factor for atherosclerotic cardiovascular disease [26, 27], men and women are likely to show a different pattern in these relationships. Additionally, although few studies have investigated the relationships between job-related factors and physical activity, sex differences in neighborhood environment and physical activity have been reported [28]. Thus, it is reasonable to consider the relationship between occupational environment and physical activity separately by sex. In this study, we analyzed the relationships among job resources, vigor, exercise habit (i.e., one aspect of physical activity), and serum lipids in Japanese employees in a manufacturing company. We employed a multiple group path analysis to assess sex differences in these relationships. This study could provide an opportunity to integrate previous evidence into one study by using multivariate analysis and demonstrate the effects of job resources on serum lipids. In addition, this study will encourage occupational health staff to pay attention to job-related factors in order to prevent coronary heart disease. It could thus be hypothesized that employees who have higher levels of job resources at worksites would be vigorous and have more intensive exercise habit. Increasing the intensity of exercise habit would in turn lead to improvement of serum lipid levels.

Methods

Participants

Data were collected at a medical checkup conducted by a manufacturing company that makes beverages in February and March of 2012. A total of 5141 employees were asked to participate in this study, and 4543 employees (men=4018, women=525, mean age=38.7, SD=9.9) who completed the examination and had no missing data during the analysis (valid response rate=88.4 %) were included in the study. We obtained informed consent from all participants before the medical checkup, through an instruction on the questionnaire. The instruction assured protection of personal information, citing that the data would only be used by health care professionals, and only anonymized data would be offered to others strictly for academic use. The study protocol was approved by the ethical committee of the University of Occupational and Environmental Health, Japan (No. H25-120).

Measurements

The self-answered questionnaire was distributed, and blood sample was collected from all participants simultaneously.

The questionnaire assessed the following fields.

Job Resources

The questions on job control (three items, e.g., "I can work at my own pace"), skill utilization (one item [reversed], "My knowledge and skills are rarely used at work"), suitable jobs (one item, "This job suits me well"), and meaningfulness of work (one item, "My job is worth doing") from the Brief Job Stress Questionnaire (BJSQ) were used for assessment [29]. All items were rated on a 4-point Likert scale, ranging from 1

(strongly disagree) to 4 (strongly agree). The internal consistency of job control in this study measured by Cronbach's alpha was 0.66.

Vigor

Vigor also was measured by questions from the BJSQ (three items, e.g., "I have been very active") [29]. The items were rated on a 4-point Likert scale, ranging from 1 (hardly) to 4 (almost). Cronbach's alpha of vigor was 0.89.

Exercise Habit

Exercise habit was measured by a single question that was scored along three points, "(1) do not exercise," "(2) do modest exercise (walking, golf, etc.)," and "(3) do intensive exercise (sports, match, etc.)." Participants were instructed to "please check a point that was most appropriate." A higher point meant a more intensive exercise habit.

Serum Lipids, i.e., Triglyceride, HDL-C, and LDL-C

Triglyceride, HDL-C, and LDL-C levels measured as part of the medical checkup were used. Blood samples were collected from antecubital veins, using fasting measurements. Participants were instructed in advance to fast before the medical checkup. Following the separation of serum, lipids were measured by an enzymatic procedure.

Analyses

Data were analyzed using a multiple group path analysis, stratified by sex (men=4018, women=525). Before the analysis, we conducted an ex ante analysis on all participants (N=4543) to compare our hypothesized model with the reversed relationships model between vigor and exercise habit (Fig. 1). After the ex ante analysis, we conducted the multiple group path analysis. First, four paths were drawn from job control, skill utilization, suitable jobs, and meaningfulness of work to vigor. Then, we drew a path from vigor to exercise habit. Finally, the three physiological outcomes (triglyceride, HDL-C, and LDL-C) were positioned as dependent variables of exercise habit. Covariances were assumed among four job resources.

We presumed equality constraints to parameters between male and female groups and constructed six different models. The greater the model number was, the stronger the constraints were. Model 1 was not constrained by any parameter. Constraints were added for paths coefficients in model 2, variances of job resources in model 3, covariances in model 5, and all parameters in model 6. In model 4, constraints were assumed partially for covariances (refer to Table 2 for details of model constraints). We adopted the best model, using

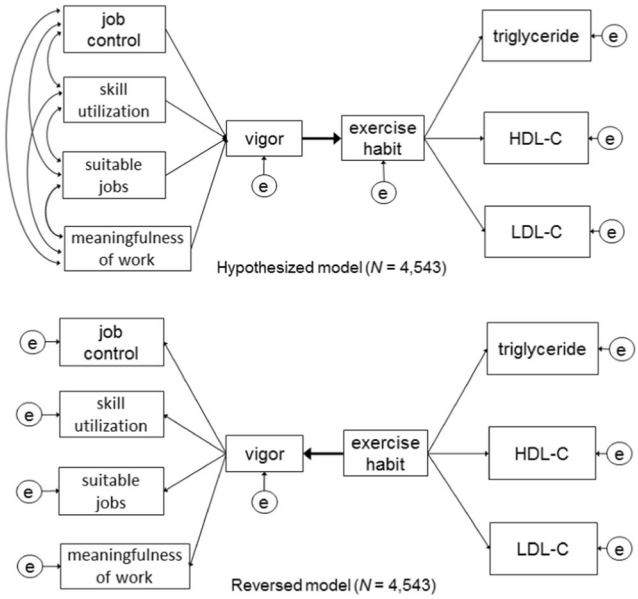

Fig. 1 Two models of the ex ante analysis on the association between vigor and exercise habit in all participants (N=4543)

results of a likelihood ratio test and model fit indices. We calculated the goodness of fit index (GFI), adjusted goodness of fit index (AGFI), root mean square error of approximation (RMSEA), and Akaike's information criterion (AIC) for the same. All paths in the model were standardized. SPSS and Amos 22.0 version were used for analyses.

Results

Internal Validity Analysis

At first, we conducted a t test and a χ^2 test between participants who completed the study and those who did not to confirm whether non-response bias was present. Employees who completed were significantly younger (M=38.7) than those who did not (M=45.3, t (5139)=14.65, p<0.05). In addition, the proportion of men in the completed group (88.4 %) was significantly higher than that in those who did not complete (42.3 %, χ^2 (1)=800.06, p<0.05).

Descriptive Statistics and Differences in Study Variables Between Sexes

Table 1 shows the descriptive statistics of variables measured in this study by sex. Mean scores of meaningfulness of work, vigor, and exercise habit in men were significantly higher than those in women. Regarding serum lipids, means of triglyceride and LDL-C were significantly higher in men than they were in women. On the other hand, mean HDL-C was significantly lower in men than it was in women.

Table 1 Descriptive statistics of age, job resources, vigor, exercise habit, and values of serum lipids, and mean differences between sexes (N=4543)

Variables	Mean (SD) men (N=4018)	Mean (SD) women (N=525)	t value (df=4541)
Age	38.73 (9.9)	38.04 (9.9)	1.52 n.s.
Job resources			
Job control (3 items)	8.06 (1.8)	8.03 (1.8)	0.40 n.s.
Skill utilization (1 item)	2.78 (0.7)	2.73 (0.7)	1.50 n.s.
Suitable jobs (1 item)	2.81 (0.7)	2.88 (0.8)	−1.92 n.s.
Meaningfulness of work (1 item)	2.79 (0.8)	2.71 (0.8)	2.26*
Vigor (3 items)	6.98 (2.2)	6.67 (2.3)	2.94*
Exercise habit (1 item)	1.64 (0.7)	1.45 (0.6)	6.11*
Serum lipids			
Triglyceride (mg/dl)	120.83 (107.2)	86.39 (252.9)	5.60*
HDL-C (mg/dl)	58.33 (14.9)	72.93 (17.3)	−20.66*
LDL-C (mg/dl)	120.98 (31.5)	110.91 (30.3)	6.91*

df degree of freedom, *n.s.* not significant

*p<0.05

Multiple Group Path Analysis

First, we conducted the ex ante analysis in all participants (N=4543). Path coefficients between vigor and exercise habit were almost identical between our hypothesized model (0.13, p<0.05) and the reversed model (0.11, p<0.05). However, the χ^2 value and AIC of our hypothesized model ($\chi^2(22)$=774.17, AIC=820.17) were slightly better than those of the reversed model ($\chi^2(22)$=781.13, AIC=827.13). Accordingly, we adopted our hypothesized model and conducted the multiple group path analysis.

Table 2 shows the results of the likelihood ratio test and model fit indices across the six models (with different equality constraints as described previously). Model 5 had a significantly worse model fit than model 4 (χ^2 (1)=6.46, p<0.05), as did model 6 in comparison to model 5 (χ^2 (5)=1064.43, p<0.05). Models 1, 2, 3, and 4 did not have any significant differences. In addition, model 4 had the strongest quality constraints to the parameters. Although model 4 had poorer GFI (0.956) than the other three models, its AGFI, RMSEA, and AIC were the best (AGFI=0.936, RMSEA=0.056, AIC=988.10). We prioritized statistical parsimony and adopted model 4 as the best model. Therefore, we estimated the covariance between suitable jobs and meaningfulness of work, and residual variances between the groups.

Results of the multiple group path analysis using job resources, vigor, exercise habit, and serum lipids are shown in Fig. 2 (men) and Fig. 3 (women). Most parameters estimated in this model were equal between the groups. All paths in the model were standardized.

Table 2 Likelihood ratio test and model fit indices of models with equality constraints of parameters (grouping=men and women)

Model	Equality constraints	χ^2 (df)	$\Delta\chi^2$ (df)	GFI	AGFI	RMSEA	AIC
1	No constraints (free estimation)	912.41 (44)	–	0.957	0.913	0.066	1004.41
2	Path coefficients	921.13 (52)	10.89 (8) n.s.	0.957	0.925	0.061	997.13
3	Variances of job resources	923.30 (56)	2.16 (4) n.s.	0.957	0.930	0.058	991.30
4	Covariances of job resources except between suitable jobs and meaningfulness of work	930.10 (61)	6.81 (5) n.s.	0.956	0.936	0.056	988.10
5	Covariances of job resources	936.56 (62)	6.46 (1)*	0.956	0.936	0.056	992.56
6	Residual variances	2000.99 (67)	1064.43 (5)*	0.875	0.833	0.080	2046.99

Note. We used Amos version 22.0 for model comparison

df degree of freedom, *n.s.* not significant

*p<0.05

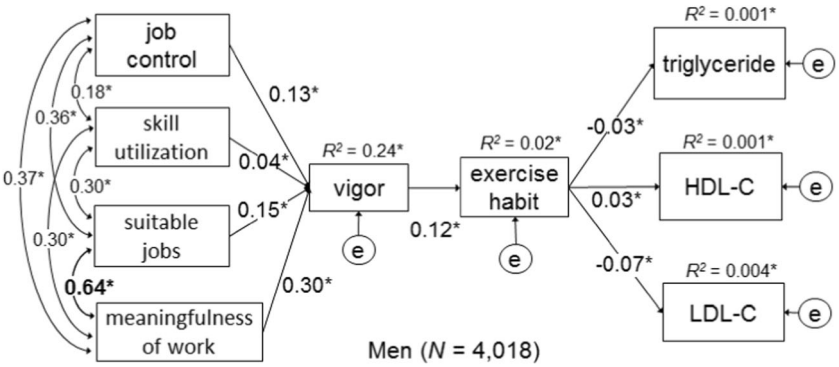

Fig. 2 Results of the multiple group path analysis using job resources, vigor, exercise habit, and serum lipid levels in men. Note: All paths were standardized. Parameters differing between the groups are shown in *bold*. GFI = 0.956, AGFI = 0.936, RMSEA = 0.056, AIC = 988.10. *$p<0.05$

All paths from job resources to vigor were significantly positive in both sexes. Of the job resources, meaningfulness of work had the strongest association with vigor (0.30 in men and 0.27 in women). All covariances among job resources were positively associated with each other, with suitable jobs and meaningfulness of work having the strongest association (0.64 in men and 0.58 in women). The four job resources explained 24 % of the variance of vigor in men and 19 % in women ($ps<0.05$). The path coefficients from vigor to exercise had significant positive relationships (0.12 in men and 0.14 in women). Exercise habit and triglyceride had significant negative relationships in both men and women (−0.03 in men and −0.01 in women). LDL-C had significant negative relationships in both sexes (−0.07). Further, exercise habit and HDL-C had significant positive relationships in both sexes (0.03). Exercise habit could explain 0.0–0.4 % of the variances of serum lipids in both sexes ($ps<0.05$).

Discussion

The purpose of this study was to analyze the relationships among job resources, vigor, exercise habit (i.e., one aspect of physical activity), and serum lipids in Japanese employees. We also analyzed sex differences of these relationships, using the multiple group path analysis. Results indicated that a comprehensive series of relationships from job resources, through vigor and exercise habit, to serum lipid levels, exist in both sexes, integrating previous evidence into one study. Higher levels of job resources were associated with greater vigor, vigor to more intensive exercise habit, and this in turn led to improve serum lipid levels in both sexes.

The associations between exercise habit and serum lipid levels were in line with the preceding studies [4–7]. Our results may provide additional evidence that more intensive exercise habit likely improves serum lipid levels in both sexes. However, R^2 values of serum lipids were very small (0.000–0.004). Although these values were statistically significant, the explanatory power of exercise habit was not high. Accordingly, many other factors, in addition to exercise habit, could be associated with serum lipids. Lower level of dyslipidemia among the Japanese in comparison with the European or American population may also explain the weak associations found in this study. For example, the prevalence of abnormal HDL-C levels in Japan was 8.3 % [30], which was much lower than those in European countries and in the USA (30–49 %) [31–33]. Therefore, associations between exercise habit and serum lipids may be weaker in our sample, owing to the fact that majority of our sample had healthy values in serum lipids.

Vigor and exercise habit had a significant positive association in both sexes. In addition, our hypothesized direction model had slightly better model fit than did its reversed model in the ex ante analysis. Vigor has been found to positively

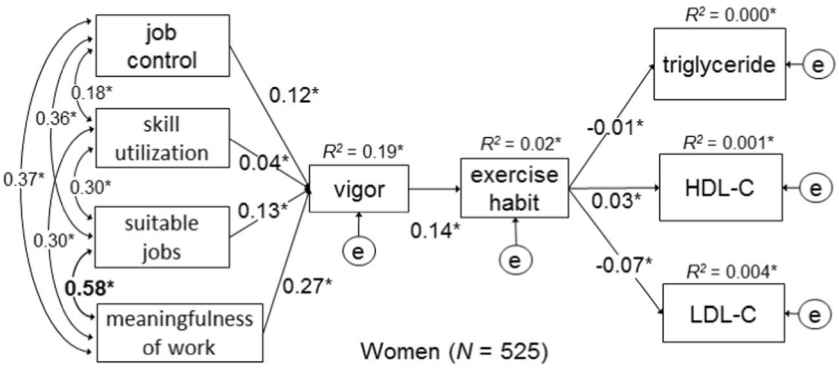

Fig. 3 Results of the multiple group path analysis using job resources, vigor, exercise habit, and serum lipid levels in women. Note: All paths were standardized. Parameters differing between the groups are shown in *bold*. GFI=0.956, AGFI=0.936, RMSEA=0.056, AIC=988.10. *$p<0.05$

predict employees' good health and proactive behaviors in occupational health studies [34]. It has been suggested that one of the major reasons for this association is that people who are vigorous could be more motivated for physical activity so that they can experience an enhanced vigorous feeling [16, 35]. Therefore, although several studies have mentioned reverse, reciprocal, or other relationships between physical activity and vigor [36–38], being vigorous is also likely to be a determinant of intensive exercise habit. Because this study was cross-sectional, further investigation is needed to establish a causal relationship among these variables.

All four job resources had a positive relationship with vigor in both sexes. This finding validates the results of preceding studies [17, 19–21] and our hypothesis. An increase in job control, which was one of the job resources measured in this study, has been suggested to be predictive of an increase in vigor [19]. It is likely that employees with higher levels of job resources are better able to choose adequate strategies to handle their tasks and reach their goals, and thus have high vigor [20].

Meaningfulness of work, in particular, had a strong association with vigor. Meaningfulness of work, considered to be an intrinsic reward for employees [21], has been proposed to play an important role in increasing positive affect and vigor [20]. A sense of significance for employees' task could serve to energize, reinforce, and maintain work behavior, and may be associated with vigor [39].

Contrary to our expectations, these relationships did not differ between men and women. Although mean scores were significantly different, especially for HDL-C, our results indicated that these associations are almost identical. Sex differences in the effect of exercise or physical activity on improving serum lipid levels have often been discussed [40, 41]. For example, Skoumas et al. [41] indicated that the benefits of being physically active on serum lipids were significant in women but not in men. However, physical activity with sufficient amount, frequency, and duration improved various kinds of serum lipids, regardless of sex [6, 42]. For example, Suzuki et al. [42] revealed that a physical activity routine of 180 min/week had beneficial effects on serum lipids in both sexes, but one of 90 min/week had beneficial effects only in women. Our study could suggest that the association between exercise habit intensity and serum lipid levels is linear, and that more intensive exercise habit is associated with lower triglyceride and LDL-C and higher HDL-C, regardless of sex. The relationships between job resources, vigor, and exercise habit did not differ by sex. This is inconsistent with findings from a community-based survey [28] that showed sex difference in the patterns of relationships between neighborhood environment and physical activity. This discrepancy may be explained by the fact that all participants in our study were employed, while the community-based survey [28] included unemployed people. The unemployment rate in the community-based survey [28] was much higher among women (48.9 %) than among men (16.7 %). Because unemployed people may be more exposed to neighborhood environment than employed people, nonworking women may be more influenced by neighborhood environment than men outside the home. Therefore, the sex difference in the relationship between environment and physical activity observed in the community-based survey [28] may be explained by the sex difference in employment status. Sex differences in the relationship between environment and physical activity may be much smaller among the working population than in the general population.

Although our study design was cross-sectional, it has several practical implications. Enhancing job resources could improve exercise habit and thereby improve serum lipid levels. This possibility, suggested by our study, could encourage occupational health staff to pay attention to job-related factors in order to prevent coronary heart disease. Kouvonen et al. [43] suggested that lack of job resources could spill over to leisure time and may be connected to feelings of helplessness, and insufficient physical activity. Job resources may improve exercise habit through a positive affect like vigor. Although interventions in job resources in order to promote employees' health have been already conducted [44], only few have tried to promote physical activity. In studies focusing on other factors related to physical activity [45], employees are likely to be reluctant to put additional efforts due to time constraints [46]. On the other hand, job resources and employees' vigor are primarily effective in improving performance and commitment towards work and productivity [47], thus directly benefitting the organization.

This study being cross-sectional, a causal relationship could not be established. In particular, the causal relationship between vigor and exercise habit needs to be further investigated. The generalizability of our results needs to be further examined, given that the sample consisted of predominantly male employees from a single company. Available job resources may differ considerably across companies, worksites, job types, and departments. In addition, the small percentage of women (11.6 %) in this study may make it difficult to accurately compare sex differences in the relationships. The internal validity of this study could be questionable due to non-response bias, as participants who completed this study were significantly younger and predominantly male. The path coefficients may have been confounded by age and educational, economic, and marital status. We did not include these covariates in order to avoid complexity of the model. The validity and reliability of the scales used in this study could be questioned, as a single item measured some variables. In particular, we did not measure the frequency, duration, and maintenance of exercise habit. The present findings may also be explained by an incorrect measurement of exercise habit. The frequency, duration, and maintenance of exercise habit

might have led to more accurate measurement. The internal consistency of job control was not too high (Cronbach's alpha=0.66). These limitations need to be addressed in future studies. Moreover, physical activity is constituted not only by exercise in leisure time. The Japan Ministry of Health, Labour and Welfare [3] suggests that all types of activities are effective for health. Future studies also need to assess how worksite support can be effective even to marginally increase physical activity of employees.

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Compliance with Ethical Standards

Informed Consent All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all participants for being included in the study.

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

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Original

Changes in the psychosocial work characteristics and insulin resistance among Japanese male workers: a three-year follow-up study

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Abstract: Objective: This study investigated the impact of changes in psychosocial work characteristics on insulin resistance (IR) among Japanese male workers. **Methods:** Subjects were 1,815 male workers who received a comprehensive health examination and requested measurement of their serum insulin level in Fiscal Years (FY) 2008 and 2011. Psychosocial work characteristics, including job demands, job control, and workplace social support (from supervisors and coworkers), were assessed in each of the job demands-control and demand-control-support models. Psychosocial work characteristics were assessed by the Brief Job Stress Questionnaire. Changes in the psychosocial work characteristics were measured by creating a four-category variable for each of the psychosocial work characteristics: (1) stable low group, (2) increased group, (3) decreased group, and (4) stable high group. We defined IR as a value of 2.5 or more on the homeostasis model assessment of insulin resistance (HOMA-IR), or having a diagnosis of diabetes. A series of multiple logistic regression analyses were conducted. **Results:** The group experiencing a decrease in supervisor support had a significantly higher risk of having IR compared to the stable high group with an odds ratio (OR) of 2.44; 95% CI: 1.48-4.02. After adjusting for covariates, this significant association was unchanged; the OR was 2.19; 95% CI: 1.23-3.91. On the other hand, there was no significant association of changes in the psychosocial work characteristics, expect for decrease in supervisor support, with IR. **Conclusions:** A decrease in supervisor support was found to be an independent risk factor for worsening IR.
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Key words: Homeostasis model assessment of insulin resistance (HOMA-IR), Japan, Job demands-control (JD-C) model, Longitudinal studies, Psychosocial work characteristics, Type 2 diabetes

Introduction

The number of people with Type 2 diabetes is growing rapidly worldwide. According to the International Diabetes Federation (IDF) report[1,2], 387 million (8.3%) adults have been diagnosed with diabetes (including Type 1 and Type 2 diabetes) worldwide. Furthermore, the number of people with diabetes will exceed 592 million within 25 years. The Western Pacific Region, including Japan, has the largest number (138 million) of diabetic adults, and the number of diabetic adults in this region is expected to increase to 218 million by 2035. In Japan, 7.2 million people have diabetes and Japan has the 10th highest prevalence rate of diabetes in the world. Diabetes imposes a large economic burden on individuals and their families, on national health systems, and on countries. Health spending on diabetes accounted for 11% of total health expenditures worldwide in 2014[2]. Global health spending to treat diabetes and manage complications totaled at least USD 612 billion in 2014[2]. Notably, half of all adults with diabetes are between the ages of 40 and 59 years and almost all diabetic adults in this age group suffer from Type 2 diabetes[3]. This middle-aged group, who are in the

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prime of life, will continue to comprise the greatest number of people with diabetes in the coming years. Therefore, the prevention of diabetes among middle-aged people is important in the workplace.

Preceding studies have investigated the risk factors for Type 2 diabetes, including obesity[4], physical inactivity[5,6], smoking[7], heavy alcohol use[8], and inadequate duration of sleep[9]. In addition, psychosocial stress resulting from specific psychosocial characteristics in occupational settings has also been hypothesized to increase the risk of Type 2 diabetes[10]. To explain the effects of psychosocial work characteristics on Type 2 diabetes, two kinds of underlying mechanisms are hypothesized. The first mechanism known as a "direct effect", suggests that psychosocial stress has been linked to increasing serum glucose levels and poor glucose tolerance among diabetic patients[11]. The second mechanism known as an "indirect effect," suggests that psychosocial stress has been linked to well-established Type 2 diabetes risk factors, such as obesity[12], metabolic syndrome[13], smoking, alcohol consumption, and physical inactivity[14].

Studies investigating the impact of psychosocial work characteristics on Type 2 diabetes or glucose tolerance, have been conducted using either the job demands-control (JD-C) model or the demand-control-support (DCS) model, which includes measures of job demands, job control, supervisor support, and coworker support[15,16]. These studies have been conducted both in domestic and overseas settings, however, their findings have been inconsistent and inconclusive[10]. For example, a cross-sectional study of Japanese male workers showed a significant association of job strain (i.e., the combination of high job demands and low job control) and workplace social support with hemoglobin A_{1c} (HbA_{1c})[17]. On the contrary, another study of Japanese male workers failed to show a significant association of psychosocial work characteristics with Type 2 diabetes assessed by an oral glucose tolerance test[18]. In foreign countries (i.e., other than Japan), two longitudinal studies showed a significant association of high job strain with self-reported or doctor diagnosed Type 2 diabetes[19,20]. Two other longitudinal studies showed a significant association of high job strain with Type 2 diabetes assessed by an oral glucose tolerance test among women[21,22]. Furthermore, two cross-sectional studies showed a significant association of high job strain and low job control with Type 2 diabetes among women[23,24]. However, other three longitudinal and cross-sectional studies failed to show a significant association of psychosocial work characteristics with Type 2 diabetes among men and women[25-27].

In addition to this inconclusive evidence for the association of psychosocial work characteristics with Type 2 diabetes or glucose tolerance, all of the longitudinal studies introduced above only assessed the psychosocial work characteristics at a single point in time (i.e., baseline) even though these characteristics may change over time. Therefore, when we focus on the psychosocial work characteristics associated with Type 2 diabetes or glucose tolerance, "time-dependent change" of these characteristics should be taken into account.

Furthermore, early diagnosis and treatment of Type 2 diabetes are the most important ways to prevent its progression and its associated complications. Insulin resistance (IR) occurs prior to the onset of Type 2 diabetes; therefore, improving IR may delay or prevent the onset and/or progression of Type 2 diabetes and identifying the psychosocial work characteristics associated with IR is of great importance for workers. In order to quantify IR, the gold standard is the glucose clamp technique. However, this technique is procedurally complicated and is difficult to complete[28]. Alternatively, a homeostasis model assessment of insulin resistance (HOMA-IR) has been introduced as one of the most convenient indices to determine IR level. HOMA-IR has a strong correlation with the results of the glucose clamp technique[29]. HOMA-IR has been used as a measure of IR to determine the association of IR with the onset of coronary heart disease[30] as well as to determine the association between IR and circulating adipocytokines such as plasma resistin or leptin[31]. To the best of our knowledge, however, the association of psychosocial work characteristics with IR measured by HOMA-IR has not been fully investigated.

The purpose of this study was to investigate the impact of changes in the psychosocial work characteristics on IR. Changes in psychosocial work characteristics were measured using the JD-C and DCS models over three years. We hypothesized that; (1) workers who experienced a favorable change in psychosocial work characteristics would decrease their risk of elevating their level of IR, and (2) workers who experienced an unfavorable change in psychosocial work characteristics would increase their risk of elevating their level of IR.

Methods

Participants

Participant data was collected from annual comprehensive health examinations conducted on workers in a health care center in the Kanto (east coast) region of Japan in fiscal years (FY) 2008 and 2011. A comprehensive health examination has been conducted on workers for 35 years or more in this health care center, and at the time of examination, the examinees could request a check on their serum insulin level. The data were collected on serum analysis of IR, a physical examination, and a self-administered questionnaire, which included scales on job demands, job control, and social support in the workplace.

Due to the small sample size of female workers, we used the health examination data of male workers only,

which included the serum insulin level measured in FY 2008 and 2011. At baseline (FY 2008), 29,586 male workers underwent a comprehensive health examination, and of these, a total of 6,128 workers requested to measure the serum insulin level. Of 6,128 workers, 1,359 workers were excluded from the study for the following reasons: past history of diabetes, fasting plasma glucose (FPG) level ≥126 mg/dL and HbA_{1c} ≥6.5 %, as indicated by the National Glycohemoglobin Standardization Program (NGSP) units (diagnostic criteria for diabetes)[32], or an IR value ≥2.5, as measured by HOMA-IR. Moreover, we excluded 520 shift workers, who are reported to have a higher risk of diabetes[33]. After further excluding 877 workers who had one or more missing questionnaire responses, 3,372 workers were eligible for a follow-up survey. Of 3,372 workers, 1,816 workers requested to measure the serum insulin levels at the time of the follow-up survey, in FY 2011. Furthermore, we excluded one worker who had FPG levels of 140 mg/dL or more, because the association of FPG with IR is weakened when the FPG is 140 mg/dL or more[32]. Therefore, the number of study participants was 1,815. Compared to the final sample (n=1,815), the dropout sample (n=1,557) had significantly higher prevalence of current smokers, lower alcohol consumption, lower BMI, higher HbA_{1c}, and lower job demands. Demographic and occupational characteristics and lifestyle behaviors of participants are shown in Table 1. Glucose metabolism and psychosocial work characteristics of participants are shown in Table 2.

The study protocol was approved by the Ethics Committee of the Hitachi, Limited Ibaraki Hospital Group (Ibaraki, Japan) in 2008 and 2011. Written informed consent was obtained from all participants.

Measures

1) Psychosocial work characteristics

Based on the JD-C or DCS model[15,16], psychosocial work characteristics included job demands, job control, and workplace social support (i.e., supervisor support and coworker support). We assessed psychosocial work characteristics using the Brief Job Stress Questionnaire (BJSQ)[34]. The BJSQ includes four three-item scales: (1) the job demands scale (Cronbach's α coefficient was 0.67 and 0.72 at baseline and follow-up, respectively), (2) the job control scale (Cronbach's α coefficient was 0.78 and 0.77 at baseline and follow-up, respectively), (3) the supervisor support scale (Cronbach's α coefficient was 0.84 and 0.85 at baseline and follow-up, respectively), and (4) the coworker support scale (Cronbach's α coefficient was 0.79 and 0.82 at baseline and follow-up, respectively), each with a response range of 3-12. We also calculated the job demands/control ratio (range 0.25-4.00) to quantify the degree of job strain[35]. High exposure to job control and workplace social support, and low exposure to job demands and job strain were considered beneficial. The participants were dichotomized into high and low groups relative to the median of each scale score or job demands/control ratio at baseline and follow-up, respectively.

According to a preceding study[36], changes in psychosocial work characteristics were measured by creating a four-category variable for each psychosocial work characteristic: (1) stable low group (low group at both baseline and follow-up), (2) increased group (low group at baseline with high group at follow-up), (3) decreased group (high group at baseline with low group at follow-up), and (4) stable high group (high group at both baseline and follow-up). We defined the decreased group as a favorable change group in terms of job demands and job strain, whereas we defined the increased group as a favorable change group in terms of job control, supervisor support, and coworker support. Detailed demographic and occupational characteristics and lifestyle behaviors of participants at baseline, according to changes in psychosocial work characteristics, are shown in Appendices A-E.

2) Glucose metabolism

All participants were assessed for FPG, HbA_{1c}, and immuno-reactive insulin (IRI) levels. We calculated HOMA-IR using the HOMA model (HOMA-IR = FPG [mg/dL] * IRI [μU/mL] / 405)[29]. Participants were dichotomized using the recommended cut-off value of HOMA-IR for the Japanese population[32] into those with IR (≥2.5 on HOMA-IR) and those without IR (<2.5 on HOMA-IR).

The quality of each biochemical test was assessed by internal and external quality control methods. For the internal quality control method, we first calculate the mean (M), standard deviation (SD), and coefficient variation (CV) for the control sample. Subsequently, we measured the control sample daily before measuring the specimen sample to check the difference between M, SD, and CV scores of the specimen sample and the control score set in advance. FPG was measured using the electrode method (GA082, A&T Corporation, Kanagawa, Japan) and the reagent was calibrated once per day. HbA_{1c} was measured by high performance liquid chromatography (HPLC) method (G9, Tosoh Corporation, Tokyo, Japan) and the reagent was calibrated once per week. IRI was measured by the chemiluminescence immunoassay (CLIA) method (i1000SR, Abbott Japan, Co., Ltd, Tokyo, Japan) and the reagent was calibrated once a month. For the external quality control method, we measured M, SD and CV in control samples sent from quality control organizations (e.g., Japan Medical Association, Tokyo, Japan) and reported the results to the organizations. Subsequently, we received feedback from the organizations on the M, SD, and CV scores.

3) Other covariates

Other covariates included demographic characteristics (i.e., age and marital status), occupational characteristics

Table 1. Demographic and occupational characteristics and lifestyle behaviors of participants

	Total (n=1,815)	
	Mean (SD)	n (%)
Baseline (FY 2008)		
Age	50.16 (7.58)	
35-39 years old		215 (11.8)
40-49 years old		538 (29.6)
50-59 years old		942 (51.9)
60 years old or more		120 (6.6)
Marital status		
Currently married		1581 (87.1)
Never married		187 (10.3)
Divorced/widowed		47 (2.6)
Department		
Design engineering department		538 (29.6)
Inspection department		197 (10.9)
Production assembling department		346 (19.1)
Production control department		144 (7.9)
Transportation department		17 (0.9)
General affairs department		279 (15.4)
Sales department		51 (2.8)
Data input department		4 (0.2)
Research department		85 (4.7)
Medical department		4 (0.2)
Service department		13 (0.7)
Others		137 (7.5)
Employment position and occupation		
Manager		767 (42.3)
Main career track		552 (30.4)
General clerk		107 (5.9)
Non-clerical workers		360 (19.8)
Others		29 (1.6)
Smoking history		
Non smoker		1146 (63.1)
Current smoker		669 (36.9)
Alcohol consumption [g/wk]	126.48 (121.52)	
0-44		663 (36.5)
45-154		554 (30.5)
155 or more		598 (32.9)
Exercise habits		
Yes		738 (40.7)
No		1077 (59.3)
Sleeping hours		
<5 hours		104 (5.7)
≥5 hours to <6 hours		808 (44.5)
≥6 hours to <7 hours		740 (40.8)
≥7 hours		163 (9.0)
Body mass index [kg/m^2]	23.67 (2.54)	
Low (22.5 or less)		601 (33.7)
Middle (22.6-24.6)		615 (33.9)
High (24.7 or more)		589 (32.5)

Table 1. Demographic and occupational characteristics and lifestyle behaviors of participants (continued)

	Total (n=1,815)	
	Mean (SD)	n (%)
Follow-up (FY 2011)		
Smoking history		
Non smoker		1295 (71.3)
Current smoker		520 (28.7)
Alcohol consumption [g/wk]	125.70 (123.33)	
0-44		581 (32.0)
45-154		652 (35.9)
155 or more		582 (32.1)
Exercise habits		
Yes		744 (41.0)
No		1071 (59.0)
Sleeping hours		
<5 hours		107 (5.9)
≥5 hours to <6 hours		788 (43.4)
≥6 hours to <7 hours		725 (39.9)
≥7 hours		195 (10.7)

(i.e., department and employment position and occupation), psychosocial work characteristics (i.e., job demands, job control, supervisor support, and coworker support), lifestyle behaviors (i.e., smoking history, alcohol consumption, exercise habits, and sleeping hours), and body mass index (BMI) at baseline, and changes in lifestyle behaviors during the follow-up period. Except for the BMI, these covariates were assessed using a self-administered questionnaire.

Age was classified into four groups: 35-39 years old, 40-49 years old, 50-59 years old, and 60 years old or older. Marital status was classified into three groups: currently married, never married, and divorced or widowed. Department was classified into 12 groups using the original classification in the questionnaire (see Table 1). Employment position and occupation was classified into five groups: manager, main career track, general clerk, non-clerical workers, and others. Psychosocial work characteristics at baseline, such as scores of job demands, job control, supervisor support, and coworker support, were used as continuous variables. Smoking history was classified into two groups: non smoker and current smoker. Alcohol consumption was classified into three groups using the tertile: 44 g/wk or less, 45-154 g/wk, and 155 g/wk or more. Exercise habits were classified into two groups: yes or no. Sleeping hours were classified into four groups: <5 hours, ≥5 hours to <6 hours, ≥6 hours to <7 hours, and ≥7 hours. BMI was classified into three groups using the tertile: 22.5 kg/m^2 or less, 22.6-24.6 kg/m^2, and 24.7 kg/m^2 or more. Changes in lifestyle behaviors were classified into three or four categories using data from each lifestyle behavior at baseline and follow-up. Changes in smoking history were classified into four groups: continuing smoker, continuing non-smoker, quitter, and initiator or relapsed quitter. Changes in alcohol consumption were classified into three groups: no change, increased, and decreased. Changes in exercise habits were classified into four groups: continual exercising, never exercised, stopped exercising, and commenced exercise. Changes in sleeping hours were classified into three groups: no change, increased, and decreased.

Statistical analysis

According to a preceding study[36], using the stable low group or stable high group as a reference, a series of multiple logistic regression analyses were conducted to estimate the ORs and 95% confidence intervals (CIs) of IR (defined as having a diagnosis of diabetes, meeting the diabetes diagnostic criteria described earlier, or having a value of 2.5 or more on HOMA-IR at follow-up) for increased or decreased group of each psychosocial work characteristic. In the analyses, we first calculated the crude ORs (i.e., without any adjustment) (Model 1). We then adjusted for demographic characteristics (i.e., age and marital status) (Model 2), and subsequently for occupational characteristics (i.e., department and employment position and occupation) (Model 3), for psychosocial work characteristics at baseline (i.e., scores of job demands, job control, supervisor support, and coworker support) (Model 4), for lifestyle behaviors at baseline (i.e., sleeping hours, smoking history, alcohol consumption, and exercise habits) (Model 5), for BMI (Model 6), and

Table 2. Glucose metabolism and psychosocial work characteristics of participants (n=1,815)

Glucose metabolism	Baseline		Follow-up	
	Mean (SD)		Mean (SD)	
Fasting plasma glucose (FPG) [mg/dl]	99.66 (7.99)		105.27 (9.31)	
Hemoglobin A_{1c} (HbA_{1c}) [%]	5.26 (0.28)		5.20 (0.37)	
Immuno-reactive insulin (IRI) [μU/ml]	5.00 (2.10)		5.40 (2.40)	
Homeostasis model assessment-insulin resistance (HOMA-IR)	1.24 (0.54)		1.42 (0.68)	
Scale scores (BJSQ)†	Mean (SD)	Cronbach's α	Mean (SD)	Cronbach's α
Job demands	8.39 (1.82)	0.67	7.98 (1.94)	0.72
Job control	9.00 (1.93)	0.78	9.20 (1.86)	0.77
Job strain (job demands/control ratio)	1.00 (0.42)	–	0.93 (0.41)	–
Supervisor support	7.43 (1.87)	0.83	7.38 (1.87)	0.85
Coworker support	7.86 (1.67)	0.79	7.83 (1.73)	0.82

† BJSQ, Brief Job Stress Questionnaire.

Table 3. Prevalence of insulin resistance at follow-up by changes in psychosocial work characteristics†

	n	No. of case (%)
Job demands		
Stable low	735	56 (7.6)
Increased	165	6 (3.6)
Decreased	337	32 (9.5)
Stable high	578	42 (7.3)
Job control		
Stable low	971	81 (8.3)
Increased	250	12 (4.8)
Decreased	183	10 (5.5)
Stable high	411	33 (8.0)
Job strain (job demands/control)		
Stable low	715	58 (8.1)
Increased	169	8 (4.7)
Decreased	307	26 (8.5)
Stable high	624	44 (7.1)
Supervisor support		
Stable low	749	57 (7.6)
Increased	220	11 (5.0)
Decreased	250	33 (13.2)
Stable high	596	35 (5.9)
Coworker support		
Stable low	889	67 (7.5)
Increased	223	15 (6.7)
Decreased	216	21 (9.7)
Stable high	487	33 (6.8)

† Insulin resistance was defined as a value of 2.5 or more on the HOMA-IR at follow-up.

finally for changes in lifestyle behaviors (Model 7). The level of significance was 0.05 (two-tailed). Statistical analyses were performed using IBM SPSS Statistics version 22.

Results

The mean score of HOMA-IR was 1.24 (SD=0.54) at baseline and 1.42 (SD=0.68) at follow-up, respectively (Table 2). The prevalence of workers with IR at follow-up was 7.5% (n=136). Of 136 workers with IR, 111 workers had a value of 2.5 or more on HOMA-IR, nine workers had a diagnosis of diabetes, two workers met the diabetes diagnostic criteria, and 14 workers met these requirements redundantly. The prevalence of IR at follow-up by changes in psychosocial work characteristics is shown in Table 3.

For supervisor support, the multiple logistic regression analyses revealed that the decreased group had a significantly higher OR for IR compared to the stable high group (Model 1) (OR=2.44; 95% CI: 1.48-4.02) (Table 4). This pattern was unchanged after adjusting for demographic characteristics (Model 2), occupational characteristics (Model 3), psychosocial work characteristics at baseline (Model 4), and lifestyle behaviors at baseline (Model 5). After also adjusting for BMI and changes in lifestyle behaviors, the association was attenuated but still statistically significant (Models 6 and 7).

For job control, the increased group had a marginally significantly lower OR for IR compared to the stable low group (OR=0.55; 95% CI: 0.30-1.03). After adjusting for covariates (Models 2-7), however, this association was no longer marginally significant.

There was no significant association of change in job demands, job strain (job demands/control), or coworker support with IR before or after adjusting for any covariates.

Table 4. Association of changes in psychosocial work characteristics with insulin resistance: logistic regression analysis†

	Odds ratio (95% confidence interval)			
	Model 1‡	Model 2§	Model 3 ‖	Model 4¶
Job demands				
Increased [a]	0.46 (0.19-1.08)	0.45 (0.19-1.07)	0.45 (0.19-1.09)	0.47 (0.19-1.14)
Decreased [b]	1.34 (0.83-2.17)	1.29 (0.79-2.12)	1.28 (0.77-2.13)	1.26 (0.75-2.13)
Job control				
Increased [a]	0.55 (0.30-1.03)	0.59 (0.32-1.11)	0.59 (0.31-1.12)	0.55 (0.29-1.06)
Decreased [b]	0.66 (0.32-1.37)	0.75 (0.36-1.58)	0.79 (0.37-1.68)	0.76 (0.35-1.65)
Job strain (job demands/control)				
Increased [a]	0.56 (0.26-1.20)	0.56 (0.25-1.21)	0.56 (0.25-1.22)	0.56 (0.25-1.25)
Decreased [b]	1.22 (0.74-2.02)	1.33 (0.79-2.24)	1.25 (0.73-2.15)	1.29 (0.74-2.27)
Supervisor support				
Increased [a]	0.64 (0.33-1.24)	0.65 (0.33-1.28)	0.65 (0.33-1.28)	0.66 (0.33-1.31)
Decreased [b]	2.44 (1.48-4.02)	2.45 (1.48-4.06)	2.35 (1.40-4.00)	2.59 (1.50-4.46)
Coworker support				
Increased [a]	0.89 (0.50-1.58)	0.85 (0.48-1.53)	0.86 (0.48-1.56)	0.84 (0.46-1.54)
Decreased [b]	1.48 (0.84-2.63)	1.45 (0.81-2.59)	1.53 (0.84-2.80)	1.54 (0.83-2.88)

	Odds ratio (95% confidence interval)		
	Model 5**	Model 6††	Model 7‡‡
Job demands			
Increased [a]	0.44 (0.18-1.08)	0.46 (0.19-1.15)	0.49 (0.20-1.24)
Decreased [b]	1.22 (0.72-2.06)	1.16 (0.68-1.99)	1.13 (0.65-1.97)
Job control			
Increased [a]	0.55 (0.28-1.06)	0.58 (0.30-1.13)	0.67 (0.34-1.33)
Decreased [b]	0.79 (0.36-1.73)	0.75 (0.34-1.68)	0.78 (0.34-1.79)
Job strain (job demands/control)			
Increased [a]	0.52 (0.23-1.18)	0.57 (0.25-1.28)	0.57 (0.25-1.31)
Decreased [b]	1.23 (0.70-2.17)	1.22 (0.68-2.17)	1.24 (0.68-2.29)
Supervisor support			
Increased [a]	0.66 (0.33-1.32)	0.68 (0.34-1.38)	0.66 (0.32-1.37)
Decreased [b]	2.40 (1.37-4.19)	2.18 (1.24-3.86)	2.19 (1.23-3.91)
Coworker support			
Increased [a]	0.86 (0.47-1.59)	0.90 (0.48-1.67)	0.93 (0.49-1.75)
Decreased [b]	1.54 (0.82-2.91)	1.76 (0.92-3.39)	1.78 (0.90-3.49)

† Insulin resistance was defined as having a score of 2.5 or more on HOMA-IR at follow-up.
‡ Crude (i.e., without any adjustment).
§ Adjusted for demographic characteristics (i.e., age and marital status).
‖ Additionally adjusted for occupational characteristics (i.e., job department, employment position and occupation).
¶ Additionally adjusted for psychosocial work characteristics at baseline.
** Additionally adjusted for lifestyle behaviors (i.e., sleeping hours, smoking history, alcohol consumption, and exse habits) at baseline.
†† Additionally adjusted for body mass index.
‡‡ Additionally adjusted for changes in lifestyle behaviors.
[a] Comparison group is stable low group.
[b] Comparison group is stable high group.

Discussion

In this study, we found a significant association between decreasing supervisor support and IR. This significant association was unchanged after adjusting for any covariates. There was no significant association observed between changes in the other psychosocial work characteristics and IR.

In the present study, the group that experienced a decrease in supervisor support had a significantly higher OR for IR compared to the group in which supervisor support remained stable and high. This finding is consistent with a preceding cross-sectional study of Japanese male workers, which showed a negative and significant association of workplace social support with HbA_{1c}[17]. The present study replicated this evidence using a longitudinal design especially in the situation where unfavorable change in supervisor support occurs.

As we introduced earlier, the mechanism of the effect of psychosocial stress on Type 2 diabetes has been hypothesized to have one of two effects, and these include a "direct effect" or an "indirect effect"[11-14]. We investigated these effects by adjusting for lifestyle behavior at baseline and for BMI, as well as for changes in lifestyle behaviors as covariates, in a series of multiple logistic regression analyses. As a result, the significant association of decreasing supervisor support with IR was observed even after adjusting for lifestyle behaviors at baseline (Model 5) (OR=2.40; 95% CI: 1.37-4.19), BMI (Model 6) (OR= 2.18; 95% CI: 1.24-3.86), and changes in lifestyle behaviors (Model 7) (OR=2.19; 95% CI: 1.23-3.91) while the association was slightly attenuated compared to Model 4 (OR=2.59; 95% CI: 1.50-4.46). Similar trends were observed in a preceding study on UK civil servants[21]. These findings suggest that the association of decreasing supervisor support with increasing IR is partially mediated by BMI and lifestyle behaviors as well as by their time dependent changes, and that such a mediation (or indirect) effect is minimal.

In contrast, we found no significant association of increasing supervisor support with IR. This finding may be explained by a traditional two-factor theory, sometimes known as Herzberg's motivation-hygiene theory[37]. In this theory, hygiene factors, including the workers relationship with a supervisor, does not provide positive satisfaction, though dissatisfaction results from their absence. For that reason, a decrease in supervisor support would affect IR, but an increase in supervisor support would not necessarily prevent IR. Furthermore, we did not find a significant association of changes in coworker support with IR. The vertical principle in Japanese society may explain this finding[38]. In the Japanese workplace, vertical relationships remains deeply rooted while horizontal relationships are relatively weak compared to those in other countries, therefore, changes in supervisor support rather than changes in coworker support, may have a greater impact on IR.

There was no significant association between changes in job demands, job control, or job strain and IR. Since we surveyed middle-aged male workers who received a comprehensive health examination and had their level of serum insulin checked, they may have had enough time and money to have an "optional" health examination and had higher levels of health awareness. In fact, the present sample had lower levels of job demands and job strain and higher levels of job control, compared to those who received only mandatory annual health examination in the same health care center (data not shown), which may lead to non-significant association of a change in job demands, job control, or job strain with IR.

Our study has several strengths. First, this is the first longitudinal study based on the JD-C or DCS models, which investigates the association of changes in psychosocial work characteristics with IR. Most preceding longitudinal studies measured the psychosocial work characteristics only once, which would not assess whether psychosocial work characteristics had changed. Second, we demonstrated the association of psychosocial work characteristics with IR measured by HOMA-IR as an objective variable. Almost all the preceding studies showed the association of psychosocial work characteristics with Type 2 diabetes. We focused on the earlier and reversible level of worsening glucose metabolism using HOMA-IR. High supervisor support may prevent worsening IR in occupational settings.

Some possible limitations to this study must be considered. First, as mentioned earlier, we surveyed only male workers who received a comprehensive health examination and requested to have their serum insulin level checked. In a future study, we need to reduce the potential for selection bias by measuring the serum insulin level at random among all of the people (men and women) who completed an annual health examination. Second, the present sample came from one big manufacturing company group in Japan. Therefore, generalization of the findings should be done with caution. Third, although we excluded those who had been diagnosed with diabetes at baseline from the study, past history of other kinds of diseases could not be considered or adjusted, which may mask the true association because those who had suffered from some kind of disease might have experienced higher levels of job resources (especially supervisor support). Future research should consider the effects of various medical history and/or workplace consideration on the present findings. Fourth, while we adjusted for exercise habits during leisure time, this was assessed by a single item questionnaire with a dichotomous option. Furthermore, the level of physical activity during working hours could not be adjusted; however, it might be possi-

ble to partially adjust for this variable by including occupational characteristics in the statistical model. In the future, we would measure the occupational and leisure time metabolic equivalents (METs) to assess physical activity levels more precisely. Fifth, we could not discriminate Type 1 diabetes and Type 2 diabetes clearly. In the present study, we surveyed middle-aged male workers, excluding those who had been diagnosed with diabetes at baseline to reduce the influence of juvenile-onset Type 1 diabetes as possible. Finally, due to investigational circumstances (i.e., difficulties with following up subjects over a longer period of time), we investigated the association of changes in psychosocial work characteristics with IR over a three-year period only. Earlier longitudinal studies on the association of psychosocial work characteristics with Type 2 diabetes used a 6-15 year follow-up period[19-21,27]. However, since our outcome variable was IR, which is a preliminary stage of Type 2 diabetes, the three-year follow-up period (i.e., shorter than six years) is reasonable and valid. However, further studies are needed to confirm a more appropriate duration of follow-up periods for assessing the effects of changes in psychosocial work characteristics on IR.

Conclusions

To the best of our knowledge, this is the first study to assess the association of changes in psychosocial work characteristics with IR. The present study showed that a decrease in supervisor support was an independent risk factor of worsening IR. Furthermore, high supervisor support may reduce the risk of Type 2 diabetes in the future. Further research should reveal the psychological and biological mechanisms underlying the association of a change in supervisor support over time with IR.

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Appendix A. Demographic and occupational characteristics and lifestyle behaviors of participants at baseline by change in job demands

Change in job demands	Stable low (*n*=735)		Increased (*n*=165)		Decreased (*n*=337)		Stable high (*n*=578)		*p*
	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	
Age	52.11 (7.42)		47.91 (7.28)		51.60 (7.28)		47.47 (7.27)		<0.001/<0.001[a]
35-39 years old		57 (7.8)		30 (18.2)		23 (6.8)		105 (18.2)	
40-49 years old		176 (23.9)		53 (32.1)		95 (28.2)		214 (37.0)	
50-59 years old		416 (55.6)		80 (48.5)		199 (59.1)		247 (42.7)	
60 years old or more		86 (11.7)		2 (1.2)		20 (2.1)		12 (2.1)	
Marital status									0.094
Currently married		634 (86.3)		141 (85.5)		300 (89.0)		506 (87.5)	
Never married		72 (9.8)		21 (12.7)		31 (9.2)		63 (10.9)	
Divorced/widowed		29 (3.9)		3 (1.8)		6 (1.8)		9 (1.6)	
Department									0.003
Design engineering department		175 (23.8)		56 (33.9)		103 (30.6)		204 (35.3)	
Inspection department		79 (10.7)		16 (9.7)		44 (13.1)		58 (10.0)	
Production assembling department		149 (20.3)		35 (21.2)		60 (17.8)		102 (17.6)	
Production control department		58 (7.9)		8 (4.8)		31 (9.2)		47 (8.1)	
Transportation department		11 (1.5)		1 (0.6)		3 (0.9)		2 (0.3)	
General affairs department		127 (17.3)		33 (20.0)		46 (13.6)		73 (12.6)	
Sales department		31 (4.2)		1 (0.6)		7 (2.1)		12 (2.1)	
Data input department		1 (0.1)		0 (0)		2 (0.6)		1 (0.2)	
Research department		35 (4.8)		5 (3.0)		12 (3.6)		33 (5.7)	
Medical department		0 (0)		0 (0)		1 (0.3)		3 (0.5)	
Service department		6 (0.8)		1 (0.6)		4 (1.2)		2 (0.3)	
Others		63 (8.6)		9 (5.5)		24 (7.1)		41 (7.1)	
Employment position and occupation									<0.001
Manager		286 (38.9)		59 (35.8)		164 (48.7)		258 (44.6)	
Main career track		186 (15.3)		68 (41.2)		94 (27.9)		204 (35.3)	
General clerk		60 (8.2)		5 (3.0)		19 (5.6)		23 (4.0)	
Non-clerical workers		179 (24.4)		32 (19.4)		58 (17.2)		91 (15.7)	
Others		24 (3.3)		1 (0.6)		2 (0.3)		2 (0.3)	
Smoking history									0.763
Non smoker		464 (63.1)		105 (63.6)		205 (60.8)		372 (64.4)	
Current smoker		271 (36.9)		60 (36.4)		132 (39.2)		206 (35.6)	

Appendix A. Demographic and occupational characteristics and lifestyle behaviors of participants at baseline by change in job demands (continued)

Change in job demands	Stable low (*n*=735)		Increased (*n*=165)		Decreased (*n*=337)		Stable high (*n*=578)		*p*
	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	
Alcohol consumption [g/wk]	133.42 (124.57)		127.56 (130.44)		129.98 (119.86)		115.32 (115.28)		0.056/0.110[a]
0-44		258 (35.1)		64 (38.8)		116 (34.4)		225 (38.9)	
45-154		210 (28.6)		49 (29.7)		106 (31.5)		189 (32.7)	
155 or more		267 (36.3)		52 (31.5)		115 (34.1)		164 (28.4)	
Exercise habits									0.035
Yes		324 (44.1)		71 (43.0)		133 (39.5)		210 (36.3)	
No		411 (55.9)		94 (57.0)		204 (60.5)		368 (63.7)	
Sleeping hours									<0.001
<5 hours		21 (2.9)		8 (4.8)		24 (7.1)		51 (8.8)	
≥5 hours to <6 hours		268 (36.5)		77 (46.7)		161 (47.8)		302 (52.2)	
≥6 hours to <7 hours		351 (47.8)		64 (38.8)		126 (37.4)		199 (34.4)	
≥7 hours		95 (12.9)		16 (9.7)		26 (7.7)		26 (4.5)	
Body mass index [kg/m^2]	23.58 (2.53)		23.44 (2.61)		23.79 (2.56)		23.79 (2.50)		0.239/0.573[a]
Low (22.5 or less)		254 (34.6)		66 (40.0)		105 (31.2)		186 (32.2)	
Middle (22.6-24.6)		247 (33.6)		50 (30.3)		118 (35.0)		200 (34.6)	
High (24.7 or more)		234 (31.8)		49 (29.7)		114 (33.8)		192 (33.2)	

[a] *p* values for continuous variables are shown in left side; *p* values for categorical variables are shown in right side

Appendix B. Demographic and occupational characteristics and lifestyle behaviors of participants at baseline by change in job control

Change in job control	Stable low (*n*=971)		Increased (*n*=250)		Decreased (*n*=183)		Stable high (*n*=411)		*p*
	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	
Age	48.72 (7.33)		50.82 (7.77)		49.84 (8.14)		53.29 (6.81)		<0.001/<0.001[a]
35-39 years old		139 (14.3)		28 (11.2)		28 (15.3)		20 (4.9)	
40-49 years old		340 (35.0)		69 (27.6)		48 (26.2)		81 (19.7)	
50-59 years old		457 (47.1)		132 (52.8)		94 (51.4)		259 (63.0)	
60 years old or more		35 (3.6)		21 (8.4)		13 (7.1)		51 (12.4)	
Marital status									<0.001
Currently married		807 (83.1)		222 (88.8)		165 (90.2)		387 (94.2)	
Never married		141 (14.5)		20 (8.0)		14 (7.7)		14 (7.7)	
Divorced/widowed		23 (2.4)		8 (3.2)		4 (2.2)		4 (2.2)	
Department									0.001
Design engineering department		313 (32.2)		65 (26.0)		56 (30.6)		104 (25.3)	
Inspection department		117 (12.0)		27 (10.8)		10 (5.5)		43 (10.5)	
Production assembling department		193 (19.9)		43 (17.2)		31 (16.9)		79 (19.2)	
Production control department		75 (7.7)		22 (8.8)		13 (7.1)		34 (8.3)	
Transportation department		7 (0.7)		7 (2.8)		1 (0.5)		2 (0.5)	
General affairs department		128 (13.2)		46 (18.4)		27 (14.8)		78 (19.0)	
Sales department		24 (2.5)		10 (4.0)		10 (5.5)		7 (1.7)	
Data input department		3 (0.3)		0 (0)		0 (0)		1 (0.2)	
Research department		42 (4.3)		9 (3.6)		14 (7.7)		20 (4.9)	
Medical department		1 (0.1)		0 (0)		0 (0)		3 (0.7)	
Service department		9 (0.9)		1 (0.4)		0 (0)		3 (0.7)	
Others		59 (6.1)		20 (8.0)		21 (11.5)		37 (9.0)	
Employment position and occupation									<0.001
Manager		364 (37.5)		114 (45.6)		86 (47.0)		203 (49.4)	
Main career track		339 (34.9)		73 (29.2)		55 (30.1)		85 (20.7)	
General clerk		58 (6.0)		6 (2.4)		12 (6.6)		31 (7.5)	
Non-clerical workers		201 (20.7)		53 (21.2)		27 (14.8)		79 (19.2)	
Others		9 (0.9)		4 (1.6)		3 (1.6)		13 (3.2)	
Smoking history									0.078
Non smoker		592 (61.0)		157 (62.8)		116 (63.4)		281 (68.4)	
Current smoker		379 (39.0)		93 (37.2)		67 (36.6)		130 (31.6)	

Appendix B. Demographic and occupational characteristics and lifestyle behaviors of participants at baseline by change in job control (continued)

Change in job control	Stable low (n=971)		Increased (n=250)		Decreased (n=183)		Stable high (n=411)		p
	Mean (SD)	n (%)	Mean (SD)	n (%)	Mean (SD)	n (%)	Mean (SD)	n (%)	
Alcohol consumption [g/wk]	123.20 (124.31)		130.06 (122.83)		133.07 (124.10)		129.12 (112.78)		0.647/0.091[a]
0-44		382 (39.3)		90 (36.0)		58 (31.7)		133 (32.4)	
45-154		282 (29.0)		73 (29.2)		68 (37.2)		131 (31.9)	
155 or more		307 (31.6)		87 (34.8)		57 (31.1)		147 (35.8)	
Exercise habits									<0.001
Yes		324 (33.4)		117 (46.8)		95 (51.9)		202 (49.1)	
No		647 (66.6)		133 (53.2)		88 (48.1)		209 (50.9)	
Sleeping hours									<0.001
<5 hours		104 (5.7)		16 (6.4)		8 (4.4)		12 (2.9)	
≥5 hours to <6 hours		808 (44.5)		112 (44.8)		79 (43.2)		137 (33.3)	
≥6 hours to <7 hours		740 (40.8)		103 (41.2)		82 (44.8)		193 (47.0)	
≥7 hours		163 (9.0)		19 (7.6)		14 (7.7)		69 (16.8)	
Body mass index [kg/m^2]	23.66 (2.66)		23.42 (2.20)		23.80 (2.40)		23.81 (2.46)		0.250/0.068[a]
Low (22.5 or less)		344 (35.4)		90 (36.0)		54 (29.5)		123 (29.9)	
Middle (22.6-24.6)		305 (31.4)		93 (37.2)		72 (39.3)		145 (35.3)	
High (24.7 or more)		322 (33.2)		67 (26.8)		57 (31.1)		143 (34.8)	

[a] p values for continuous variables are shown in left side; p values for categorical variables are shown in right side

Appendix C. Demographic and occupational characteristics and lifestyle behaviors of participants at baseline by change in job strain

Change in job strain (job demands/control)	Stable low (*n*=715)		Increased (*n*=169)		Decreased (*n*=307)		Stable high (*n*=624)		*p*
	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	
Age	52.74 (7.16)		48.05 (7.05)		51.17 (7.23)		47.27 (7.21)		<0.001/<0.001[a]
35-39 years old		47 (6.6)		26 (15.4)		26 (8.5)		116 (18.8)	
40-49 years old		151 (21.1)		62 (36.7)		84 (27.4)		241 (28.6)	
50-59 years old		433 (60.6)		78 (46.2)		175 (57.0)		256 (41.0)	
60 years old or more		84 (11.7)		3 (1.8)		22 (7.2)		11 (1.8)	
Marital status									<0.001
Currently married		637 (89.1)		145 (85.8)		274 (89.3)		525 (84.1)	
Never married		50 (7.0)		21 (12.4)		27 (8.8)		89 (14.3)	
Divorced/widowed		28 (3.9)		3 (1.8)		6 (2.0)		10 (1.6)	
Department									0.031
Design engineering department		177 (24.8)		57 (33.7)		91 (29.6)		213 (34.1)	
Inspection department		73 (10.2)		22 (13.0)		41 (13.4)		61 (9.8)	
Production assembling department		137 (19.2)		28 (16.6)		58 (18.9)		123 (19.7)	
Production control department		57 (8.0)		11 (6.5)		20 (6.5)		56 (9.0)	
Transportation department		9 (1.3)		2 (1.2)		4 (1.3)		2 (0.3)	
General affairs department		136 (19.0)		24 (14.2)		40 (13.0)		79 (12.7)	
Sales department		26 (3.6)		3 (1.8)		11 (3.6)		11 (1.8)	
Data input department		1 (0.1)		0 (0)		2 (0.7)		1 (0.2)	
Research department		34 (4.8)		6 (3.6)		9 (2.9)		36 (5.8)	
Medical department		1 (0.1)		0 (2)		1 (0.3)		2 (0.3)	
Service department		5 (0.7)		1 (0.6)		3 (1.0)		4 (0.6)	
Others		59 (8.3)		15 (8.9)		27 (8.8)		36 (5.8)	
Employment position and occupation									<0.001
Manager		314 (43.9)		69 (40.8)		146 (47.6)		238 (38.1)	
Main career track		174 (24.3)		58 (34.3)		83 (27.0)		237 (38.0)	
General clerk		52 (7.3)		7 (4.1)		18 (5.9)		30 (4.8)	
Non-clerical workers		152 (21.3)		34 (20.1)		57 (18.6)		117 (18.8)	
Others		23 (3.2)		1 (0.6)		3 (1.6)		2 (0.3)	
Smoking history									0.050
Non smoker		461 (64.5)		113 (66.9)		173 (56.4)		399 (63.9)	
Current smoker		254 (35.5)		56 (33.1)		134 (43.6)		225 (36.1)	

Appendix C. Demographic and occupational characteristics and lifestyle behaviors of participants at baseline by change in job strain (continued)

Change in job strain (job demands/control)	Stable low (n=715)		Increased (n=169)		Decreased (n=307)		Stable high (n=624)		p
	Mean (SD)	n (%)	Mean (SD)	n (%)	Mean (SD)	n (%)	Mean (SD)	n (%)	
Alcohol consumption [g/wk]	139.14 (123.16)		125.13 (123.08)		119.93 (122.44)		115.57 (117.64)		0.003/0.004[a]
0-44		232 (32.4)		60 (35.5)		120 (39.1)		251 (40.2)	
45-154		207 (29.0)		59 (34.9)		96 (31.3)		192 (30.8)	
155 or more		276 (38.6)		50 (29.6)		91 (29.6)		181 (29.0)	
Exercise habits									<0.001
Yes		332 (46.4)		81 (47.9)		114 (37.1)		211 (33.8)	
No		383 (53.6)		88 (52.1)		193 (62.9)		413 (66.2)	
Sleeping hours									<0.001
<5 hours		17 (2.4)		7 (4.1)		20 (6.5)		60 (9.6)	
≥5 hours to <6 hours		247 (34.5)		79 (46.7)		151 (49.2)		331 (53.0)	
≥6 hours to <7 hours		348 (48.7)		68 (40.2)		116 (37.8)		208 (33.3)	
≥7 hours		103 (14.4)		15 (8.9)		20 (6.5)		25 (4.0)	
Body mass index [kg/m^2]	23.70 (2.56)		23.68 (2.49)		23.58 (2.67)		23.69 (2.58)		0.925/0.817[a]
Low (22.5 or less)		229 (32.0)		57 (33.7)		111 (36.2)		214 (34.3)	
Middle (22.6-24.6)		249 (34.8)		62 (36.7)		100 (32.6)		204 (32.7)	
High (24.7 or more)		237 (33.1)		50 (29.6)		96 (31.3)		206 (33.0)	

[a] p values for continuous variables are shown in left side; p values for categorical variables are shown in right side

Appendix D. Demographic and occupational characteristics and lifestyle behaviors of participants at baseline by change in supervisor support

Change in supervisor support	Stable low (*n*=749)		Increased (*n*=220)		Decreased (*n*=250)		Stable high (*n*=596)		*p*
	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	
Age	50.19 (7.23)		49.83 (7.43)		48.92 (7.65)		50.75 (7.99)		<0.001/0.025[a]
35-39 years old		80 (10.7)		29 (13.2)		37 (14.8)		69 (11.6)	
40-49 years old		227 (30.3)		66 (30.0)		87 (34.8)		158 (26.5)	
50-59 years old		403 (53.8)		114 (51.8)		108 (43.2)		317 (53.2)	
60 years old or more		39 (5.2)		11 (5.0)		18 (7.2)		52 (8.7)	
Marital status									0.006
Currently married		630 (84.1)		192 (87.3)		216 (86.4)		543 (91.1)	
Never married		95 (12.7)		26 (11.8)		26 (10.4)		40 (6.7)	
Divorced/widowed		24 (3.2)		2 (0.9)		8 (3.2)		13 (2.2)	
Department									0.194
Design engineering department		224 (29.9)		59 (26.8)		86 (34.4)		169 (28.4)	
Inspection department		92 (12.3)		23 (10.5)		26 (10.4)		56 (9.4)	
Production assembling department		151 (20.2)		41 (18.6)		38 (15.2)		116 (19.5)	
Production control department		65 (8.7)		20 (9.1)		13 (5.2)		46 (7.7)	
Transportation department		7 (0.9)		1 (0.5)		4 (1.6)		5 (0.8)	
General affairs department		100 (13.4)		38 (17.3)		40 (16.0)		101 (16.9)	
Sales department		15 (2.0)		7 (3.2)		11 (4.4)		18 (3.0)	
Data input department		0 (0)		0 (0)		2 (0.8)		2 (0.3)	
Research department		33 (4.4)		9 (4.1)		7 (2.8)		36 (6.0)	
Medical department		2 (0.3)		0 (0)		0 (0)		2 (0.3)	
Service department		4 (0.5)		2 (0.9)		0 (0)		7 (1.2)	
Others		56 (7.5)		20 (9.1)		23 (9.2)		38 (6.4)	
Employment position and occupation									<0.001
Manager		273 (36.4)		81 (36.8)		128 (51.2)		285 (47.8)	
Main career track		241 (32.2)		71 (32.3)		73 (29.2)		167 (28.0)	
General clerk		53 (7.1)		11 (5.0)		12 (4.8)		31 (5.2)	
Non-clerical workers		175 (23.4)		53 (24.1)		34 (13.6)		98 (16.4)	
Others		7 (0.9)		4 (1.8)		3 (1.2)		15 (2.5)	
Smoking history									0.187
Non smoker		459 (61.3)		131 (59.5)		163 (65.2)		393 (65.9)	
Current smoker		290 (38.7)		89 (40.5)		87 (34.8)		203 (34.1)	

Appendix D. Demographic and occupational characteristics and lifestyle behaviors of participants at baseline by change in supervisor support (continued)

Change in supervisor support	Stable low (*n*=749)		Increased (*n*=220)		Decreased (*n*=250)		Stable high (*n*=596)		*p*
	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	
Alcohol consumption [g/wk]	120.24 (124.44)		126.54 (118.51)		121.08 (125.44)		136.58 (116.77)		0.086/0.002[a]
0-44		305 (40.7)		81 (36.8)		102 (40.8)		175 (29.4)	
45-154		217 (29.0)		65 (29.5)		73 (29.2)		199 (33.4)	
155 or more		227 (30.3)		74 (33.6)		75 (30.0)		222 (37.2)	
Exercise habits									0.036
Yes		275 (36.7)		92 (41.8)		109 (44.0)		262 (44.0)	
No		474 (63.3)		128 (58.2)		141 (56.4)		334 (56.0)	
Sleeping hours									0.001
<5 hours		53 (7.1)		8 (3.6)		22 (8.8)		21 (3.5)	
≥5 hours to <6 hours		345 (46.1)		91 (41.4)		116 (46.4)		256 (43.0)	
≥6 hours to <7 hours		300 (40.1)		103 (46.8)		91 (36.4)		246 (41.3)	
≥7 hours		51 (6.8)		18 (8.2)		21 (8.4)		73 (12.2)	
Body mass index [kg/m^2]	23.60 (2.59)		23.29 (2.46)		24.23 (2.62)		23.68 (2.42)		<0.001/0.005[a]
Low (22.5 or less)		271 (36.2)		85 (38.6)		67 (26.8)		188 (31.5)	
Middle (22.6-24.6)		249 (33.2)		76 (34.5)		78 (31.2)		212 (35.6)	
High (24.7 or more)		229 (30.6)		59 (26.8)		105 (42.0)		196 (32.9)	

[a] *p* values for continuous variables are shown in left side; *p* values for categorical variables are shown in right side

Appendix E. Demographic and occupational characteristics and lifestyle behaviors of participants at baseline by change in coworker support

Change in coworker support	Stable low (*n*=889)		Increased (*n*=223)		Decreased (*n*=216)		Stable high (*n*=487)		*p*
	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	
Age	50.27 (7.39)		50.30 (6.97)		49.40 (7.65)		50.22 (8.15)		0.488/0.005[a]
35-39 years old		102 (11.5)		15 (6.7)		28 (13.0)		70 (14.4)	
40-49 years old		256 (28.8)		83 (37.2)		78 (36.1)		121 (24.8)	
50-59 years old		477 (53.7)		110 (49.3)		95 (44.0)		260 (53.4)	
60 years old or more		54 (6.1)		15 (6.7)		15 (6.9)		36 (7.4)	
Marital status									0.011
Currently married		750 (84.4)		194 (87.0)		192 (88.9)		445 (91.4)	
Never married		110 (12.4)		26 (11.7)		18 (8.3)		33 (6.8)	
Divorced/widowed		29 (3.3)		3 (1.3)		6 (2.8)		9 (1.8)	
Department									0.003
Design engineering department		271 (30.5)		71 (31.8)		58 (26.9)		138 (28.3)	
Inspection department		117 (13.2)		21 (9.4)		16 (7.4)		43 (8.8)	
Production assembling department		166 (18.7)		46 (20.6)		36 (16.7)		98 (20.1)	
Production control department		76 (8.5)		20 (9.0)		19 (8.8)		29 (6.0)	
Transportation department		12 (1.3)		3 (1.3)		0 (0)		2 (0.4)	
General affairs department		125 (14.1)		30 (13.5)		36 (16.7)		88 (18.1)	
Sales department		17 (1.9)		3 (1.3)		14 (6.5)		17 (3.5)	
Data input department		1 (0.1)		0 (0)		2 (0.9)		1 (0.2)	
Research department		39 (4.4)		9 (4.0)		11 (5.1)		26 (5.3)	
Medical department		0 (0)		0 (0)		1 (0.5)		3 (0.5)	
Service department		3 (0.3)		1 (0.4)		3 (1.4)		6 (1.2)	
Others		62 (7.0)		19 (8.5)		20 (9.3)		36 (7.4)	
Employment position and occupation									0.008
Manager		347 (39.0)		91 (40.8)		102 (47.2)		227 (46.6)	
Main career track		293 (33.0)		60 (26.9)		64 (29.6)		135 (27.7)	
General clerk		56 (6.3)		9 (4.0)		12 (5.6)		30 (6.2)	
Non-clerical workers		186 (20.9)		56 (25.1)		33 (15.3)		85 (17.5)	
Others		7 (0.8)		7 (3.1)		5 (2.3)		10 (2.1)	
Smoking history									0.382
Non smoker		576 (64.8)		131 (58.7)		134 (62.0)		305 (62.6)	
Current smoker		313 (35.2)		92 (41.3)		82 (38.0)		182 (37.4)	

Appendix E. Demographic and occupational characteristics and lifestyle behaviors of participants at baseline by change in coworker support (continued)

Change in coworker support	Stable low (*n*=889)		Increased (*n*=223)		Decreased (*n*=216)		Stable high (*n*=487)		*p*
	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	Mean (SD)	*n* (%)	
Alcohol consumption [g/wk]	122.02 (120.43)		137.18 (128.02)		128.94 (134.35)		128.65 (114.23)		0.366/0.442[a]
0-44		346 (38.9)		75 (33.6)		81 (37.5)		161 (33.1)	
45-154		262 (29.5)		73 (32.7)		63 (29.2)		156 (32.0)	
155 or more		281 (31.6)		75 (33.6)		72 (33.3)		170 (34.9)	
Exercise habits									0.029
Yes		336 (37.8)		88 (39.5)		90 (41.7)		224 (46.0)	
No		553 (62.2)		135 (60.5)		126 (58.3)		263 (54.0)	
Sleeping hours									0.127
<5 hours		55 (6.2)		6 (2.7)		13 (6.0)		30 (6.2)	
≥5 hours to <6 hours		407 (45.8)		102 (46.8)		101 (46.8)		198 (40.7)	
≥6 hours to <7 hours		356 (40.0)		100 (44.8)		79 (36.6)		205 (42.1)	
≥7 hours		71 (8.0)		15 (6.7)		23 (10.6)		54 (11.1)	
Body mass index [kg/m^2]	23.59 (2.50)		23.34 (2.63)		23.79 (2.54)		23.92 (2.53)		0.019/0.012[a]
Low (22.5 or less)		315 (35.4)		91 (40.8)		69 (31.9)		136 (27.9)	
Middle (22.6-24.6)		301 (33.9)		64 (28.7)		80 (37.0)		170 (34.9)	
High (24.7 or more)		273 (30.7)		68 (30.5)		67 (31.0)		181 (37.2)	

[a] *p* values for continuous variables are shown in left side; *p* values for categorical variables are shown in right side

JOB DEMANDS, JOB CONTROL, SOCCIAL SUPPORT, AND DRINKING HABITS AMONG JAPANESE EMPLOYEES

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Introduction: Excessive drinking may cause physical, psychological, and/or behavioral impairments. Drinking habit may be formed by high strain status caused by job stressors. We conducted a pilot study to reveal the associations between job demands, job control, social support, and drinking habit among Japanese employees.
Methods: A total of 5141 employees who worked in a manufacturing company in Japan were invited to participate in a self-administered questionnaire survey. After excluding 641 employees who had at least one missing response, data of 4500 employees were used for statistical analyses. Job demands, job control, and social support from supervisor, coworkers, or families were measured by the Brief Job Stress Questionnaire. Drinking habit was selected with six categories: 1) none, 2) sometimes, 3) everyday (<180ml), 4) everyday (180-360ml), 5) everyday (360-540ml), and 6) everyday (>540ml).
Results: As drinking habit was considered as dependent variable, hierarchical regression analyses were conducted: step 1. sex and age (control variables) as independent variables, step 2. job demands, job control, and social support, as independent variables step 3. interactions among step 2 variables. Analyses were conducted three times by three social support resources. Among all resources, high job demands and high job control had positive association with increased drinking consumption. There was a marginal interactive effect between job control and supervisor support ($p=.067$) suggesting that a combination of high job control with high supervisor support was associated with increased drinking consumption.
Conclusions: High job control had positive association with high drinking habit among Japanese workers.

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Electrical Perception of "Death Message" in *Chara*: The Role of Turgor Pressure

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Summary Specimens consisting of two tandem internodal cells were prepared using *Chara corallina* in order to analyze the electrical response upon wounding. When one cell (victim cell) was killed by severing, another cell (receptor cell) generated a sharp spike followed by a long-lasting depolarization at the nodal end. It has been reported that K^+ released from the victim cell is involved in generation of the long-lasting depolarization (Shimmen 2006). In the present study, the trigger to induce the sharp spike was studied. When the turgor pressure of the victim cell was decreased by adding 1 M methanol to the external medium, a sharp spike similar to that induced by wounding was generated at the nodal end of the receptor cell. It is suggested that the sudden disappearance of the turgor pressure of the victim cell due to severing is a trigger to induce the sharp spike of the receptor cell.

Key words *Chara*, Death message, Membrane potential, Turgor pressure, Wounding response.

Generation of electrical responses upon wounding is a general phenomenon in plants (Mertz and Higinbotham 1976, Meyer and Weisenseel 1997, Stahlberg and Cosgrove 1994, Frachisse *et al.* 1985, Fromm and Eschrich 1993, Julien *et al.* 1991, Julien and Frachisse 1992, Rhodes *et al.* 1996, Robin 1985, Robin and Bonnemain 1985). Wildon *et al.* (1992) found that the electrical signal is involved in systemic synthesis of a proteinase inhibitor in tomato. It was also reported that both variation potential and action potential induced synthesis of proteinase inhibitor in tomato (Stankovic and Davies 1996). Thus, the electrical signal seems to play a pivotal role in wounding response of plants.

It is suggested that the neighbor cells first perceive the message from the killed cells and generate the electrical signal. However, it will be hard to selectively monitor the electrical signal from the target cells in higher plants because of their complex morphology. Using *Chara corallina*, a simple model system to analyze the wound-induced electrical response was developed (Shimmen 2001). A specimen consisting of tandem two internodal cells was prepared. One cell (victim cell) was killed and the electrical response of the neighbor cell (receptor cell) was monitored. When the victim cell was severed, two kinds of electrical response were induced at the nodal end of the receptor cell: A shape spike and a following long-lasting depolarization. During the long-lasting depolarization, action potentials were generated due to activation of the voltage-dependent ion channels (Shimmen 2002). Involvement of K^+ released from the victim cell in generating the long-lasting depolarization was suggested (Shimmen 2005, 2006). We came to a hypothesis that the sharp spike is induced *via* disappearance of the turgor pressure of the victim cell upon cutting, based on the following experience. Some species of *Spirogyra* differentiate the terminal cell to be a rhizoid for anchoring to the substratum. When algal filaments of such species were severed, a new terminal cell recognizes its terminal position and starts the rhizoid differentiation (Nagata 1973). We noticed that the distal end of the terminal cell became convex due to its turgor pressure. Before cutting the algal filaments, the septum between cells is flat due to balancing of turgor pressure. It was indicated that the terminal cell recognizes its terminal position *via* stretching of the membrane at the distal end (Inoue *et al.* 2002). On the analogy to *Spirogyra*, we speculated that a stretching force is applied to the nodal end of the receptor cell when the turgor pressure of the victim cell was lost upon severing in *C. corallina*. However, we cannot observe stretching of the end membrane of an internodal cell, since the cell end is surrounded by nodal cells. It was suggested that the membrane depolarization is induced at the nodal end of the receptor cell by lowering the turgor pressure of the victim cell, without severing the cell. Upon addition of 0.2 M sorbitol to the external medium of the victim cell, a significant membrane depolarization was induced. However, a sharp spike was not observed (Shimmen 2001).

Recently, we came to a hypothesis that more rapid

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decrease in the turgor pressure of the V cell may induce a sharp spike of the R cell, since the turgor pressure should be suddenly lost upon cutting the victim cell. Hayama *et al.* (1979) applied 4% methanol to the external medium in order to induce strong transcellular osmosis in *Nitella flexilis*. In the present study, we found that addition of 1 M methanol to the external medium of the victim cell induced a sharp spike in the victim cell.

Materials and methods

Chara corallina was cultured in an air-conditioned room (25°C) as described previously (Mimura and Shimmen 1994). Specimens consisting of two tandem internodal cells were prepared by removing neighbor internodal and branchlet cells with scissors (Shimmen 2001). They were kept in artificial pond water (APW) containing 0.1 mM KCl, 1 mM NaCl and 0.1 mM $CaCl_2$ (pH about 5.6) at least 2 d before use.

The electrical measurements were carried out using a chamber composed of three pools (Fig. 1). A specimen consisting of two tandem internodal cells was mounted on the chamber so that the node between internodal cells was located in pool B. Partitions (P) between pools were electrically sealed with white Vaseline. The length of P was about 10 mm. The node was attached to P separating pools A and B. Hereafter, an internodal cell separated between pools A and B and that separated between pools B and C were called receptor cell (R cell) and victim cell (V cell), respectively. The length of pool B was about 3 mm. All pools were filled with APW buffered with 5 mM HEPES–NaOH (pH 7.0). Hereafter, buffered APW was simply called APW. The potential difference between pools A and B was measured with agar electrodes which were connected to Ag–AgCl wire *via* 3 M KCl solution (E_A, E_B). Under such measuring configuration, the electrical response generated at the nodal end of the R cell was selectively monitored (Shimmen 2001, 2002, 2003).

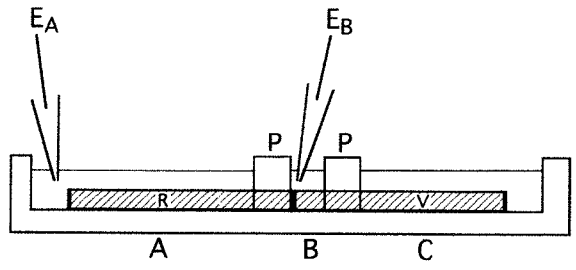

Fig. 1. Setup for analysis of methanol-induced depolarization. A specimen consisting of two tandem internodal cells was mounted on a chamber having three pools, A, B and C. The node was attached to the partition separating pools A and B (P). An internodal cell separated between pools A and B and another separated between pools B and C were receptor cell (R) and victim cell (V), respectively. The potential difference between two pools, A and B, was measured with agar electrodes (E_A, E_B). Under such measuring configuration, response generated at the nodal end of the R cell was selectively monitored.

The change of turgor pressure of internodal cells upon addition of either 1 M methanol or 0.2 M sorbitoll to the external medium was measured according to Ogata (2010).

Electrical signals were amplified with an amplifier (Microelectrode Amplifier MEZ7101, Nihon Kohden, Tokyo, Japan) and recorded using a pen-writing recorder (VP-6358A, National Panasonic, Tokyo, Japan).

Experiments were carried out at room temperature (23–27°C) under dim light (about 90 lux).

Results

Some organic solvents, permeable to the biological membrane, transiently decrease the turgor pressure of *Chara* cells (Steudle and Tyerman 1983). The effect of methanol was confirmed according to Ogata (2010). At the beginning, the external medium was APW (Fig. 2 upper). Upon replacement of the external medium with APW supplemented with 1 M methanol (meth), the turgor pressure began to decrease and attained a minimum value after about 5 s. Then the turgor pressure started to recover. After about 2 min of methanol application, turgor pressured recovered the original level (data not shown). Thus, decrease in turgor pressure upon addition of 1 M methanol was quick and transient. The external medium was replaced with APW lacking methanol. The effect of 0.2 M sorbitol on the turgor pressure was examined using the same cell (Fig. 2 lower). Upon replacement of the external medium with APW supplemented with 0.2 M sorbitol (sorb), the turgor pressure slowly decreased. It stayed at a low level and recovered upon replacement of the external medium with APW lacking sorbitol (data not shown). It must be noticed that the initial speed of turgor pressure decrease induced by 1 M methanol was significantly higher than that induced by 0.2 M sorbitol (Fig. 2).

Since 1 M methanol is significantly hypertonic to the intracellular osmolarity of *Chara* cells: 247±3 mOsM (Shimmen 2001), any toxic effect on the cell was suspected. This was examined by a microscopic observation of cells (Fig. 3). First, an internodal cell was bathed in APW (0 s). The external medium was replaced with APW supplemented with 1 M methanol. However, morphology of the cell did not change at all (Fig. 3 left, 10, 60 s) and cytoplasmic streaming continued without cessation (not shown). When 5 internodal cells were incubated in APW supplemented with 1 M methanol for 2 d, all cells maintained a normal morphology and active cytoplasmic streaming (data not shown). Thus, 1 M methanol did not exert severe ill effects on internodal cells with respect to morphology, cytoplasmic streaming and survival. Next, the effect of 1 M sorbitol was examined (Fig. 3, right). Just after replacement of the external medium with APW supplemented with 1 M sorbitol

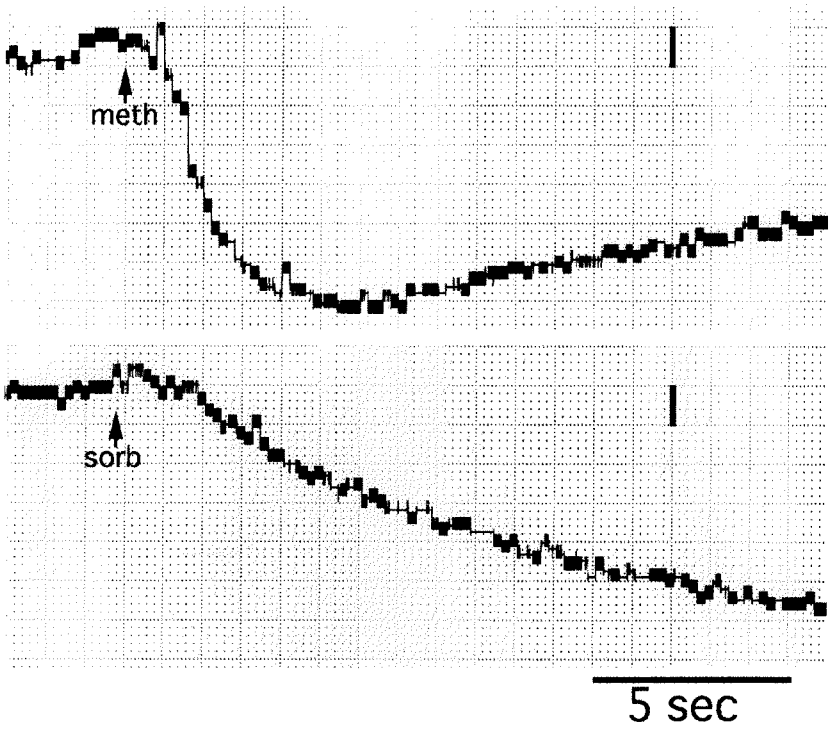

Fig. 2. Change of turgor pressure upon addition of either methanol or sorbitol to the external medium of an internodal cell. Measurement was carried out according to Ogata (2010). First, the external medium was APW. Upon replacement of the external medium with APW supplemented with 1 M methanol (meth), turgor pressure gradually decreased and then started to recover after about 5 s. After the end of measurement, the external medium was replaced with APW lacking methanol. When the turgor pressure restored an original level (lower trace), the external medium was replaced with APW supplemented with 0.2 M sorbitol (sorb). Turgor pressure gradually decreased. For further explanation, see the text. Vertical bar represents 0.1 MPa.

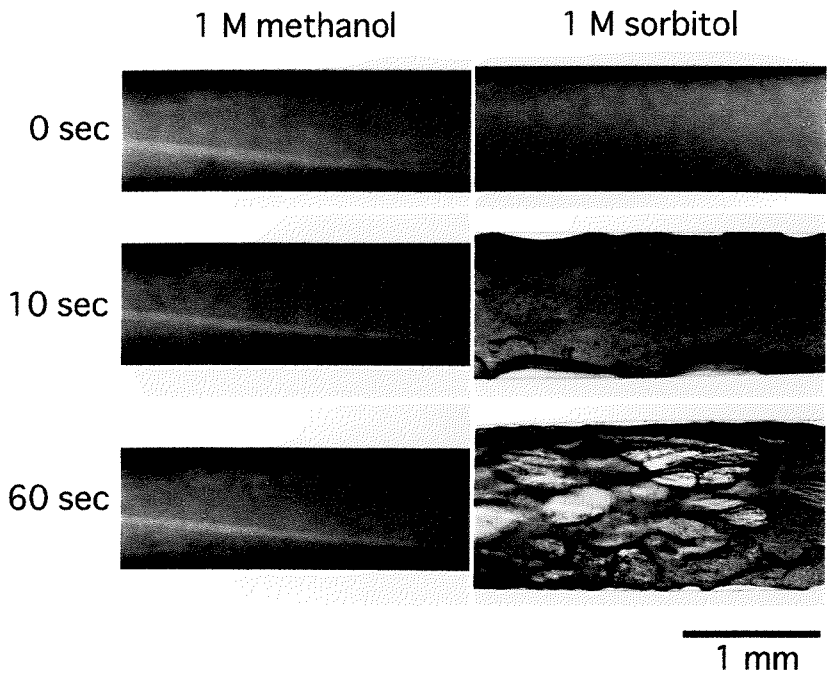

Fig. 3. Effect of methanol and sorbitol on morphology of internodal cell. Internodal cells were treated with APW supplemented with either 1 M methanol or 1 M sorbitol. After 10 and 60 s, photographs were taken. For further explanation, see the text.

(10 s), plasmolysis started and cytoplasmic streaming stopped (not shown). After 60 s, morphology of the cell was miserable due to severe plasmolysis (60 s).

The effect of 1 M methanol on membrane potential was examined. In the experiment to analyze the wounding response, the V cell was severed and the electrical

response of the R cell was monitored (Shimmen 2001). In Fig. 1, left cell corresponds to R cell and right one V cell. The external medium was first APW. When the external medium of pool C was replaced with APW supplemented with 1 M methanol, two types of depolarization were generated after a delay. Hereafter, replacement of the external medium with APW supplemented with methanol is described as "addition of methanol". In some specimens, addition of 1 M methanol to pool C induced a sharp spike followed by a slow repolarization (Fig. 4a). In other specimens, a slow and transient depolarization without sharp spike was generated upon addition of methanol to pool C (Fig. 4b). A similar response lacking a sharp spike was generated when the turgor pressure of the V cell was moderately decreased by adding 0.2 M sorbitol (Shimmen 2001). Among 21 specimens examined, a sharp spike was generated in 19 cells. However, the number of specimens generating a sharp spike was variable between culture batches. In some culture batches, all specimens generated only a slow depolarization without sharp spike.

After the methanol-induced depolarization, a wound-induced depolarization was induced by severing the V cell in order to compare the mode of these responses. This experiment was carried out in 13 specimens, which generated a sharp spike upon methanol-treatment. In some specimens, the initial part of the methanol-induced depolarization was quite similar to that of the wound-induced depolarization. Figure 5 shows a typical example. Upon addition of 1 M methanol (meth), a sharp spike was generated (1) and then an action potential was (2). The action potential was transmitted to the cell part in pool A (3), resulting in generation of a bipolar action potential. After the membrane potential recovered to the level before the methanol addition, the V cell was served in pool C. After a sharp spike (4), a bipolar action potential (5, 6) and a long-lasting depolarization (7) were generated. When an action potential was not generated in the methanol-stimulation as shown in Fig. 4b, it was also absent in the wound-induced depolarization (data not shown).

The effect of 1 M sorbitol on membrane potential was examined (Fig. 6). Upon addition of 1 M sorbitol to the

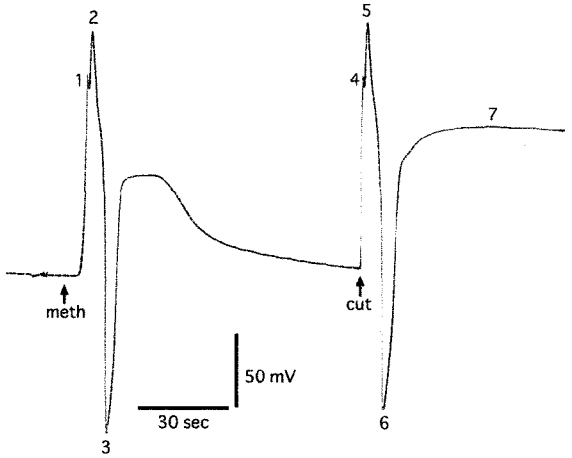

Fig. 5. Comparison of methanol- and wound-induced responses. A specimen was mounted as shown in Fig. 1. When the external medium of pool C was replaced with APW supplemented with 1 M methanol (meth), a rapid spike was generated (1). After generation of a bi-polar action potential (2, 3), the membrane repolarized. When the victim cell was cut in pool C (cut), an initial spike (4), a bi-polar action potential (5, 6) and a long-lasting depolarization (7) were successively generated. For further explanation, see the text.

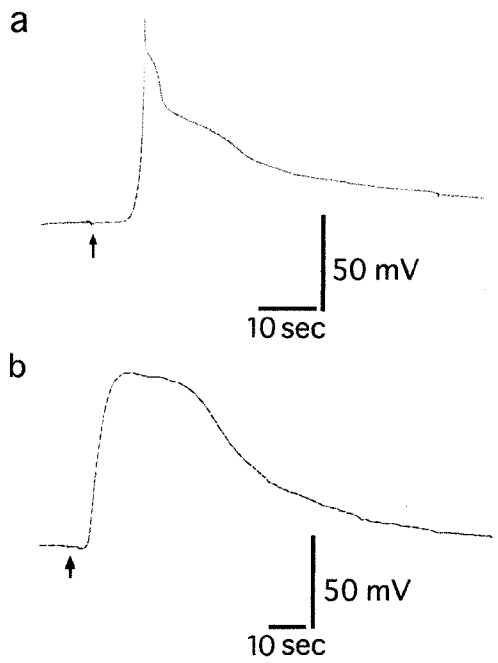

Fig. 4. Methanol-induced depolarization. Experiments were carried out using a chamber shown in Fig. 1. The external medium was first APW. When the external medium of pool C was replaced with APW supplemented with 1 M methanol (arrows), two types of response were generated at the end of the receptor cell (a and b). For further explanation, see the text.

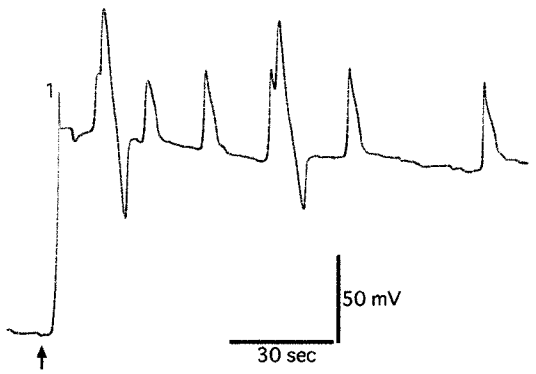

Fig. 6. Sorbitol-induced depolarization. Experiments were carried out using a chamber shown in Fig. 1. The external medium was first APW. When the external medium of pool C was replaced with APW supplemented with 1 M sorbitol (arrow), a rapid spike (1) and then a long-lasting depolarization were generated. Irregular spikes were superimposed on the long-lasting depolarization. For further explanation, see the text.

external medium of the V cell (arrow), a sharp spike (1) was generated after a delay and then a long-lasting depolarization with irregular spikes was induced. The mode of the response generated by 1 M sorbitol was similar to that generated upon severing the V cell.

Discussion

A sharp spike was generated by decreasing the turgor pressure of the V cell with 1 M methanol (Fig. 4a), supporting our hypothesis that generation of the sharp spike is triggered by the sudden disappearance of the turgor pressure of the V cell upon cutting. The sharp spike generated upon methanol-treatment was quite similar to that induced by wounding (Fig. 5). As shown in Fig. 2, the initial rate of turgor decrease upon treatment of the V cell with 1 M methanol was higher than that induced by treatment with 0.2 M sorbitol. Treatment with 0.2 M sorbitol never induced a sharp spike (Shimmen 2001), indicating that the initial rate of turgor decrease must be higher to induce the sharp spike. In some specimens, however, the sharp spike was not induced by treatment with 1 M methanol (Fig. 4b). In these specimens, however, the R cell generated a sharp spike upon severing the V cell (data not shown). In methanol stimulation, turgor gradually decreases, depending on the water efflux across the plasma membrane of the V cell. On the other hand, upon cutting the V cell, turgor pressure is expected to suddenly be lost. It is suggested that susceptibility of the nodal end of the R cell to the decrease in turgor pressure of the V cell might be different among specimens. At present, the reason remains unsolved.

In the previous paper (Shimmen 2002), activation of chloride channels at the beginning of the wound-induced depolarization was reported in *Chara*. In mesophyll cells of *Arabidopsis thaliana*, it was suggested that activation of the mechano-sensitive anion channel is mediated by convex curvature of the plasma membrane (Qi *et al.* 2004). The nodal end of *Chara* is equipped with a function to transduce the pressure signal into an electrical signal (Shimmen 2003). Upon decrease or loss of turgor pressure of the V cell, the nodal end might become convex, resulting in activation of chloride channel. Transduction of pressure signal into electrical one upon wounding has been suggested in tomato (Stankovic and Davies 1998) and sunflower (Stankovic *et al.* 1997). When the flank region of internodal cells was mechanically stimulated, a depolarization is induced due to activation of Cl^- channels (Shimmen 1997). Kaneko *et al.* (2005) showed increase in cytoplasmic Ca^{2+} upon mechanical stimulation of flank region, suggesting possible activation of the Ca^{2+} channel in addition to Cl^- channel upon mechanical stimulation at the flank region of the internodal cell. Amplitude of the rapid spike upon severing the V cell decreased to some extent, when the extracellular Ca^{2+} was decreased (Shimmen 2002). Thus, possible activation of Ca^{2+} channel at the nodal region upon wounding is a target of the future study.

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Associations Between Selected Xenobiotics and Antinuclear Antibodies in the National Health and Nutrition Examination Survey, 1999–2004

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Background: Potential associations between background environmental chemical exposures and autoimmunity are understudied.

Objectives: Our exploratory study investigated exposure to individual environmental chemicals and selected mixtures in relation to the presence of antinuclear antibodies (ANA), a widely used biomarker of autoimmunity, in a representative sample of the U.S. population.

Methods: This cross-sectional analysis used data on 4,340 participants from the National Health and Nutrition Examination Survey (1999–2004), of whom 14% were ANA positive, to explore associations between ANA and concentrations of dioxins, dibenzofurans, polychlorinated biphenyls, organochlorines, organophosphates, phenols, metals, and other environmental exposures and metabolites measured in participants' serum, whole blood, or urine. For dioxin-like compounds with toxic equivalency factors, we developed and applied a new statistical approach to study selected mixtures. Lognormal models and censored-data methods produced estimates of chemical associations with ANA in males, nulliparous females, and parous females; these estimates were adjusted for confounders and accommodated concentrations below detectable levels.

Results: Several associations between chemical concentration and ANA positivity were observed, but only the association in males exposed to triclosan remained statistically significant after correcting for multiple comparisons (mean concentration ratio = 2.8; 95% CI: 1.8, 4.5; $p < 0.00001$).

Conclusions: These data suggest that background levels of most xenobiotic exposures typical in the U.S. population are not strongly associated with ANA. Future studies should ideally reduce exposure misclassification by including prospective measurement of the chemicals of concern and should track changes in ANA and other autoantibodies over time.

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Introduction

Autoimmune diseases are characterized by pathologic inflammation and autoantibodies or self-directed T lymphocyte responses. These acquired, often incurable, disorders affect up to 8% of the U.S. population, and many are rapidly increasing in prevalence for reasons that are unclear [Bach 2002; Jacobson et al. 1997; National Institutes of Health (NIH) 2005]. These diseases are major causes of death and disability among young and middle-aged women and have an enormous public health impact in the United States and worldwide (NIH 2005). Little is known about the causes of autoimmune diseases and the autoantibodies associated with them, but both genetic and environmental factors are likely to be involved (Ellis et al. 2014).

Although animal and human studies provide evidence of immunosuppression in relation to certain early- and later-life chemical exposures (e.g., low vaccine responses, thymic atrophy), autoimmune responses are less well-studied (Heilmann et al. 2010; Jusko et al. 2012; Lawrence and Kerkvliet 2006; Looker et al. 2014). However, a few studies indicate that some environmental factors, including drugs, tobacco smoke, silica, and various chemicals, are associated with autoimmune diseases and other immune effects (Miller 2011). Specific examples include polychlorinated biphenyls (PCBs) (Langer et al. 2008), hexachlorobenzene (Daniel et al. 2001; Loose et al. 1978; Michielsen et al. 1999; Queiroz et al. 1998a, 1998b; Schielen et al. 1993), and mercury (Bagenstose et al. 1999; Pollard et al. 2001; Via et al. 2003).

Among the most commonly measured biomarkers of autoimmunity are antinuclear antibodies (ANA), which are traditionally assessed by indirect immunofluorescence and are a heterogeneous group of autoantibodies targeting both nuclear and cytoplasmic components of cells (Tan 1989). Although ANA are associated with a number of autoimmune diseases, they can also develop in apparently healthy individuals after infections or following the use of medications; furthermore, their prevalence tends to be higher in parous females and the elderly (Hollingsworth et al. 1996; Parks et al. 2014; Satoh et al. 2007, 2012). Many persistent organic pollutants (POPs) exhibit hormone-disruption properties that could lead to increased ANA, and exposure to POPs has been hypothesized to increase the risk of systemic lupus erythematosus (Cooper et al. 1998). Indeed, some research has evaluated the prevalence of ANA in relation to

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1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl) ethenyl]benzene (Cooper et al. 2004), PCBs (Gallagher et al. 2013), asbestos (Pfau et al. 2005), and mercury (Bernhoft 2012; Gallagher and Meliker 2012; Lubick 2010; Nyland et al. 2011; Somers et al. 2015). However, to date, few studies have considered a broad range of background chemical exposures in relation to ANA.

In light of the limited information available about the effects of xenobiotics on autoimmunity, and given the availability of both ANA and chemical data for a large number of individuals in the National Health and Nutrition Examination Survey (NHANES), we assessed ANA associations with selected xenobiotics and mixtures by evaluating NHANES data from 1999 to 2004.

Methods

Study participants. The NHANES data were collected by the U.S. Centers for Disease Control and Prevention (CDC) in 2-year cycles; we analyzed data from 1999–2000, 2001–2002, and 2003–2004 (http://www.cdc.gov/nchs/nhanes/nhanes_questionnaires.htm). From these cycles, NHANES staff used a multistage strategy to select a representative sample of 7,106 participants ≥ 12 years old for a substudy to assess serum levels of organochlorines. Of these, 4,754 had both chemical and ANA samples available for analysis. We excluded pregnant women and participants who self-reported as "other non-Hispanic race" (including non-Hispanic multiracial), reducing our sample size to 4,340. The NHANES data set provided extensive self-reported sociodemographic information and other health-related data. Constructed variables such as body mass index (BMI) and poverty index ratio (PIR) were also included (Lohman et al. 1988). We found no appreciable differences in demographic profiles between the larger substudy and our study sample (data not shown). The NHANES protocol was approved by the NCHS Research Ethics Review Board, and written informed consent was obtained from all participants (http://www.cdc.gov/nchs/nhanes/irba98.htm).

Determination of ANA status. ANA were measured in serum specimens with a standard immunofluorescence assay using commercial HEp-2 ANA slides (Inova Diagnostics) with 1:80 dilutions of sera (Satoh et al. 2012) and staining with DyLight 488-conjugated donkey antihuman IgG antibodies (Jackson ImmunoResearch) (Jakymiw et al. 2006). Staining intensities were graded from 0 to 4 relative to a standard reference gallery (http://www.cdc.gov/nchs/nhanes/nhanes1999-2000/SSANA_A.htm); intensities of 3 and 4 were defined as positive based on findings from commercial ANA reference laboratories (Chan et al. 2007; Satoh et al. 2012).

Chemical measurements. Given the exploratory nature of this study, we analyzed a diverse set of both persistent and nonpersistent chemicals. These included broad classes of compounds such as dioxins, dibenzofurans, PCBs, and other organochlorines, as well as metals, phenols, chloroacetanilides, organophosphates, pyrethroids, carbamate metabolites, cotinine, and other compounds and metabolites (Tables 1 and 2). The exception to this exploratory approach was compounds with dioxin-like activity, which have well-documented immunotoxic effects in animal studies (Lawrence and Vorderstrasse 2004). We decided *a priori* to include any chemical with a toxic equivalency factor (TEF) from the World Health Organization (WHO) (Van den Berg et al. 2006) (Table 1); other chemicals evaluated in the present study are listed in Table 2.

Although we aimed to be as broad as possible in our assessment of exposures, there were several complicating factors. First, some chemicals were undetectable in nearly all participant samples, with concentrations below the assay's limit of detection (LOD) (Browne and Whitcomb 2010). Although we used statistical methods developed to handle large proportions of nondetects (Dinse et al. 2014; Helsel 2012), we excluded chemicals for which the overall proportion (across all cycles) of undetectable concentrations exceeded 90% [e.g., 1,2,3,7,8-pentachlorodibenzofuran (PnCDF); Table 1] because statistical estimates could become unstable in these cases. Second, measured concentrations of some compounds of interest, such as perfluoroalkyl substances, were not determined in the NHANES participants with ANA data, further limiting the number of environmental chemicals available for the present study.

Chemicals or their metabolites were measured in the serum, whole blood, or urine of NHANES participants. All specimens were analyzed by the Division of Laboratory

Table 1. Available data for dioxin-like chemicals for 4,340 participants studied in the 1999–2004 National Health and Nutrition Examination Surveys (NHANES).

		Number of observations (percent < LOD)[b]		
Chemical (pg/g serum lipid)	TEF[a]	Cycle 1: 1999–2000	Cycle 2: 2001–2002	Cycle 3: 2003–2004
Chlorinated dibenzo-*p*-dioxins				
2,3,7,8-TCDD	1.00000	1,565 (100)	1,092 (87)	1,683 (63)
1,2,3,7,8-PnCDD	1.00000	1,554 (87)	1,087 (64)	1,683 (47)
1,2,3,4,7,8-HxCDD	0.10000	0	1,090 (65)	1,665 (75)
1,2,3,6,7,8-HxCDD	0.10000	1,523 (61)	1,086 (6)	1,673 (19)
1,2,3,7,8,9-HxCDD	0.10000	1,514 (87)	1,088 (58)	1,672 (73)
1,2,3,4,6,7,8-HpCDD	0.01000	1,519 (42)	1,070 (1)	1,677 (3)
1,2,3,4,6,7,8,9-OCDD	0.00030	1,544 (40)	1,033 (18)	1,656 (16)
Chlorinated dibenzofurans				
2,3,4,7,8-PnCDF	0.30000	1,546 (62)	1,081 (33)	1,675 (35)
2,3,7,8-TCDF[c]	0.10000	1,546 (100)	1,084 (99)	1,673 (97)
1,2,3,4,7,8-HxCDF	0.10000	1,530 (64)	1,078 (17)	1,670 (40)
1,2,3,6,7,8-HxCDF	0.10000	1,538 (80)	1,089 (28)	1,671 (51)
1,2,3,7,8,9-HxCDF[c]	0.10000	1,519 (100)	1,078 (100)	1,668 (100)
2,3,4,6,7,8-HxCDF[c]	0.10000	1,527 (98)	1,083 (89)	1,669 (95)
1,2,3,7,8-PnCDF[c]	0.03000	1,559 (100)	1,085 (99)	1,671 (98)
1,2,3,4,6,7,8-HpCDF	0.01000	1,372 (57)	1,071 (10)	1,661 (10)
1,2,3,4,7,8,9-HpCDF[c]	0.01000	0	1,073 (100)	1,656 (94)
1,2,3,4,6,7,8,9-OCDF	0.00030	1,516 (99)	1,058 (100)	1,654 (73)
Dioxin-like polychlorinated biphenyls				
3,3′,4,4′,5-Pentachlorobiphenyl (PCB126)	0.10000	1,544 (51)	1,079 (11)	1,664 (7)
3,3′,4,4′,5,5′-Hexachlorobiphenyl (PCB169)	0.03000	1,526 (53)	1,076 (11)	1,668 (42)
3,4,4′,5-Tetrachlorobiphenyl (PCB81)	0.00030	1,528 (99)	1,070 (100)	1,664 (64)
2,3,3′,4,4′-Pentachlorobiphenyl (PCB105)	0.00003	1,510 (89)	1,092 (76)	1,637 (3)
2,3′,4,4′,5-Pentachlorobiphenyl (PCB118)	0.00003	1,520 (60)	1,092 (24)	1,642 (0)
2,3,3′,4,4′,5-Hexachlorobiphenyl (PCB156)	0.00003	1,501 (71)	1,087 (40)	1,645 (18)
2,3,3′,4,4′,5′-Hexachlorobiphenyl (PCB157)	0.00003	1,497 (97)	1,086 (90)	1,631 (36)
2,3′,4,4′,5,5′-Hexachlorobiphenyl (PCB167)	0.00003	1,504 (95)	1,085 (87)	1,636 (42)
2,3,3′,4,4′,5,5′-Heptachlorobiphenyl (PCB189)	0.00003	0	1,090 (100)	1,596 (76)

A lone zero in the 1999–2000 column indicates that the chemical in that row was excluded from the mixtures analyses owing to missing data in at least one cycle (i.e., Cycle 1).

Abbreviations: ANA, antinuclear antibodies; HpCDD, heptachlorodibenzo-*p*-dioxin; HpCDF, heptachlorodibenzofuran; HxCDD, hexachlorodibenzo-*p*-dioxin; HxCDF, hexachlorodibenzofuran; LOD, limit of detection; OCDD, octachlorodibenzo-*p*-dioxin; OCDF, octachlorodibenzofuran; PCB, polychlorinated biphenyl; PnCDD, pentachlorodibenzo-*p*-dioxin; PnCDF, pentachlorodibenzofuran; TCDD, tetrachlorodibenzo-*p*-dioxin; TCDF, tetrachlorodibenzofuran; TEF, toxic equivalency factor.

[a]The TEF values are the 2005 World Health Organization estimates (Van den Berg et al. 2006). [b]The percent below the LOD can vary over time because it is a function of the concentration distribution, the volume of sample available for analysis, and the analytic method used to evaluate the sample. [c]For survey years 1999–2004 combined, the overall percent below the LOD was ≥ 90%.

Sciences, National Center for Environmental Health, Atlanta, Georgia (CDC 2005, 2009). For quantitative summaries of exposure levels, see the tables in CDC (2009) and Crinnion (2010). In addition, quantitative summaries of LOD values are given in Appendix D of CDC (2009).

Individual chemicals and dioxin-like mixtures. We investigated various individual chemicals, as well as several mixtures of dioxin-like chemicals that have TEFs. Three mixture groupings (chlorinated dibenzo-*p*-dioxins, chlorinated dibenzofurans, and dioxin-like PCBs) and the TEFs of their component chemicals are shown in Table 1. When assessing these mixtures, TEFs are used as adjustment factors to transform component concentrations to a common potency scale relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Each TEF is based on expert judgment of the relative potency of a given dioxin-like chemical to that of TCDD, derived predominantly from *in vivo* rodent experiments that assessed responses induced by the aryl hydrocarbon (Ah) receptor (Van den Berg et al. 2006). Once the component concentrations have been expressed in equal potency units, they are summed to create a toxic equivalent (TEQ) concentration for the mixture.

Selection of confounders. Our previously reported analyses (Parks et al. 2014; Satoh et al. 2012) showed a greater prevalence of ANA in female versus male, parous versus nulliparous female, old versus young, normal weight versus overweight/obese, and non-Hispanic black versus non-Hispanic white. The present study confirms these associations (see Supplemental Material, Table S1) in addition to an association between ANA and time period. These factors are often predictors of chemical concentrations in NHANES (Chen et al. 2010; Ye et al. 2014) and were associated with many chemicals investigated in the present study; therefore, we considered them as possible confounders in our analyses. We also included poverty index ratio (PIR) because socioeconomic status is associated with autoimmune diseases (Calixto and Anaya 2014) and with many chemicals in our study.

Statistical model. Large proportions of nondetectable concentrations, which was the case for many chemicals in our study, can complicate the usual modeling of ANA positivity as a function of chemical concentration and various confounders. We addressed this problem by treating analyte concentration as the dependent variable and ANA status as a covariate (Dinse et al. 2014), incorporating nondetects as left-censored data and applying conventional survival methods that adjust for confounders and incorporate quantifiable analyte measurements. Our main analysis assumed a lognormal distribution for chemical concentration, a standard choice (Ott 1994) that implies log concentration is normally distributed, although we also performed parametric sensitivity analyses based on exponential, Weibull, gamma, and log-logistic distributions, and a semi-parametric sensitivity analysis based on a reverse-scale Cox method (Dinse et al. 2014). The mean log concentration was modeled by

Table 2. Available data for chemicals without a toxic equivalency factor for 4,340 participants studied in the 1999–2004 National Health and Nutrition Examination Surveys (NHANES).

Chemical or metabolite [units]	Matrix	Number of observations (percent < LOD)[a] Cycle 1: 1999–2000	Cycle 2: 2001–2002	Cycle 3: 2003–2004
Non–dioxin-like polychlorinated biphenyls [ng/g]				
2,4,4′-Trichlorobiphenyl (PCB28)	S	1,458 (98)	0	1,642 (0)
2,2′,3,5′-Tetrachlorobiphenyl (PCB44)	S	0	0	1,645 (0)
2,2′,4,5′-Tetrachlorobiphenyl (PCB49)	S	0	0	1,632 (1)
2,2′,5,5′-Tetrachlorobiphenyl (PCB52)	S	1,506 (99)	892 (90)	1,652 (0)
2,3′,4,4′-Tetrachlorobiphenyl (PCB66)	S	1,523 (97)	1,078 (89)	1,653 (1)
2,4,4′,5-Tetrachlorobiphenyl (PCB74)	S	1,515 (62)	1,092 (28)	1,653 (0)
2,2′,3,4,5′-Pentachlorobiphenyl (PCB87)	S	0	1,085 (99)	1,653 (17)
2,2′,4,4′,5-Pentachlorobiphenyl (PCB99)	S	1,493 (70)	1,077 (34)	1,632 (0)
2,2′,4,5,5′-Pentachlorobiphenyl (PCB101)	S	1,522 (99)	1,092 (96)	1,653 (3)
2,3,3′,4′,6-Pentachlorobiphenyl (PCB110)	S	0	1,085 (99)	1,639 (2)
2,2′,3,3′,4,4′-Hexachlorobiphenyl (PCB128)[b]	S	1,526 (99)	1,085 (100)	1,651 (76)
2,2′,3,4,4′,5′-Hexachlorobiphenyl (PCB138+158)	S	1,521 (65)	1,089 (5)	1,651 (0)
2,2′,3,4′,5,5′-Hexachlorobiphenyl (PCB146)	S	1,514 (76)	1,087 (48)	1,651 (2)
2,2′,3,4′,5′,6-Hexachlorobiphenyl (PCB149)	S	0	1,092 (100)	1,631 (5)
2,2′,3,5,5′,6-Hexachlorobiphenyl (PCB151)	S	0	1,092 (99)	1,632 (22)
2,2′,4,4′,5,5′-Hexachlorobiphenyl (PCB153)	S	1,518 (60)	1,092 (3)	1,651 (0)
2,2′,3,3′,4,4′,5-Heptachlorobiphenyl (PCB170)	S	1,422 (62)	1,089 (20)	1,648 (3)
2,2′,3,3′,4,5,5′-Heptachlorobiphenyl (PCB172)	S	1,499 (96)	1,066 (81)	1,647 (36)
2,2′,3,3′,4,5′,6′-Heptachlorobiphenyl (PCB177)	S	1,482 (93)	1,078 (80)	1,645 (20)
2,2′,3,3′,5,5′,6-Heptachlorobiphenyl (PCB178)	S	1,523 (91)	1,087 (77)	1,651 (25)
2,2′,3,4,4′,5,5′-Heptachlorobiphenyl (PCB180)	S	1,517 (56)	1,090 (9)	1,652 (1)
2,2′,3,4,4′,5′,6-Heptachlorobiphenyl (PCB183)	S	1,522 (86)	1,092 (65)	1,648 (12)
2,2′,3,4′,5,5′,6-Heptachlorobiphenyl (PCB187)	S	1,520 (61)	1,092 (29)	1,644 (2)
2,2′,3,3′,4,4′,5,5′-Octachlorobiphenyl (PCB194)	S	0	1,083 (33)	1,607 (22)
2,2′,3,3′,4,4′,5,6-Octachlorobiphenyl (PCB195)	S	0	1,072 (100)	1,601 (46)
2,2′,3,3′,4,4′,5,6′-Octachlorobiphenyl (PCB196+203)	S	0	1,088 (39)	1,642 (14)
2,2′,3,3′,4,5,5′,6′-Octachlorobiphenyl (PCB199)	S	0	1,083 (36)	1,627 (14)
2,2′,3,3′,4,4′,5,5′,6-Nonachlorobiphenyl (PCB206)	S	0	1,050 (86)	1,631 (7)
Decachlorobiphenyl (PCB209)	S	0	0	1,618 (7)
Organochlorines				
1-Chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]benzene (o,p′-DDT) [pg/g][b]	S	1,323 (99)	1,076 (99)	0
1-Chloro-4-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]benzene (p,p′-DDT) [pg/g]	S	1,332 (70)	1,092 (60)	0
1-Chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethenyl]benzene (p,p′-DDE) [pg/g]	S	1,549 (0)	1,090 (0)	0
Hexachlorobenzene [pg/g][b]	S	1,345 (98)	1,077 (91)	0
2,4,5-Trichlorophenol (2,4,5-TCP) [µg/g]	U	0	0	1,648 (64)
2,4,6-Trichlorophenol [µg/g]	U	1,045 (16)	1,053 (49)	1,648 (68)
Pentachlorophenol [µg/g]	U	0	0	1,536 (64)
Aldrin [ng/g][b]	S	0	1,070 (100)	0
beta-Hexachlorocyclohexane [ng/g]	S	1,501 (36)	1,077 (25)	0
Dieldrin [ng/g]	S	0	1,021 (32)	0
Endrin [ng/g][b]	S	0	1,028 (100)	0
gamma-Hexachlorocyclohexane [ng/g][b]	S	1,428 (97)	1,070 (99)	0
Heptachlor epoxide [ng/g]	S	1,265 (66)	1,065 (37)	0
Mirex [ng/g][c]	S	1,451 (92)	1,078 (64)	0
Oxychlordane [ng/g]	S	1,321 (46)	1,057 (16)	0
trans-Nonachlor [ng/g]	S	1,527 (30)	1,075 (9)	0
Metals				
Cadmium [µg/L]	WB	1,564 (23)	1,091 (26)	1,681 (23)
Lead [µg/dL]	WB	1,564 (0)	1,091 (0)	1,681 (0)
Mercury, total blood [µg/L][d]	WB	369 (7)	276 (5)	1,681 (8)
Mercury, inorganic blood [µg/L][d]	WB	369 (97)	272 (93)	1,656 (74)
Mercury, urinary [µg/g][e]	U	358 (11)	266 (13)	0
Phenols [µg/g]				
Bisphenol A (BPA)	U	0	0	1,648 (7)
Triclosan	U	0	0	1,648 (25)
Benzophenone-3	U	0	0	1,648 (3)

Table continued

a linear function of covariates; thus, covariate effects on mean concentration were multiplicative (see Supplemental Material, "Statistical Model"). We assessed the association between chemical concentration and ANA via the sign, magnitude, and statistical significance of the estimated regression coefficient for ANA. A default alpha level of 0.05 was used to judge statistical significance. The regression models excluded participants with missing covariate values, reducing the sample size to 3,754 in the adjusted analyses.

The LIFEREG procedure in SAS (v9.3; SAS Institute, Cary, NC) was used to perform the lognormal regression analyses, where the outcome variable was either an individual chemical's concentration or a mixture's TEQ concentration. Concentrations of lipophilic compounds were modeled on a per-lipid basis, and those determined in urine were modeled on a creatinine basis to account for dilution. We compared the results on a per-lipid basis with results obtained when including total lipid concentration as a covariate instead of dividing analyte concentration by total lipid concentration (Schisterman et al. 2005), and we observed little difference (data not shown). Thus, we chose to model concentrations of lipophilic compounds on a per-lipid basis. We ran a similar sensitivity analysis for chemicals measured in urine, including creatinine as a covariate rather than dividing analyte concentration by creatinine concentration. The results were not materially different (data not shown); therefore, we chose to model those concentrations on a creatinine basis. Regarding the small differences cited in these sensitivity analyses, we examined each chemical's regression coefficient for ANA and obtained similar estimates using both models; among the estimated coefficients that were statistically significantly different from zero, the signs were the same under both models, and the magnitudes were very close.

Our analyses included ANA status and potential confounders as covariates. To fully adjust for sex and parity, we performed separate analyses for males, nulliparous females, and parous females. Stratification on parity simplified the modeling and was based on evidence that nulliparous and parous women differ in ANA prevalence and possibly in how ANA relates to other factors such as age (Parks et al. 2014). The potential confounders considered were race/ethnicity, time period, BMI, age, and PIR. We used categorical variables to summarize race/ethnicity (non-Hispanic white, non-Hispanic black, Hispanic), time period (1999–2000, 2001–2002, 2003–2004), and BMI (underweight, normal, overweight, obese). We treated age and PIR as quantitative (continuous) variables, using a restricted cubic spline (Harrell 2001) for age and a linear term for PIR. Allowing confounder categories to act as ANA effect modifiers generally did not provide a statistically significant improvement in model fit, so we did not include ANA-by-confounder interactions in our primary analyses. However, as a post hoc analysis to further investigate the association between ANA and one particular chemical (triclosan), we fitted several expanded models, with each adding a two-way interaction between ANA and a given confounder.

Appropriate statistical interpretations depend on having adequate data. Thus, within each sex/parity group, we excluded any chemical for which fewer than six ANA-positive participants had a detectable concentration. This procedure eliminated one chemical in males (urinary mercury) and two in nulliparous females (mirex and alachlor mercapturate).

Assessing chemical–ANA associations. Associations between chemical concentration and ANA were estimated using the ANA regression coefficient. Because ANA effects in nulliparous and parous females were often similar, we simplified the reporting of some results by calculating combined estimates for all females as weighted averages of parity-specific estimates using inverse variance estimates as weights. However, rather than work directly with the ANA regression coefficient, we exponentiated it to obtain a parameter that was interpretable as the ratio of mean concentrations for ANA-positive versus ANA-negative participants (see Supplemental Material, "Statistical Model"). Estimates of this mean concentration ratio (MCR) > 1 corresponded to positive associations between chemical concentration

Table 2. Continued.

Chemical or metabolite [units]	Matrix	Number of observations (percent < LOD)[a] Cycle 1: 1999–2000	Cycle 2: 2001–2002	Cycle 3: 2003–2004
Chloroacetanilides [µg/g]				
Acetochlor mercapturate[b]	U	0	1,055 (98)	0
Alachlor mercapturate[c]	U	1,026 (66)	0	0
Metolachlor mercapturate[b]	U	0	1,067 (97)	0
Organophosphates [µg/g]				
Dimethylphosphate (DMP)	U	0	0	1,631 (49)
Diethylphosphate (DEP)	U	0	0	1,598 (47)
Dimethylthiophosphate (DMTP)	U	0	0	1,631 (20)
Diethylthiophosphate (DETP)	U	0	0	1,610 (48)
Dimethyldithiophosphate (DMDTP)	U	0	0	1,610 (58)
Diethyldithiophosphate (DEDTP)[b]	U	0	0	1,631 (91)
3-Chloro-7-hydroxy-4-methyl-2H-chromen-2-one/ol[b]	U	0	1,039 (97)	0
3,5,6-Trichloro-2-pyridinol	U	1,050 (7)	1,050 (28)	0
Diethylaminomethylpyrimidinol/one[b]	U	0	1,047 (95)	0
Malathion dicarboxylic acid	U	1,023 (46)	0	0
para-Nitrophenol	U	1,049 (76)	1,038 (51)	0
Oxypyrimidine	U	956 (68)	1,067 (96)	0
Pyrethroids [µg/g]				
4-Fluoro-3-phenoxybenzoic acid[b]	U	1,024 (96)	1,068 (100)	0
cis-3-(2,2-Dibromovinyl)-2,2-dimethylcyclopropane carboxylic acid[b]	U	895 (100)	1,068 (99)	0
cis-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (cis-Cl2CA)	U	1,029 (56)	1,068 (66)	0
3-Phenoxybenzoic acid	U	1,052 (29)	1,068 (25)	0
trans-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (trans-Cl2CA)	U	1,042 (66)	1,063 (75)	0
Carbamates [µg/g]				
2-Isopropoxyphenol[b]	U	1,007 (97)	1,053 (100)	1,556 (100)
Carbofuranphenol[b]	U	1,049 (87)	1,061 (100)	1,557 (100)
Tobacco smoke exposure [ng/mL]				
Cotinine	S	1,548 (37)	1,085 (25)	1,681 (16)
Other compounds [µg/g]				
Atrazine mercapturate[b]	U	1,000 (95)	1,042 (99)	0
2,4-Dichlorophenol	U	0	0	1,648 (16)
N,N-Diethyl-3-methylbenzamide (DEET)	U	1,036 (84)	1,067 (88)	0
ortho-Phenylphenol	U	0	0	1,648 (45)
2,5-Dichlorophenol	U	0	0	1,648 (1)
2,4-Dichlorophenoxyacetic acid	U	1,041 (46)	1,022 (74)	0
2,4,5-Trichlorophenoxyacetic acid[b]	U	969 (96)	1,067 (100)	0

Abbreviations: ANA, antinuclear antibodies; LOD, limit of detection; PCB, polychlorinated biphenyl; S, serum; U, urine; WB, whole blood.
[a]The percent below the LOD can vary over time because it is a function of the concentration distribution, the volume of sample available for analysis, and the analytic method used to evaluate the sample. [b]For survey years 1999–2004 combined, the overall proportion below the LOD was ≥ 90%. [c]< 6 nulliparous female participants were ANA positive and had a detectable concentration. [d]No data were available for males in survey years 1999–2002. [e]No data were available for females in survey years 2003–2004 or for males in any survey years.

and ANA (i.e., persons with higher concentrations had a higher prevalence of ANA). Similarly, an MCR < 1 corresponded to a negative chemical-ANA association, such that persons with higher concentrations had a lower prevalence of ANA. An MCR = 1 corresponded to no association between ANA and the chemical. Logarithmic distance from 1 reflects association strength.

Accounting for censoring. Nondetectable concentrations were left-censored, known only to be less than the LOD, and a mixture TEQ was interval-censored if some component concentrations were below the LOD and others were not, in which case the TEQ was known to be between a lower limit and an upper limit (see Supplemental Material, "Accounting for censoring"). If all information on a component chemical was missing, the TEQ censoring interval ranged from zero to infinity and was uninformative. Rather than exclude such persons, however, we calculated their TEQs by treating missing concentrations as censored in the interval from zero to the largest observed concentration for that chemical. As a sensitivity analysis, we compared the results obtained when excluding and including those with missing component concentrations; both analyses yielded similar results (data not shown).

Accounting for sampling. The NHANES data were obtained from a multistage stratified cluster sample. The LIFEREG procedure does not incorporate information on sampling strata and clusters; therefore, although it properly estimates regression coefficients, it does not account for the correlation structure when estimating variances. Thus, when constructing confidence intervals (CIs) for regression coefficients, we used a jackknife procedure to provide standard errors appropriate for complex survey data (see Supplemental Material, "Accounting for sampling"). We ignored probability sampling weights to improve efficiency for assessing chemical–ANA associations, exploiting the fact that our analysis conditions on variables that influenced the sampling (Korn and Graubard 1999).

Accounting for multiple comparisons. Because many chemicals were investigated, we used a Bonferroni correction to adjust statistical significance for multiple comparisons. We report both uncorrected and corrected results. Consistent with the exploratory nature of our study, uncorrected results with $p < 0.05$ can be used to generate hypotheses for future investigation, although many may later prove to be false positives. Bonferroni correction is fairly conservative; therefore, associations that remain statistically significant after adjustment are more likely to be true positives. We also applied the false discovery rate approach (Benjamini and Hochberg 1995) for comparison, which is less conservative than the Bonferroni method, and obtained similar results (data not shown).

Results

Participant descriptors. Of the 4,340 NHANES participants in our analysis, 623 (14.4%) were ANA positive, which is consistent with previous ANA prevalence estimates of 13.8% (Satoh et al. 2012), 13.3% (Tan et al. 1997), and 12.9% (Mariz et al. 2011). In addition, of these 4,340 participants, 51% were males, 29% were parous females, 17% were nulliparous females, and 3% were females with no information on parity (see Supplemental Material, Table S1). The distribution of participants across categories of race/ethnicity, time period, age, PIR, and BMI, as well as category-specific ANA positivity percentages, are also shown in the Supplemental Material, Table S1. Multiple logistic regression produced odds ratios that confirmed an association between ANA and several of the covariates in our analysis (see Supplemental Material, Table S1).

Dioxin-like chemicals. We investigated 26 dioxin-like chemicals for which information was available in NHANES; these chemicals were classified into 7 chlorinated dibenzo-*p*-dioxins, 10 chlorinated dibenzofurans, and 9 dioxin-like PCBs (Table 1). We analyzed 21 of the chemicals individually after excluding 5 chlorinated dibenzofurans because > 90% of their concentrations were below the LOD. We also analyzed mixtures of chemicals within categories as well as an overall mixture of dioxin-like chemicals. The mixture analyses excluded 3 chemicals without data in one NHANES cycle, 1 of which had already been eliminated because of heavy censoring. Therefore, the mixture analyses involved 19 dioxin-like chemicals, comprising 6 chlorinated dibenzo-*p*-dioxins, 5 chlorinated dibenzofurans, and 8 dioxin-like PCBs.

Overall, there was little evidence that ANA were associated with any of the dioxin-like chemicals or their mixtures. Only two dioxin-like chemicals were statistically significantly associated with ANA at the 0.05 level (Figure 1). Those chemicals were 1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin (OCDF) in males (MCR = 1.3; 95% CI: 1.0, 1.8; $p = 0.04$) and 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin (HpCDD) in males (MCR = 0.9; 95% CI: 0.8, 1.0; $p = 0.05$). Among all of the dioxin-like chemicals, the only one with an MCR larger than the 1.3 observed for 1,2,3,4,6,7,8,9-OCDF in males was PCB189 in nulliparous females (MCR = 3.1; 95% CI: 0.6, 15.1; $p = 0.16$), although this MCR was not significantly greater than 1. With regard to the mixture concentrations, neither the overall TEQ nor any category-specific TEQ was significantly associated with ANA regardless of sex or parity (Figure 1).

We also summarized sex-specific associations between chemical concentration and ANA in terms of statistical significance (Figure 2). The two associations noted above (1,2,3,4,6,7,8,9-OCDF and 1,2,3,4,6,7,8-HpCDD, both in males) were statistically significant at the uncorrected 0.05 level, but not after correcting for multiple comparisons.

Non–dioxin-like chemicals. We investigated 83 non–dioxin-like chemicals, which were subdivided into 10 categories: 29 non–dioxin-like PCBs, 16 organochlorines, 5 metals, 3 phenols, 3 chloroacetanilides, 12 organophosphates, 5 pyrethroids, 2 carbamates, 1 biomarker of tobacco smoke exposure, and 7 other compounds (Table 2). Excluding chemicals with > 90% of their concentrations below the LOD left 66 non–dioxin-like chemicals in 9 categories: 28 PCBs, 11 organochlorines, 5 metals, 3 phenols, 1 chloroacetanilide, 9 organophosphates, 3 pyrethroids, 1 biomarker of tobacco smoke exposure, and 5 other compounds.

For each non–dioxin-like chemical, an estimate of the MCR and its 95% CI are shown in Figure 1 for each sex/parity group. Without correcting for multiple comparisons, 15 non–dioxin-like chemicals showed some evidence of an association with ANA ($p < 0.05$). Of these, 11 associations were in males: triclosan (MCR = 2.8; 95% CI: 1.8, 4.5; $p < 0.00001$), PCB101 (MCR = 0.8; 95% CI: 0.7, 0.9; $p = 0.001$), PCB44 (MCR = 0.9; 95% CI: 0.8, 1.0; $p = 0.01$), oxypyrimidine (MCR = 1.8; 95% CI: 1.1, 3.1; $p = 0.02$), PCB110 (MCR = 0.9; 95% CI: 0.7, 1.0; $p = 0.03$), 2,4-dichlorophenoxyacetic acid (MCR = 0.6; 95% CI: 0.4, 1.0; $p = 0.03$), PCB52 (MCR = 0.9; 95% CI: 0.8, 1.0; $p = 0.04$), 1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethenyl]benzene (*p,p′*-DDE) (MCR = 0.8; 95% CI: 0.7, 1.0; $p = 0.04$), PCB66 (MCR = 0.9; 95% CI: 0.8, 1.0; $p = 0.04$), PCB74 (MCR = 0.9; 95% CI: 0.8, 1.0; $p = 0.04$), and oxychlordane (MCR = 0.9; 95% CI: 0.8, 1.0; $p = 0.04$). There were 4 suggestive associations in females: 2,4-dichlorophenol (MCR = 0.7; 95% CI: 0.5, 0.9; $p = 0.01$), PCB151 (MCR = 0.8; 95% CI: 0.7, 1.0; $p = 0.02$), 2,5-dichlorophenol (MCR = 0.7; 95% CI: 0.5, 1.0; $p = 0.03$), and dimethylthiophosphate (MCR = 1.3; 95% CI: 1.0, 1.7; $p = 0.03$).

Not only does Figure 1 illustrate the sex-specific associations mentioned above, but it also shows the parity-specific associations in females. Although none of these associations was statistically significant after correcting for multiple testing, 4 associations were suggestive ($p < 0.05$) in nulliparous females: *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (trans-Cl2CA) (MCR = 2.1;

95% CI: 1.2, 3.9; p = 0.01), oxypyrimidine (MCR = 0.5; 95% CI: 0.3, 0.9; p = 0.01), PCB138 (MCR = 0.9; 95% CI: 0.8, 1.0; p = 0.02), and PCB74 (MCR = 0.9; 95% CI: 0.8, 1.0; p = 0.05). There were also two suggestive associations in parous females, dimethylthiophosphate (MCR = 1.6; 95% CI: 1.1, 2.2; p = 0.01) and alachlor mercapturate (MCR = 3.8; 95% CI: 1.1, 13.7; p = 0.04); the former was also noted for all females combined (MCR = 1.3; 95% CI: 1.0, 1.7; p = 0.03).

The statistical significance of associations between ANA and non–dioxin-like chemicals was plotted separately for males and females (Figure 2). Of the 15 non–dioxin-like chemicals associated with ANA at the 0.05 level in either sex, only one association remained statistically significant after correcting for multiple comparisons: triclosan in males (MCR = 2.8; 95% CI: 1.8, 4.5; p < 0.00001), where creatinine-adjusted concentrations were higher in ANA-positive participants than in ANA-negative participants (see Supplemental Material, Figure S1). The nonparametric curves in Supplemental Material Figure S1 were constructed using the methods of Kaplan and Meier (1958) with concentrations below the LOD treated as left-censored observations; these curves were not adjusted for covariates.

Our primary regression model was adjusted for confounders but did not allow ANA effects to vary with confounders because in nearly all cases, the improvement in model fit due to adding interactions was not statistically significant. However, to further investigate the association between ANA and triclosan in males, we fitted several expanded models, with each adding a two-way interaction between ANA and a given confounder. The positive association between ANA and triclosan appeared to be subject to effect modification by age but not by race/ethnicity, BMI, or PIR. The MCR estimates were 2.6 (95% CI: 1.2, 5.4) in the 12–19 age group, 1.3 (95% CI: 0.6, 2.9) in the 20–54 age group, and 7.1 (95% CI: 3.5, 14.8) in the ≥ 55 age group (overall p = 0.03).

Discussion

In general, our results did not suggest strong associations between the studied background xenobiotic exposures and ANA in this population-representative survey. These null results were consistent across classes of chemicals and across sex/parity groups. To our knowledge, this is the most comprehensive study to date of xenobiotic exposures and their possible associations with ANA.

Although our results for ANA were generally null, some chemicals showed weak associations that did not meet the Bonferroni level of significance but may warrant further

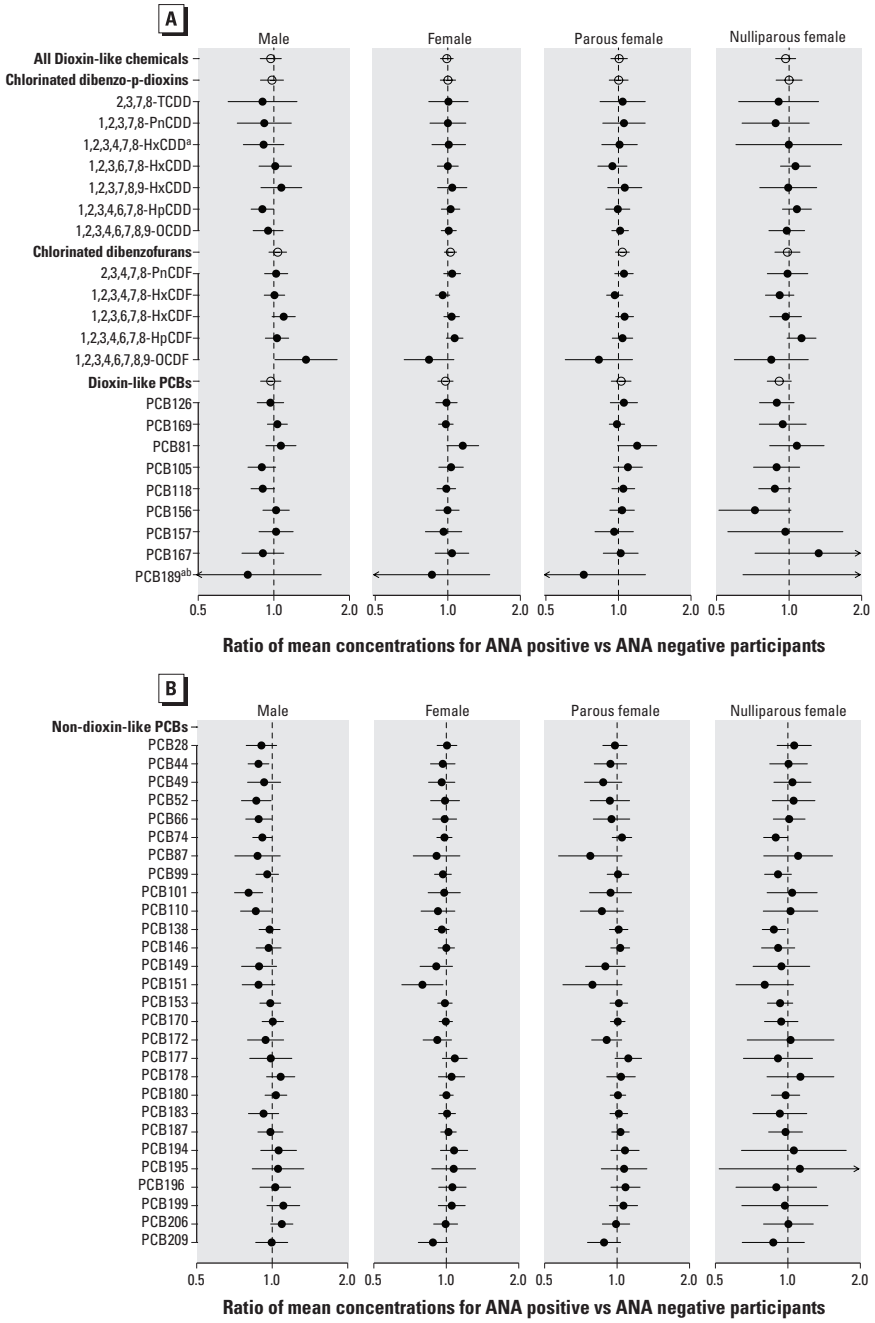

Figure 1. Estimated ANA positivity effects by sex and parity for individual chemicals and dioxin-like chemical mixtures, National Health and Nutrition Examination Survey, 1999–2004. Estimated ratios of mean concentrations (MCRs) for ANA-positive versus ANA-negative participants are plotted as solid dots for 21 dioxin-like chemicals in panel *A* and for 66 non–dioxin-like chemicals in panels *B–D*. Analogous estimates for dioxin-like chemical mixtures, both overall and within categories, are plotted as open circles in panel *A*. All estimates are adjusted for age, race/ethnicity, time period, BMI, and PIR. Values below (above) 1.0 indicate that those positive for ANA had a lower (higher) mean concentration of the chemical or mixture than those negative for ANA. The horizontal lines represent 95% confidence intervals, and left (right) arrowheads indicate that values extend below 0.5 (above 2.0). Results are shown separately by sex and parity, with overall female estimates calculated from inverse-variance weighted averages of parity-specific estimates.

[a]Two chemicals [1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin (HxCDD) and 2,3,3′,4,4′,5,5′-heptachlorobiphenyl (PCB189)] were excluded from mixture estimates because of missing data for 1999–2000. [b]Five chemicals had MCRs below 0.5 or above 2.0 in one sex/parity group; therefore, no solid dot was plotted. The unplotted MCRs were 3.1 (95% CI: 0.6, 15.1) for PCB189 in nulliparous females, 2.8 (95% CI: 1.8, 4.5) for triclosan in males, 3.8 (95% CI: 1.1, 13.7) for alachlor mercapturate in parous females, 0.5 (95% CI: 0.1, 2.4) for *para*-ntriophenol in nulliparous females, and 2.1 (95% CI: 1.8, 3.9) for *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (trans-Cl2CA) in nulliparous females. [c]For two chemicals (mirex and alachlor mercapturate), < 6 nulliparous females were ANA positive and had detectable concentrations; therefore, nothing was plotted for nulliparous females or for all females combined. [d]One chemical (urinary mercury) had no data for males; therefore, nothing was plotted for males.

Figure continued

consideration in future investigations because we cannot rule out their involvement in immune alterations that could lead to autoimmunity. The strong association between elevated triclosan concentrations and ANA positivity in males deserves comment. Triclosan is an antimicrobial used in a wide variety of consumer products such as toothpastes, soaps, and toys that works by blocking the active site of enoyl-acyl carrier protein reductase, an enzyme essential for fatty acid synthesis in bacteria (Fang et al. 2010; Yueh et al. 2014). The primary route of excretion of enoyl-acyl carrier protein reductase is via urination, and the estimated half-life of this enzyme is approximately 11 hr in urine. (Calafat et al. 2008; Fang et al. 2010; Sandborgh-Englund et al. 2006). Despite its short half-life, urinary measures of triclosan appear to be less variable over time than those of other phenols, such as bisphenol A (BPA) (Bertelsen et al. 2014; Koch et al. 2014; Meeker et al. 2013). Thus, the concentration of triclosan in a spot urine sample, such as those collected for NHANES, may serve as a reasonable biomarker of triclosan exposure. In terms of the potential immunotoxicity of triclosan, Clayton et al. (2011), using NHANES data, observed a positive association between urinary triclosan concentrations and the odds of having been diagnosed with allergies or hay fever, and others have also reported positive associations between urinary triclosan concentrations and allergic sensitization (Bertelsen et al. 2013). Similar results have been observed in female mice, where exposure to triclosan enhanced the hypersensitivity response to an allergen (Anderson et al. 2013). Although it is unclear how triclosan could be related to the development of autoimmunity, and why the association was only seen in males in our study, the enhancement of certain T-cell responses is thought to be strongly associated with the development of autoimmunity and autoimmune disease related to environmental exposures (Selmi et al. 2012). To address public health concerns, more studies are needed of populations exposed to high levels of triclosan; ideally, these studies should follow markers of immune function before, during, and after exposure.

A few small studies have reported associations between ANA positivity and various chemicals (Cebecauer et al. 2009; Cooper et al. 2004; Daniel et al. 2001; Kilburn and Warshaw 1992; Rosenberg et al. 1999), and some investigations of associations between exposures and ANA have been conducted in highly exposed individuals (e.g., miners and mercury exposure, people living in areas with substantial environmental contamination) (Bernhoft 2012; Lubick 2010; Nyland et al. 2011). In contrast, our study was based on a representative sample of the U.S. population wherein most participants presumably had only background exposures to xenobiotics. Thus, given certain limitations, we cautiously interpret our findings as somewhat reassuring from a public health perspective because there were very few statistically significant associations between xenobiotic concentrations and ANA. However, the triclosan results raise questions that will require further study.

A major limitation of our study was the assessment of exposure at a single time point. Although a single measure of serum TCDD should be reasonably reflective of body burden or long-term exposure, owing to its half-life of approximately a decade in adults (Wolfe et al. 1994), spot urine concentrations of some nonpersistent compounds are unlikely to provide good representations of long-term, average exposure. For instance, multiple spot urine specimens taken from women during pregnancy typically demonstrate low reproducibility for exposure to BPA (Jusko et al. 2014) and organophosphate pesticide metabolites (Spaan et al. 2015). Consequently, some chemicals may be more susceptible to exposure misclassification than others, and this misclassification is

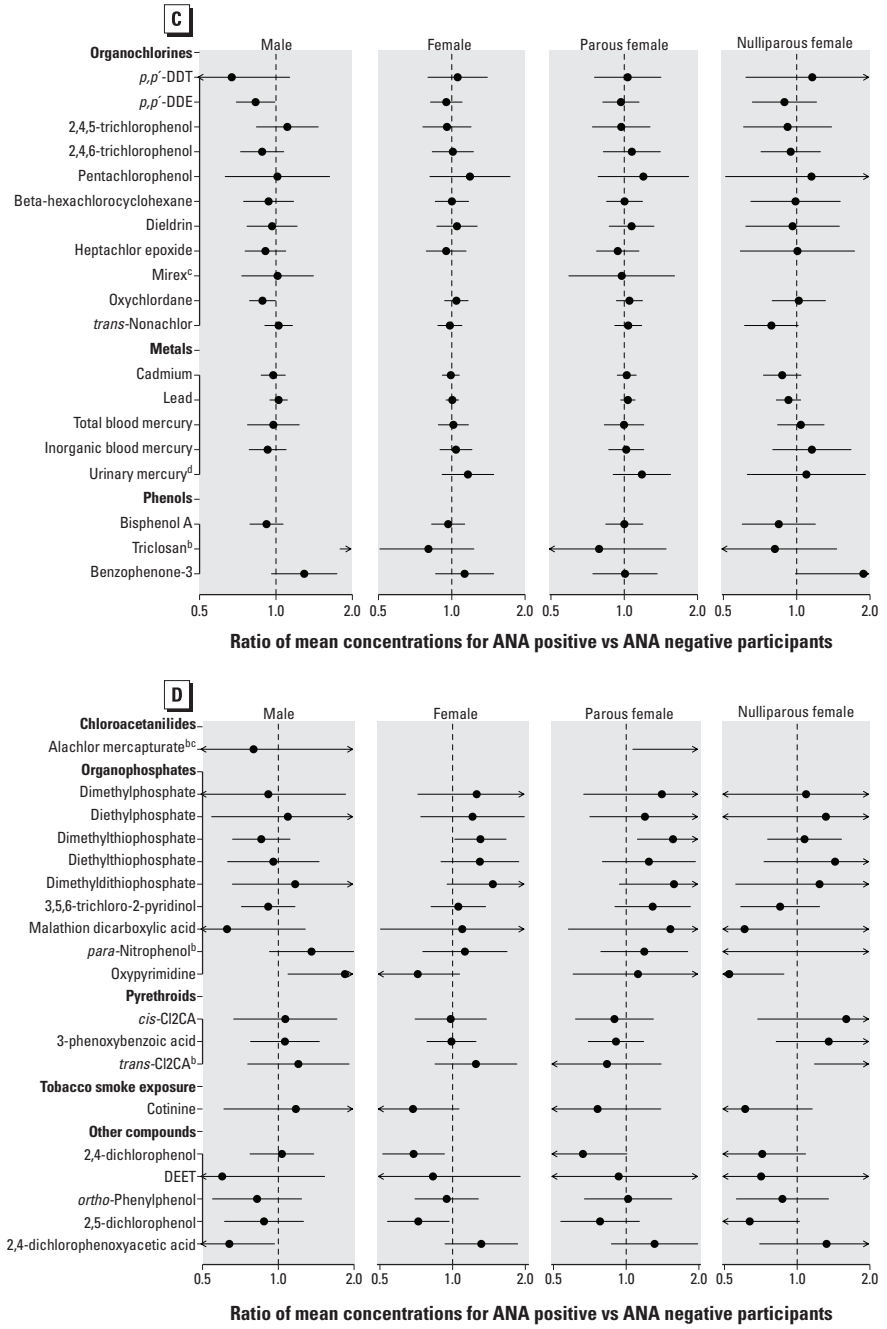

Figure 1. Continued.

largely dependent on their persistence in the matrices used to assess exposure. In addition to problems with variability, temporality is an issue for chemicals with short half-lives because ANA positivity would have developed before biospecimen collection, and we had no historical exposure information. Prospective cohort studies with measurements taken over time would be required to investigate causality (e.g., for triclosan).

Another limitation was that whereas some chemical concentrations were determined in each 2-year cycle, others were determined in only one or two of the three NHANES cycles, which reduced our statistical power to detect associations between ANA and chemical concentrations. More substantially, some chemicals of interest could not be evaluated because the CDC chemical analysis subsample did not overlap with our ANA subsample. Examples include perfluorinated alkyl substances and phthalates, both of which may exert immunotoxic effects (DeWitt et al. 2012; Grandjean et al. 2012; Hoppin et al. 2013).

A potential limitation of the present study concerns possible model misspecification with regard to confounders. The inclusion or exclusion of true confounders may have over- or underestimated our ANA associations with exposure. For example, a previous analysis of NHANES data suggested an association between ANA and a mixture of dioxin-like PCBs in females (Gallagher et al. 2013). In that analysis, all nondetects were replaced by LOD divided by the square root of 2, and two of the three NHANES cycles were ignored because the proportion of nondetects was extremely large. By comparison, our analysis did not find this association to be statistically significant ($p < 0.05$) despite our using data from all three cycles and reducing bias by treating nondetects as censored. Further investigation revealed that the significance that was originally reported depended mainly on excluding age as a predictor; see the last two columns in Table 2 of Gallagher et al. (2013), which correspond to including and excluding age, respectively. Using the data and covariates of Gallagher et al (2013), neither our lognormal analysis nor their logistic analysis showed a significant association between ANA and the PCB mixture when age was included in the model, but both analyses did show an association when age was removed from the model. For example, the lognormal analysis estimated the MCR as 1.05 (95% CI: 0.98, 1.14) when age was included and 1.17 (95% CI: 1.06, 1.29) when age was excluded. However, we believe it is important to adjust for age, particularly because age is related to both ANA and many chemical concentrations. Thus, in a similar vein, although we stratified on sex and parity, and although our regressions included age and demographic factors related to propensity to exposure, there may be important determinants of exposure and ANA that were not included in our models. For consistency and for screening purposes, we included the same covariates for every chemical, but more individualized analyses with different adjustments might reveal new insights in some cases.

Another recent analysis of NHANES data suggested that ANA were associated with total blood mercury but not with urinary mercury (Somers et al. 2015). That analysis used a weighted logistic model for ANA status and a categorical predictor for mercury after substituting LOD divided by the square root of 2 for concentrations below the LOD, but it did not examine inorganic blood mercury because of heavy censoring. Our unweighted lognormal model for mercury, with ANA as a predictor, did not find a significant association for ANA with total blood mercury, inorganic blood mercury, or urinary mercury. Although the two analyses used different models, covariates, and censoring adjustments, closer inspection suggests that the significance of the association between total blood mercury and ANA may have been

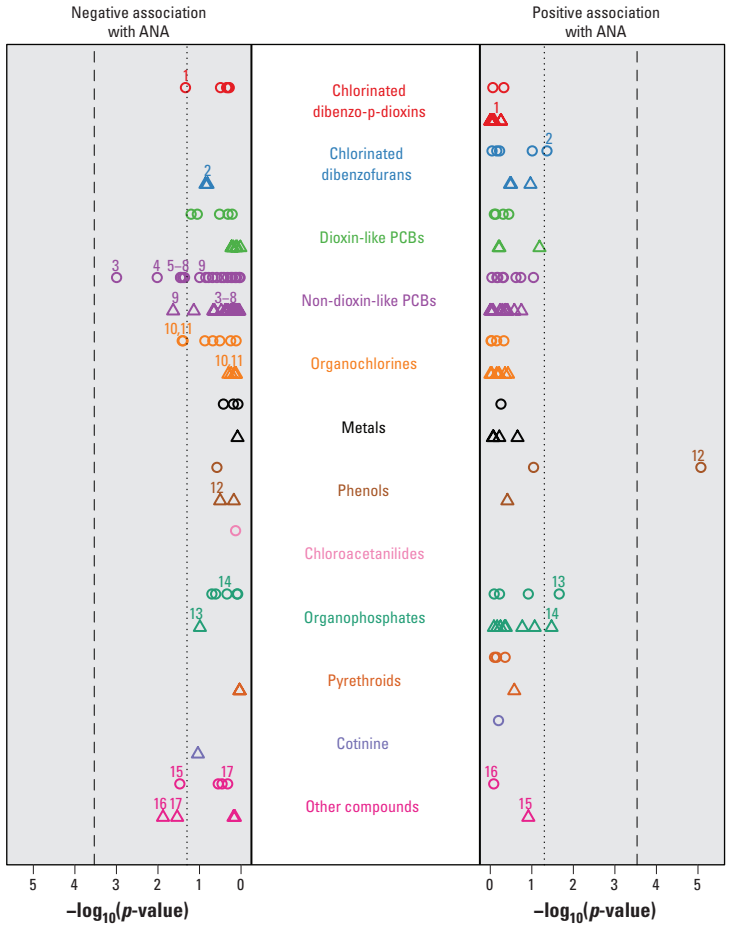

Figure 2. Statistical significance of associations between ANA and selected xenobiotics by sex, National Health and Nutrition Examination Survey, 1999–2004. For each chemical, the statistical significance of the ANA regression coefficient was calculated separately for males and females, under a lognormal concentration model adjusted for age, race/ethnicity, time period, BMI, and PIR. Chemicals are arranged within color-coded categories along the vertical axis, and negative log *p*-values are shown along the horizontal axes. Results are depicted by circles for males and triangles for females, where results for females were calculated from inverse-variance weighted averages of the parity-specific estimates. Symbols displayed on the right (left) indicate positive (negative) associations between ANA and the chemical. The dotted line corresponds to a *p*-value of 0.05 and the dashed line to the Bonferroni significance level, which is 0.05 divided by 171, the number of tests performed (86 for males and 85 for females). Chemicals significant at the uncorrected 0.05 level in at least one sex are labeled for both sexes. The chemical labels are: 1 = 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin (HpCDD); 2 = 1,2,3,4,6,7,8,9-octachlorodibenzofuran (OCDF); 3 = 2,2',4,5,5'-pentachlorobiphenyl (PCB101); 4 = 2,2',3,5'-tetrachlorobiphenyl (PCB44); 5 = 2,3,3',4',6-pentachlorobiphenyl (PCB110); 6 = 2,2',5,5'-tetrachlorobiphenyl (PCB52); 7 = 2,3',4,4'-tetrachlorobiphenyl (PCB66); 8 = 2,4,4',5-tetrachlorobiphenyl (PCB74); 9 = 2,2',3,5,5',6-hexachlorobiphenyl (PCB151); 10 = 1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethenyl]benzene (*p,p´*-DDE); 11 = oxychlordane; 12 = triclosan; 13 = oxypyrimidine; 14 = dimethylthiophosphate; 15 = 2,4-dichlorophenoxyacetic acid; 16 = 2,4-dichlorophenol; 17 = 2,5-dichlorophenol.

caused by treating the mercury variable as categorical rather than as quantitative. The main analysis by Somers et al. (2015) created four categories of total blood mercury. Relative to the first category, their 95% CIs for the ANA odds ratio under several covariate-adjusted models did not include 1 for the second and fourth categories but did for the third category, suggesting a possible relationship between ANA and total blood mercury. However, when we fitted the same covariate-adjusted logistic model, except with mercury (or log mercury) as a linear (continuous) predictor rather than as a categorical predictor, its association with ANA was not significant. In general, we recommend using our censored-data approach if a large proportion of concentrations are below the LOD; otherwise, depending on modeling preferences, using the conventional logistic analysis might be preferable if censoring is limited.

An additional concern is that our mixture analyses assumed that TEFs, which are based primarily on *in vivo* exposures in rodents, apply to assessments of the immune system in humans. TEFs are single-point potency estimates developed from an evaluation of a range of potencies for a given chemical inducing different end points. As such, TEFs may under- or overestimate the actual potency of a chemical for certain end points (Frawley et al. 2014; Trnovec et al. 2013; Van den Berg et al. 2006). To the extent that the TEF for a given chemical may differ from its actual potency for immune effects in humans, some distortion may be introduced into the mixtures analyses. In a conventional analysis, underestimating TEFs in a logistic model for ANA positivity should bias the estimated TEQ regression coefficient upward but should not affect power. However, the lognormal TEQ model focuses on the ratio of mean mixture concentrations for ANA-positive versus ANA-negative participants; thus, the estimated ANA regression coefficient should not be biased if all TEFs are underestimated by a fixed proportion because the constant bias factor would cancel out in both the numerator and the denominator of the ratio. Errors are unlikely to act as a simple scale change, however, and the estimated association between ANA and the TEQ would likely be biased toward the null. Nevertheless, although TEFs may represent an oversimplification, they provide a first approximation for an exploratory analysis of mixture data. TEFs were developed by the WHO and have been used worldwide as the *de facto* method for assessing cumulative exposures to mixtures of dioxin-like compounds and as a means of operationalizing exposure to dioxin-like compounds in human exposure–response relationships (Gallagher et al. 2013).

The present study also had several notable strengths. Environmental exposures were objectively measured (i.e., in serum, whole blood, or urine samples) instead of being assessed via self-reporting in surveys (e.g., fish consumption), and ANA were reliably determined. Unlike analyses that substitute specific values (e.g., LOD/2 or LOD divided by the square root of 2) for nondetects, our analyses did not assume that unknown values were known, thereby avoiding the biases and underestimates of variability that are common in conventional analyses. Furthermore, in contrast to approaches that discard nondetects or analyze detect/nondetect dichotomies, our method allowed full use of the available chemical concentration data. Although regression methods for left- (or right-) censored data have been used previously for individual chemicals with nondetectable concentrations (Dinse et al. 2014), and TEFs have been used to combine detectable concentrations into a mixture TEQ (Van den Berg et al. 2006), our formation of censoring intervals for the TEQ when some component concentrations are below the LOD is a new approach for handling mixtures of congeners.

Detection limits changed both across batches of some assays and over time. Although such changes could be problematic, they are of no consequence for our method if censoring is statistically noninformative about unknown concentrations. This assumption requires that simply knowing the true concentration is below the LOD provides no additional information about the magnitude of the unobserved concentration beyond the fact that it is between zero and the LOD. Noninformative censoring is plausible in the current setting, in which the LOD is primarily a function of the assay properties. Provided that the actual LOD is used for the assay that was applied, the analysis should be valid. Some LODs were found to be systematically lower in recent studies presumably because assay technologies had improved. As a consequence, conventional analyses that focus on the proportion of concentrations above the LOD or that impute using LOD divided by the square root of 2 or LOD/2 could be extremely unreliable, but our censored-data approach avoids this problem.

We performed several sensitivity analyses to validate various aspects of our approach. To evaluate robustness to the choice of concentration distribution, we analyzed the data using several other distributions, including exponential, Weibull, gamma, and log-logistic; all yielded results similar to those obtained from the lognormal distribution that we used (data not shown). We also applied the reverse-scale Cox method (Dinse et al. 2014) to the data for individual chemicals, and the results were not materially different (data not shown). With respect to reversing the roles of outcome and exposure, we refer readers to the simulations reported by Dinse et al. (2014), which showed that outcome/exposure reversal produced valid results over a range of circumstances. Finally, these same simulations showed that valid results were obtained when the proportion of concentrations below the LOD was as high as 90%, which is the value that we used as our highest permitted fraction censored in the present analysis.

Conclusions

This investigation of xenobiotics and ANA in a nationally representative sample of the U.S. population suggests that background levels of most of the environmental chemicals assessed, with the notable exception of triclosan in males, are not strongly associated with ANA. Future studies should ideally reduce exposure misclassification by including prospective measurement of the chemicals of concern and should track changes in ANA and other autoantibodies over time.

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Autoantibodies associated with systemic autoimmune rheumatic diseases in lung cancer patients

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Keywords

Autoantibodies, lung cancer, interstitial lung disease, scleroderma, polymyositis/dermatomyositis

Abbreviations

ACA, anti-centromere antibodies; CENP, centromere proteins; CI, confidence interval; DFS, dense fine speckled; ILD, interstitial lung disease; SCLC, small cell lung cancer; SARDs; systemic autoimmune rheumatic diseases; OR, Odds ratio; RR, relative risk; SIR, standardized incidence ratio; RNAP, RNA polymerase; ACA, anti-centromere antibodies; RNPC-3, RNA binding region (RNP1, RRM) containing 3;

Introduction

Lung cancer is one of the most common and serious types of cancer throughout the world. In Japan, 110 thousand people suffered from it, resulted in 70 thousand deaths in 2012. This makes it the most common cause of cancer-related death. Therefore, it is very important to detect lung cancer at early stages before it progresses to advanced stages so that the chance of curative treatment is high. There are several well-known tumor-associated serum biomarkers of lung cancer available, such as carcinoembryonic antigen (CEA), cytokeratin 19 fragment (CYFRA) and pro-gastrin releasing peptide (Pro-GRP), however, they do not have sufficient specificity and

sensitivity for use in diagnosis of early cancer. Thus, we need to continue our search for different serum biomarkers to help early detection of cancer.

Autoantibodies, especially antinuclear antibodies (ANAs) and specific autoantibody tests, have been used as serologic markers in patients with autoimmune diseases such as systemic lupus erythematosus (SLE), scleroderma (systemic sclerosis, SSc), polymyositis/dermatomyositis (PM/DM), Sjogren's syndrome and other disorders. There are many reports on ANAs and specific autoantibodies in patients with malignancies. The kind of autoantibodies found in cancer patients partly overlap with the typical specificities of patients with systemic autoimmune rheumatic diseases (SARDs).

It has been reported that ~30% of cancer patients has circulating ANAs [1]. The antigen-targets of the ANAs found in lung cancer patients and in patients with autoimmune diseases include the centromere protein (CENP), Ro52 (TRIM21) and others. In this article, literature on autoantibodies associated with SARDs in lung cancer patients is discussed.

Malignancies and autoantibodies related to scleroderma (systemic sclerosis, SSc)

Among SARDs, two conditions that are associated with increased prevalence of malignancies are dermatomyositis and SSc. A recent meta-analysis study showed that SSc patients had increased risk for cancers with relative risk (RR) of 1.75 (95% confidence interval, CI, 1.41-2.18). In particular, a risk for lung cancer was significantly increased with RR of 4.35 (95% CI, 2.08-9.09)[2]. Other meta-analysis study also reported increased risk with standardized incidence ratio (SIR) of 1.41 (95% CI, 1.18–1.68) [3] and SIR of 3.14 (95% CI: 2.02-4.89) for lung cancer [4].

Regarding the association of particular autoantibodies and risk of malignancies, reported data are quite inconsistent. Some studies reported no difference for cancer risk between three major SSc autoantibodies, anti-Scl-70/topoisomerase I, -RNA polymerase III (RNAP III) and anti-centromere antibodies (ACA) [5] while others reported an association of anti-Scl-70/topo I, anti-RNAPIII or anti-RNPC-3 (RNA binding region (RNP1, RRM) containing 3) with malignancies [6]. A recent study reported a high prevalence of anti-PM/Scl antibodies in patients with malignancy with Odds Ratio (OR) of 3.90 (95% CI, 1.31 – 11.61) but no association with anti-Scl-70, -RNAP III, or centromere by multivariate analysis [7].

Increased risk of lung cancer in patients with SSc has been reported in several countries with ~1 - 5% reported prevalence of lung cancer. Positive association of anti-Scl-70/topo I with lung cancer was shown in a few studies [8] and negative association of ACA with lung cancer also was shown [8, 9] (table 1). One study reported that among SSc patients with malignancies, lung cancer was 8.6% in anti-RNAP III positive patients (n = 70), 16.7% in anti-Scl-70/topo I positives (n = 54), and 11.5% in anti-CENP positives (n = 96)[6].

In the study by Colaci et al., prevalence of lung cancer in anti-Scl-70 positive SSc was 11.4% (12/105) whereas it was 0.7% (1/140) in ACA positive SSc. Prevalence of anti-Scl-70/topo I in SSc patients with vs. without lung cancer was 75% vs. 30.8% (p = 0.0007) whereas they were 6.2% vs. 46% (p = 0.0042) for ACA [8]. Although the available data are limited, majority of lung cancer seen in SSc patients are adenocarcinoma and squamous cell carcinoma or small lung cell cancer (SCLC) appears uncommon. Predominance of adenocarcinoma is similar to the pathology of lung cancer seen in patients with idiopathic interstitial lung disease (ILD) and may be consistent with the development of cancer from chronic inflammation via similar mechanism.

Table 1. Prevalence of autoantibodies among SSc patients with lung cancer

		Autoantibodies		
Author	**N= country**	**Anti-Scl-70/topoisomerase I**	**Anti-RNAP III**	**Anti-centromere/CENP**
Pontifex 2007 [9]	N = 20 Australia	0% (0/20)	nd	15% (3/20)
Colaci 2013[8]	N = 16 Italy	75% (12/16) 6 Ad, 1 Sq	nd	6% (1/16) 1 Ad
Moinzadeh [17]	N = 16 UK	31% (5/16)	25% (4/16)	12% (2/16)
Katzen 2015[24]	N = 17 USA	41% (7/17) 4 Ad, 1 Sq, 1* AdSq, 1 LC neuroendocrine	12% (2/17) 1 Ad, 1* AdSq	24% (4/17) 4 Ad
Shah 2017 [6]	N = 32 USA	28% (9/32)	19% (6/32)	9% (9/96)

Nd, not described; Ad, adenocarcinoma; Sq, squamous cell carcinoma; AdSq, adenosquamous

* a case that was positive for anti-Scl70 and –RNAP III

Anti-centromere protein (CENP) antibodies

Autoantibodies directed against centromere components were initially reported by Moroi Y, et al. in 1980 [10]. Centromere antigens are mainly composed of a complex of kinetochore proteins CENP (centromere protein)-A, B, C, H, I and F, and CENP-A,-B,-C are the main target antigens recognized by human autoantibodies. Anti-CENP-B and CENP-F antibodies have been most extensively studied in cancer. Anti-CENP-F antibodies were reported as a marker for non-Hodgkin lymphomas and also described in patients with various cancers [11]. Anti-CENP-B antibodies were reported in patients with breast cancer [12] and small cell lung cancer (SCLC) [13]. In SCLC patients, anti-CENP-B antibodies were detected before the diagnosis of SCLC without features of SSc. They suggested that ACA can be a biomarker in SCLC.

Anti-Scl-70/topoisomerase I antibodies

Anti-Scl-70/topo I antibodies were reported as autoantibodies to chromatin-associated molecule of 70-100kDa, known as Scl-70 in 1979 [14]. Corresponding antigen was identified as topoisomerase I in 1986 [15]. Anti-Scl-70/topo I is one of the three major autoantibodies in SSc, found in 20-40% of patients. Despite reports on the association of anti-Scl-70/topo I with lung cancer in SSc patients, there have been no reports of conventional anti-Scl-70/topo I in lung cancer patients without features of SSc.

A recent interesting study reported that the 48kD fragment of topoisomerase I (TOPO48) was specifically recognized by autoantibodies in various types of cancer and the autoantibody levels were higher in early stage of cancer vs. advanced stage [16]. In patients with non-small cell lung cancer, antibodies to the TOPO48 were detected in 67.6% (early stage) vs. 32.4% (advanced stage). Anti-TOPO48 antibodies were detected by ELISA and western blot, however, these sera with anti-TOPO48 were negative for conventional anti-Scl70/topo I antibodies. Significance of autoantibodies that do not recognize the native molecule will need to be interpreted carefully and investigated in future studies.

Anti-RNA polymerase III antibodies

Anti-RNA polymerase III antibodies that usually coexist with anti-RNAP I are other SSc-specific autoantibodies. Higher risk of malignancies including lung cancer in patients with anti-RNAP III has been reported [17]. However, there have been no reports of lung cancer patients with anti-RNAP III without features of SSc.

Autoantibodies associated with polymyositis/dermatomyositis (PM/DM)

Anti-TIF1gamma (transcriptional intermediary factor 1) antibodies

High prevalence of malignancy in DM is a classic example of an association of malignancy and SARDs. Autoantibodies to transcriptional intermediary factor 1 gamma (TIF1 gamma) have been identified as a marker for cancer-associated DM. Reported prevalence of malignancies among DM patients with anti-TIF1gamma antibodies is ~40 - 100% [18]. Among malignancies in patients with anti-TIF1gamma, percentage of lung cancer varies depending on the studies and lung cancer can be as much as ~30 - 40%, however, not all studies show high prevalence of lung cancer (table 2). Regarding the histology type of lung cancer, information is very limited but it is of interest to note that two cases reported in a Japanese study were small cell lung cancer (SCLC), in contrast to predominance of adenocarcinoma in lung cancer of patients with anti-Scl-70/topo I-positive SSc. In our cohort of lung cancer patients, a case of SCLC with anti-TIF1gamma antibodies was found, who later developed DM (manuscript in preparation).

Table 2. Prevalence of lung cancer in patients with anti-TIF1gamma antibody positive PM/DM patients

Author	Country	Anti-TIF1gamma antibody positive cancer N=	Lung cancer	Histology
Chinoy 2007[25]	UK	8	0/8	
Kaji 2007[26]	Japan	5	1/5	
Fujikawa 2009[27]	Japan	5	2/5	2 SCLC
Hamaguchi 2011 *[28]	Japan	17	7/17	
Fujimoto 2012[29]	Japan	48	14/48	
Trallero-Araguas E 2010[30]	Spain	10	1/10	
Ceribelli 2017[31]	Italy	3	1/3	1 Ad

*Include Fujikawa's cases

Other autoantibodies

Anti-Ro52 antibodies (Anti-TRIM21 antibodies)

Ro52 antigen also known as tripartite motif (TRIM) 21 protein, was characterized as an E3 ubiquity ligase. The anti-Ro52 antibodies have been reported in a wide variety of diseases including SARDs and non-autoimmune diseases such as viral infections or neoplastic diseases [19]. Ghillani et al. reported that the presence of anti-Ro52 antibody were detected in 34 patients with various pulmonary manifestations and one case of isolated anti-Ro52 was a lung cancer patient [20]. The Ro52 gene has been mapped to the end of the short arm of chromosome 11. This chromosome segment may harbor genes important in the development and progression of solid tumors. Thus, there may be more research findings coming on the association of anti-Ro52 antibodies with malignancies in future.

Anti-DFS70 antibody

Antibodies to DFS 70 (dense fine speckled 70), also known as LEDGF/p75 (lens epithelium derived growth factor p75), were originally reported as one of the anti-nuclear antibody specificities in patients with interstitial cystitis in 1994. Anti-DFS70 also has been reported in 30% of atopic dermatitis patients but recent studies suggest that anti-DFS70 has negative association with SARDs. In regards to cancer, Daniels et al. reported that ~20% of prostate cancer patients were positive for anti-DFS70 [21]. In lung cancer, Bizzaro et al. reported that one of 31 lung cancer patients have this specificity [22]. The data so far does not appear to suggest an increased prevalence of anti-DFS70 in lung cancer patients, however, whether it is associated with a particular type of lung cancer, underlying ILD, smoking or other factors will need to be evaluated.

Prevalence of autoantibodies in lung cancer patients in our cohort

Prevalence of autoantibodies tested by ELISA using recombinant proteins in our cohort of lung cancer patients is summarized (table 3). Anti-Ro52 antibodies appear to be more common in lung cancer patients than general population. Anti-Ro52 antibodies were found at a similar prevalence (15-25%) in adenocarcinoma, squamous cell carcinoma and SCLC. All other autoantibody specificities shown were detected only in 1-2 patients out of 76 patients and the prevalence does not seem to be dramatically different than those in general population [23].

Table 3. Prevalence of autoantibodies associated with systemic autoimmune disease in patients with lung cancer

	Lung cancer (n = 76)*	**General population [23]**
Anti-Ro60	1.3%	~0.5%
Anti-Ro52	15.7%	~0.5%
Anti-CENP A/B	2.6%	~0.5%
Anti-Jo-1	2.6%	~0 (<0.04%)
Anti-DFS70	2.6%	2-6%

*includes 43 adenocarcinoma, 8 squamous cell carcinoma, 20 small cell lung cancer

Summary

Strong associations between certain SARDs and lung cancer have been reported, however, the prevalence of autoantibodies associated with SARDs does not appear to be clearly different in lung cancer patients, possibly except for increased anti-Ro52 in lung cancer. Future studies may help identify new autoantibodies that can be used as biomarkers of lung cancer.

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SPECIAL ARTICLE

Report on the second International Consensus on ANA Pattern (ICAP) workshop in Dresden 2015

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The second meeting for the International Consensus on Antinuclear antibody (ANA) Pattern (ICAP) was held on 22 September 2015, one day prior to the opening of the 12th Dresden Symposium on Autoantibodies in Dresden, Germany. The ultimate goal of ICAP is to promote harmonization and understanding of autoantibody nomenclature, and thereby optimizing ANA usage in patient care. The newly developed ICAP website www.ANApatterns.org was introduced to the more than 50 participants. This was followed by several presentations and discussions focusing on key issues including the two-tier classification of ANA patterns into competent-level versus expert-level, the consideration of how to report composite versus mixed ANA patterns, and the necessity for developing a consensus on how ANA results should be reported. The need to establish on-line training modules to help users gain competency in identifying ANA patterns was discussed as a future addition to the website. To advance the ICAP goal of promoting wider international participation, it was agreed that there should be a consolidated plan to translate consensus documents into other languages by recruiting help from members of the respective communities. *Lupus* (2016) **25,** 797–804.

Key words: Antinuclear antibodies; autoantibodies; autoimmunity; consensus; standardization

Introduction

The assay for antinuclear antibodies (ANA) is commonly used in the screening of autoantibodies in systemic autoimmune diseases,[1,2] and the indirect immunofluorescence assay utilizing HEp-2 cell substrates remains the recommended methodology.[3,4] HEp-2 cells grown as a semi-confluent monolayer exhibit prominent intracellular structures and is the traditional ANA substrate of choice for most diagnostic laboratories. With appropriately equipped microscopes, these features contribute to optimal detection and ready recognition of many subcellular structures. The International Consensus on ANA staining Patterns (ICAP) initiative originated as a session of the 12th International Workshop on Autoantibodies and Autoimmunity (IWAA) held in São Paulo, Brazil, in 2014. More than 60 participants took part in the discussion during that meeting. The consensus nomenclature and representative 28 patterns are established and available on-line at the ICAP website: www.ANApatterns.org.[5] Patterns are categorized into three major groups (nuclear, cytoplasmic, and mitotic patterns) and each pattern has been defined and described in detail.[5] The second ICAP meeting was held a day prior to the 12th Dresden Symposium on Autoantibodies in Dresden, Germany on 23–26 September 2015.[6] The present report summarizes the majority of

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Figure 1 An overview of the ICAP home webpage at www.ANApatterns.org. The web link A on the toolbar connects to the ICAP nomenclature and classification tree (see Figure 2). Link B allows selection for nuclear, cytoplasmic, or mitotic patterns to provide full descriptions. Link C provides selection from the list of all 28 alphanumeric coded patterns to access specific description and images. Link D is the key word search function. Link E will access the website into selected languages still under construction. Link F will access available free posters upon user registration. Link G provides access to ICAP publications.

issues discussed and the consensus initiatives for future action. The ultimate goal of ICAP is to promote harmonization of autoantibody test nomenclature and interpretation, and to optimize ANA usage in patient care.

The www.ANApatterns.org website and free instructional posters

At the onset of the 2nd ICAP meeting, the ANApatterns.org website was introduced by Wilson de Melo Cruvinel (Brazil), who has been primarily responsible for the development and operation of this website (Figure 1). Note that the ANApatterns.org site is also formatted to be accessible on mobile devices including cell phones and tablets. A general overview of the website was provided and instructions for its use were included as an introductory chapter in the Proceedings of the Dresden Symposium.[6] Paulo Francescantonio and Marvin Fritzler had previously commented that the actual implementation of the ICAP recommendations by diagnostic service laboratories would require ICAP recommendations be taught to trainees and technologists at our respective institutions, and that there needs to be persistence in presenting the cohesive, consistent recommendation messages at both national and international scientific symposia. In keeping with these goals, additional items were introduced to the website, including several free

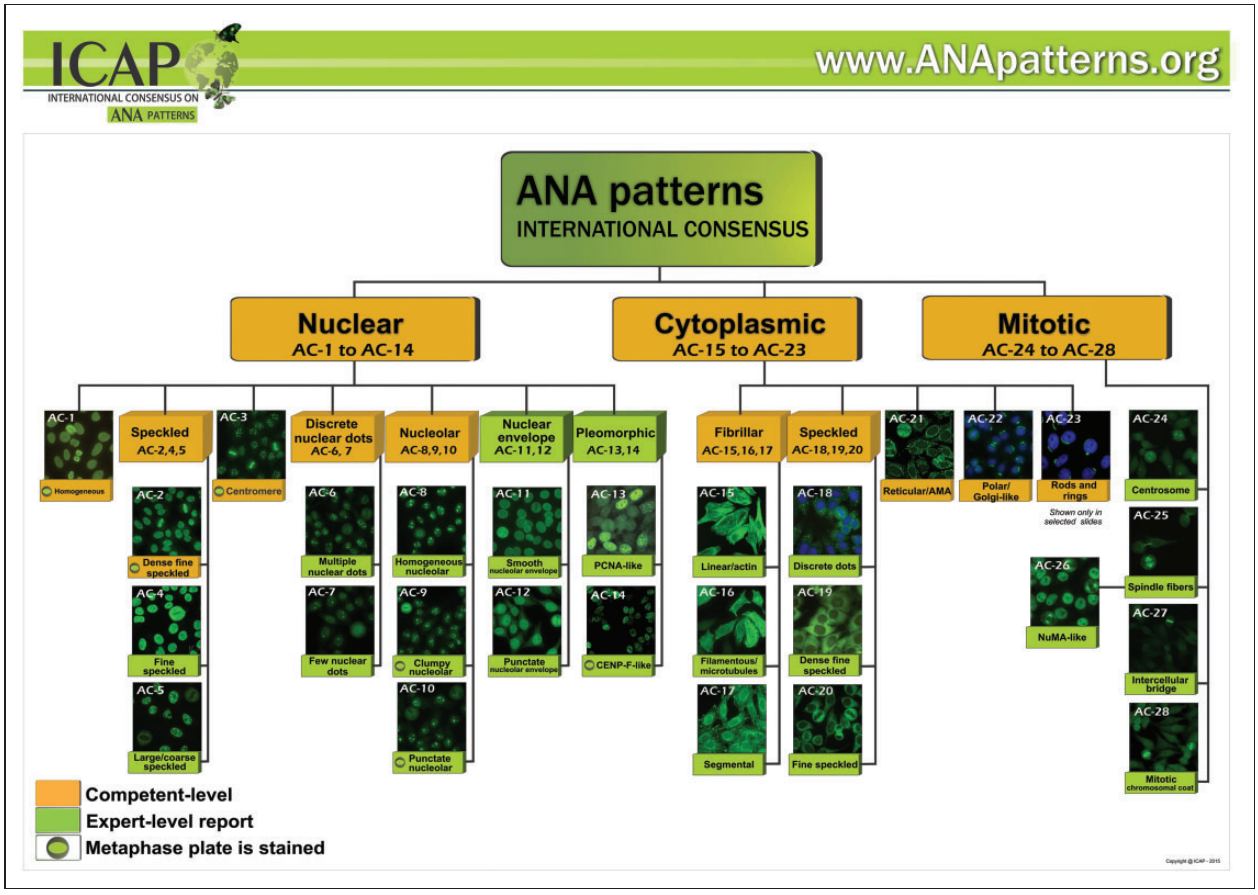

Figure 2 The nomenclature and classification tree for 28 HEp-2 cell patterns. The 28 ICAP patterns are designated with alphanumeric AC code for each from AC-1 to AC-28. Boxes with amber background are recommended as competent-level reporting, whereas those with olive green background are considered for expert-level reporting. AC, anti-cell.

posters that are available upon user registration at the website: http://anapatterns.org/download_files.php. For example, one of the figures showing the consensus nomenclature and the 28 patterns are available for download as a full-size poster (Figure 2). Additional slide sets to present the ICAP patterns will be available in the future via direct download from the website. These slide sets will be useful for teaching purposes, for example.

The website had 14,288 page views from 19 May to 11 September 2015. This represented a total of more than 3000 visits from 94 countries within these first 4 months (25 visits/day). The top three countries accessing the website during this period were USA (21.80%), Brazil (12.13%), and Germany (5.22%) (Figure 3(a)). The website usage was mostly on personal computers (91.6%); website usage on mobile cell phones and tablets was 7.61% and 0.76%, respectively (Figure 3(b)). This preliminary access data is encouraging, and clearly suggests that the ICAP website represents a useful and acknowledged source of information.

Generally, comments for the ANApatterns.org website have been highly positive regarding the overall quality of the website. There were some useful feedback comments. First, the number of representative images per anti-cell (AC) pattern provided at the initial setup for the website is limited to two. There were several requests to increase the number of images for each pattern. Hence, there are plans to obtain additional images that will be evaluated and approved by a working committee as before. The record for each image will be documented to indicate the source of images plus other relevant information. Second, a suggestion was made to provide an interpretive clinical description for each AC pattern. This description could be used as a standard comment to be included in the ANA reports and there was a consensus to move forward with this initiative. Third, it was suggested to add a comment section on the website to allow for comments from users. At a later stage, potentially these comments may be incorporated into a frequently asked questions

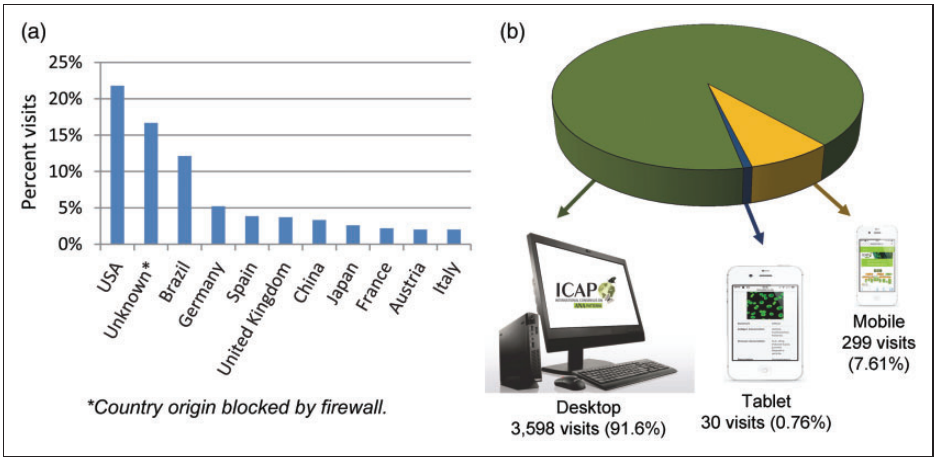

Figure 3 Summary of initial access to www.ANApatterns.org. A. Plot of countries with most visits to the website. B. Access of the website categorized based on the type of device.

(FAQ) section of the website. This latter initiative will be introduced in the coming year and will involve members of the committee as well as other interested groups.

Distinction of competent-level versus expert-level patterns

In the first ICAP report[5] patterns were divided into competent level versus expert level, with the intention that ANA readers should be trained to minimally recognize all the patterns that are listed under the competent level. Edward KL Chan (USA) and Manfred Herold (Austria) were assigned to re-address the issues regarding the decision on which patterns are to be considered competent level. Recommendations for clinical immunology laboratories to become capable of reporting patterns in the competent level include using available standard sera, such as those of the IUIS/CDC ANA standards, available from the Autoantibody Standardization Committee via www.AutoAb.org,[7] reference to images from the ICAP website at ANApatterns.org,[5] and use of additional subcellular markers (i.e. monoclonal antibodies) for co-staining validations.

The classification of patterns as competent level did not follow strict criteria, but took into consideration the clinical relevance and the morphological consistency of the patterns. Some of the competent-level patterns elicited further discussion at the second ICAP meeting. The nuclear dense fine speckled pattern (AC-2) is considered particularly important given its strong association with anti-DFS70/LEDGF antibodies and the fact that many investigators either have not heard of it or do not know the relevance of its negative correlation to systemic autoimmune rheumatic diseases.[8,9] There was some concern over the apparent lack of clinical relevance of Golgi-like pattern (AC-22)[10–12] or the rods and rings pattern (AC-23),[13–16] and yet they represent distinctive and consistent patterns that belong to the competent-level group. It has been proposed that the competent level and expert level are equivalent to the more simple description of "basic" and "advanced" levels and should not depend strictly on the clinical relevance of the pattern. It should be stated that the division between both levels may be considered rather arbitrarily and most certainly can be changed in the future as new information is available. It is acknowledged that users may not be considered as "competent" versus "expert" based solely on the ability to identify ANA patterns. Manfred Herold brought up his concerns that a critical piece of becoming "competent" in reading ANA is the ability to discriminate between positive and negative, which is a topic worthy of further discussion on the ICAP website and in subsequent ICAP meetings.

The concept of composite patterns as a separate category did not reach consensus

Luis EC Andrade (Brazil) and Karsten Conrad (Germany) were assigned to discuss whether certain patterns should be considered "composite" patterns. The discussion focused on how composite patterns are defined and the potential advantages in adopting the "composite pattern" category. Composite patterns would be defined as those in

which a single autoantibody specificity elicits the staining of more than one cell compartment. For example, NuMA (AC-26) may be considered a ''composite'' pattern because both nucleoplasmic and mitotic spindle poles are stained, with the implication that the staining in the two different compartments is characteristic of a single autoantibody specificity;[17] in this example, anti-NuMA monoclonal antibodies have shown the same staining pattern. One clear advantage of adopting the category of composite patterns can also be illustrated by the NuMA pattern, as NuMA is classified as a Mitotic Pattern in the ICAP classification tree despite the fact that all interphase cells show a strong nucleoplasmic staining. Another relevant advantage is the fact that the simultaneous occurrence of a consistent set of features in the composite patterns may increase the stringency of association with the cognate autoantibody specificities. In fact, the recognition of the multiple features of the NuMA pattern is virtually pathognomonic of anti-centrophilin/NuMA antibodies. Other patterns that could be classified as composite patterns include the CENP-F-like (AC-14), Scl-70-like (not yet classified in the ICAP classification tree), and the peculiar cytoplasmic/nucleolar staining pattern associated with anti-ribosomal P antibodies. The potential vulnerability for the category ''composite pattern'' is the confusion with ''mixed'' patterns as generated by sera that contain distinct autoantibodies to different antigens in different compartments. In addition, some specialists noted the lack of necessity to assign a distinct ''composite pattern'' category, as the subcellular localization of a given protein/antigen to different compartments at various stages of the cell cycle or under different physiological conditions is well documented. In any case, no consensus was obtained and it was decided that the category of composite patterns will not be consented at this time.

Caution on association of ANA patterns with diseases

Minoru Satoh (Japan) and Jan Damoiseaux (The Netherlands) were assigned to discuss the advantages and limitations of ANA patterns in relation to disease associations. For example, a nucleolar pattern (AC-8–AC-10) is considered to be associated with systemic sclerosis (SSc) primarily because in this disease autoimmunity may be directed to several nucleolar antigens, like Th/To,[18] U3-snoRNP/fibrillarin, and PM-Scl.[19] However, the association between SSc and the nucleolar patterns is very weak because the nucleolar pattern is often observed, even in high titer, without any clinical signs of SSc. It was further acknowledged that a given ANA pattern should suggest what the targeted autoantigens are in order to enable directed reflex testing or appropriate advice to do so for the clinician. It is the identification of autoantibodies to these self-antigens that are best associated to certain diseases, while the ANA patterns alone may be insufficiently linked to these diseases. The centromere pattern (AC-3) may be an exception, as this pattern is strongly associated with reactivity towards the CENP-B protein and, for that reason, many laboratories do not perform any antigen-specific testing for this pattern.[20,21] However, even in this case the association between the centromere pattern and SSc is not absolute. Nevertheless, the disease associations are primarily based on the target antigens recognized by autoantibodies that reveal a particular ANA pattern.[5]

Guideline for new patterns on the horizon to be considered

Prior to the second ICAP meeting, members assigned the specific task to focus on subcellular compartments were asked to re-visit their topics and provide an update for additional patterns to be considered for inclusion in the future. Karsten Conrad summarized ANA patterns that are associated with the recently defined myositis-specific autoantibodies.[22] Some of these autoantibodies do not reliably reveal novel indirect immunofluorescence (IIF) patterns and are ANA negative.[4,23,24] Karsten Conrad also made a case to include a more specific SS-A/Ro-like ANA pattern characterized by a distinctive fine speckled nuclear pattern resembling a myriad of multiple nuclear dots.[25] No consensus was achieved regarding addition of these patterns to the ICAP at this time.

Although there was no consensus on adding new patterns to the existing 28 patterns from the first ICAP, it was agreed that guidelines are needed for nomination and inclusion of novel patterns in the future. There was little discussion due to time limitation, but patterns associated with autoantibodies with clinical relevance obviously should be considered a higher priority. Other ''new'' patterns will need to await further documentation, as in publications or otherwise, with well-defined markers for validation.[26–32] The availability of multiple

consensus IIF images for new patterns is also necessary.

An unresolved issue in ANA reporting

Jan Damoiseaux, Carlos A von Mühlen (Brazil), and Ignacio Garcia-De La Torre (Mexico) were tasked to discuss how ANA reporting should come to an international consensus. With respect to the reporting of ANA, there was agreement that the test result is to be reported as negative or positive, and if positive, the IIF pattern (according to the ICAP nomenclature) and fluorescence intensity or titer are to be included. In addition, there was consensus that the report should include information on the test system applied, and, where appropriate, relevant contemporary literature provided to the clinician; for example, the laboratory might suggest that the test be repeated within a year or sooner if clinical parameters change (i.e. if ANA is at borderline positive). It was suggested that ICAP may be a good platform for preparing clinical comments to be added to the distinct patterns defined.

There was no consensus as to whether cytoplasmic and mitotic patterns are to be reported as ANA negative or positive, although it was widely agreed that some cytoplasmic patterns are clinically relevant and that this information should not be overlooked. The major concern with respect to reporting cytoplasmic patterns as ANA positive is that, in some jurisdictions, existing guidelines and diagnostic/classification criteria are based on restricting ANA to nuclear patterns. The most striking example is the diagnostic criteria for autoimmune hepatitis (1999), which is based on a scoring system.[33] A positive ANA, depending on the titer, gives positive points, while the presence of anti-mitochondrial antibodies requires subtraction of points. This evidently results in a paradox if anti-mitochondrial antibodies are reported as ANA positive. A separate report has been compiled for publication on the full discussion and proposals.[34]

On-line assessment for users to gain competence in HEp-2 cell patterns

Edward Chan and Wilson de Melo Cruvinel lead this discussion. It is acknowledged that training is needed to ensure that all laboratories are able to report patterns at the competent level (basic-level training) as well as training to help advance all laboratories to recognize expert-level patterns (advanced-level training). It is clear that multiple training programs already exist for ANA pattern recognition and reporting. However, to date, none has been adapted to the newly established ICAP nomenclature or recommendations. The general roadmap on ANA pattern training should be an open system allowing different groups to participate. Wilson de Melo Cruvinel has compiled a draft proposal for an ICAP educational program with a goal of developing an on-line assessment tool for users to gain competence in ANA determination at both basic and advanced-level training. For example, every 3 months, participants will access the ICAP website restricted by password login for the on-line immunofluorescence images. Users will examine the images provided and complete analysis by filling out a form according to ICAP guidelines. The users will be provided with a quality assessment report, which will show (1) the correct answers and results rated among the peer group; (2) the detailed characterization of the pattern; (3) autoantigen association and clinical relevance. In the US and many other countries, the program may be developed to provide Continuing Education (CE) credits and perhaps an ICAP (or IUIS/IWAA) certificate of competence. Yearly refresher courses with CE credit may also be considered.

Moving forward with ICAP internationalization

In order for ICAP to be recognized and be taken full advantage of at an international level, continuous improvement, extension, updating and maintenance of the ANApatterns.org site is essential. In addition, translation into different languages will be necessary to further promote the effort to achieve general consensus. The discussion organized by Edward Chan, Ignacio Garcia-De La Torre, and Wilson de Melo Cruvinel focused on establishing a draft guideline for each language translation project. To date, there are already projects on translation into German, Spanish, and Portuguese. The guidelines consist of: (1) for each language project, the translation should be handled by a team rather than a single individual to promote acceptance and inclusion in daily practice; (2) whenever appropriate, individuals from different countries sharing the same language should be invited to participate; (3) at different stages of the process, potential users should be involved in a "beta test" of the draft translation. This may

mean that it takes a longer time to achieve consensus in translation, but it helps to spread the message regarding the ICAP initiative. A draft letter to ''recruit'' members in the participation of translation should introduce the general description of the ICAP, the Frontiers Immunology publication[5] and the ANApatterns.org site; (4) further promotion is recommended. For example, the translation team may consider planning a manuscript/report in regional/local journals to announce the translated work. The primary target audience should be clinical immunology laboratories including organizations similar to the American Medical Laboratory Immunology.

During the Dresden Symposium on Autoantibodies, the issue of translation of the ANApatterns.org was discussed at the European Autoimmune Standardization Initiative (EASI) session where it was generally agreed that the national EASI teams[35] will help with the translation of the relevant European languages. This action also enables to invite the national EASI teams for feedback on the content of the website.

Planning of future meetings

The ICAP executive members will meet again at the time of the 10th International Congress of Autoimmunity in Leipzig, Germany, where the main agenda item will be the preparation for the 3rd ICAP meeting.

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ORIGINAL ARTICLE

International consensus on ANA patterns (ICAP): the bumpy road towards a consensus on reporting ANA results

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Abstract The International Consensus on ANA Patterns (ICAP) was initiated as a workshop aiming to thoroughly discuss and achieve consensus regarding the morphological patterns observed in the indirect immunofluorescence assay on HEp-2 cells. One of the topics discussed at the second ICAP workshop, and addressed in this paper, was the harmonization of reporting ANA test results. This discussion centered on the issue if cytoplasmic and mitotic patterns should be reported as positive or negative. This report outlines the issues that impact on two major different reporting methods. Although it was appreciated by all participants that cytoplasmic and mitotic patterns are clinically relevant, implications for existing diagnostic/classification criteria for ANA-associated diseases in particular hampered a final consensus on this topic. Evidently, a more concerted action of all relevant stakeholders is required. Future ICAP workshops may help to facilitate this action.

Keywords Anti-nuclear antibodies · Indirect immunofluorescence · Nuclear patterns · Cytoplasmic patterns · Mitotic patterns · Harmonization

Introduction

Antinuclear antibodies (ANA) are important elements in the diagnosis of a variety of autoimmune diseases, especially ANA-associated rheumatic diseases (AARD) [1–3]. While ANA originally were detected by indirect

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immunofluorescence (IIF) [4], several alternative methods have entered the market [5]. Although this has raised many discussions on the definition and positioning of ANA testing [6–8], ANA detection by IIF has the advantage of obtaining information on the IIF staining pattern, which is considered of added clinical value. The introduction of HEp-2 cells as the substrate for ANA IIF has increased the awareness that, besides nuclear patterns, cytoplasmic and mitotic cell patterns can also be recognized. As such, the term anti-cellular antibodies has been suggested to encompass the wider spectrum of these autoantibodies [8, 9]. However, because the use of the acronym ANA is firmly established and universally used, replacement by an alternative terminology will not be easy. Changes in nomenclature would have many implications, for instance for existing guidelines, disease criteria, external quality control programs, education, and reimbursement policies.

The International Consensus on ANA Patterns (ICAP) initiative started in 2014 as a workshop parallel to the 12th International Workshop on Autoimmunity and Autoantibodies (IWAA) in Sao Paulo, Brazil [10]. This first ICAP workshop was devoted to establishing a consensus on the nomenclature of the distinct ANA patterns recognized by IIF on HEp-2 cells. This resulted in a classification tree that distinguishes three major IIF staining categories: nuclear, cytoplasmic and mitotic patterns [11]. The second ICAP workshop was recently hosted in conjunction with the 12th Dresden Symposium on Autoantibodies in Dresden, Germany. Since it was recognized that standardization and harmonization in autoimmune diagnostics is of utmost importance, part of the discussion was focused on the reporting of ANA results. The discussion was prepared in advance by Carlos von Mühlen (Brazil), Ignacio Garcia De La Torre (Mexico), and Jan Damoiseaux (The Netherlands). The input included a number of (inter)national recommendations on this topic [1, 6, 8, 9, 12–18] and two proposals.

Here we register the discussion with a focus on two main points regarding the articulation of the ANA report: (1) the position of cytoplasmic and mitotic patterns, i.e., are they to be reported as ANA negative or ANA positive; and (2) the advice to clinicians on the possible significance of the patterns observed, i.e., possibly involved autoantibodies and associated diseases.

(Inter)National recommendations on ANA reporting

A non-exhaustive review of the currently available recommendations on reporting ANA results is provided in Table 1. It was generally agreed that the report should contain information on the type of immunoassay that has been used. On the other hand, if an ANA pattern is reported, this implies that ANA were determined by IIF because alternative methods do not allow pattern recognition. Surprisingly, almost none of the recommendations made a statement on reporting ANA simply as negative or positive. In the case of ANA IIF, again this may be surpassed by providing a titer and/or pattern. However, in relation to the discussion whether a cytoplasmic or mitotic apparatus staining pattern is to be considered ANA positive, a straightforward interpretation in terms of "ANA negative" or "ANA positive" was thought to be helpful. In Argentina, Belgium and Brazil, it is recommended that results for distinct cellular compartments are reported, but this does not unequivocally imply that a cytoplasmic/mitotic apparatus reactivity is to be considered ANA positive [9, 16, 18]. In the second Brazilian consensus on ANA in HEp-2 cells, however, it was decided that cytoplasmic patterns are to be considered ANA positive, but a subtitle is to be added to the report, stating that "ANA is actually a test that detects autoantibodies to cellular antigens—thus encompassing the whole cellular anatomy and all cellular structures" [9]. Also, the Italian recommendations of the Forum Interdisciplinare per la Ricerca nelle Mallattie Autoimmuni (FIRMA) explicitly state that a cytoplasmic pattern is to be considered ANA positive (http://www.gruppofirma.com). Neither the ACR nor the EASI/IUIS recommendations state a clear position towards cytoplasmic/mitotic apparatus patterns being considered ANA negative or positive [1, 6, 8]. The EASI/IUIS recommendation only states "besides nuclear patterns also cytoplasmic and mitotic apparatus patterns should be reported and specified when possible" [8]. Finally, the European Consensus Finding Study Group on Laboratory Investigation in Rheumatology (ECGSG), being part of the European League Against Rheumatism (EULAR), considers a cytoplasmic pattern as ANA negative (personal communication Johan Rönnelid, Sweden).

Recommendations about titer and pattern are obviously restricted to the IIF method for ANA detection. Titration is considered clinically relevant since a higher titer is associated with a higher positive likelihood ratio to confirm a diagnosis of an AARD [19, 20]. Furthermore, a higher titer also increases the chance of identifying the antigen that is recognized in the ANA IIF test by antigen-specific immunoassays [21, 22]. These findings, however, are challenged by the introduction of newer technologies. The added value of extremely high titers is considered to be limited as can be concluded from the fact that in several recommendations an end-point titer is defined beyond which no further dilutions are needed. Nevertheless, both the ACR and EASI/IUIS recommendations advised to perform an end-point titration [1, 8]. Alternatively, the Dutch and Italian recommendations allow reporting fluorescence intensity instead of the titer [13; http://www.gruppofirma.com]. This option may be valuable as some of

Table 1 (Inter)National recommendations on the reporting of ANA test results

	Argentina [18]	Austria [17]	Belgium [16]	Brazil [9]	Germany [12]	Italy (FIRMA)	Netherlands [10]	ACR [6]	EASI/ IUIS [8]	Remark
ANA method	+	+[a]	NS	+	NA[b]	NA[b]	+[a]	+	+[a]	[a]Tests based on a restricted number of defined nuclear antigens are excluded [b]Recommendations are restricted to IIF testing
Neg/Pos	+[a]	NS	NS[b]	NS[a]	NS	+[c]	NS	+[d]	NS	[a]Scored for all cellular compartments [b]Scored separately for nuclear and cytoplasmic staining [c]Cytoplasmic staining is considered ANA positive [d]Including % controls with similar titer
Titer (end-titer)	+[a]	+ (1:1280)	+ (1:5000)	+ (1:640)	+ (1:5120)	+[b]	+[a]	+[c]	+[c]	[a]End-titer is not defined [b]Titration is optional; staining intensity is alternative [c]Titration to highest dilution
Pattern	+[a]	+[a]	+[b]	+[a]	+	+	+[c]	NS	+[a]	[a]Including cytoplasmic and mitotic patterns [b]At least centromere and cytoplasmic patterns [c]Reporting to the clinician is optional
Advise reflex testing	NS	NS	+[a]	NS	+[b]	+[c]	+[c]	+[c]	+[c]	[a]Only if ANA is positive [b]Mention possible target antigens [c]If ANA is positive or disease associated with anti-SSA, -Jo1 (or other synthetases) and/or -RibP is suspected
Advise disease association	NS	NS	NS	+	+[a]	NS	NS	NS	NS	[a]In light of diagnostic question posed

Reflex testing may be either integrated in a testing algorithm or advised to do so

ACR American College of Rheumatology, *ANA* anti-nuclear antibodies, *EASI* European Autoimmunity Standardization Initiative, *FIRMA* Forum Interdisciplinare per la Ricerca nelle Mallattie Autoimmuni, *IIF* indirect immunofluorescence, *IUIS* International Union of Immunological Societies, *NA* not applicable, *NS* not specified

the newer automated digital ANA reading systems score the fluorescence intensity and even project end-point titers on a single well dilution, and, importantly, these intensities seem to correlate quite well with the end-point titers [5, 23, 24]. The relevance of reporting ANA patterns is underscored by all recommendations, except for the ACR recommendations. In the Netherlands, though, reporting can be decided locally upon consultation with the clinicians involved [10].

Finally, the recommendations differentially touch on the issue of advice to be added for interpretation of the results obtained. Although many laboratories use general remarks with respect to possible disease associations, most recommendations do not address this point. Only the Brazilian consensus strongly recommends the inclusion of such remarks in the ANA report [9], and in the German consensus such advice is only provided in the light of the diagnostic question posed [12]. There is, however, more consensus about the recommendations in the context of reflex testing. In Germany, it is recommended to mention possible target antigens, based on the ANA pattern and the clinical information provided [12]. Depending on the reimbursement policies as determined by individual jurisdictions, the reflex testing can be preempted in a testing algorithm. In that case, advice about reflex testing is redundant.

Two proposals for reporting of ANA test results

During the second ICAP workshop, two alternative recommendations for reporting of ANA test results were discussed. These alternatives only differed with respect to cytoplasmic and mitotic patterns being considered ANA positive or negative (Fig. 1). In these proposals the report should consist of 3 items: type of assay used, test result, and, if appropriate, advice on reflex testing. These items were chosen based on being the common denominator in the examined (inter)national recommendations (Table 1).

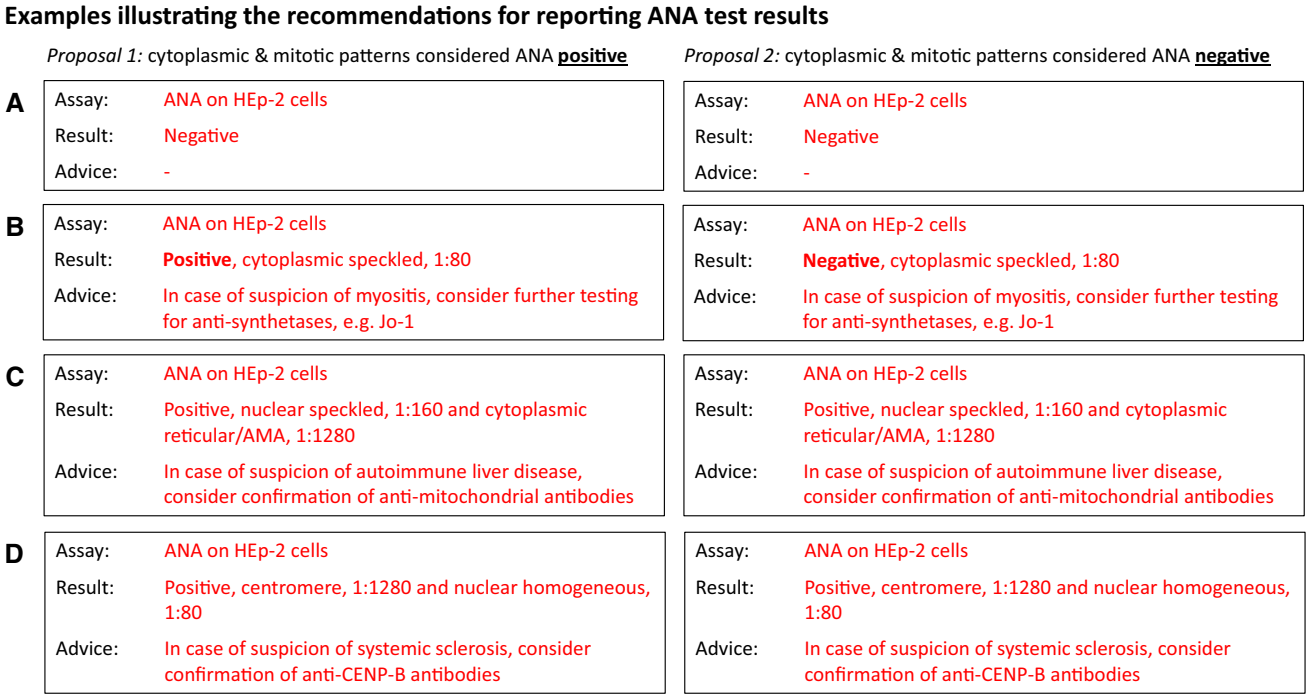

Fig. 1 Examples illustrating the recommendations for reporting of ANA test results. The report consists of three categories: the type of assay used, the test results (positive/negative, pattern, and antibody level), and the advice for the clinician. In proposal 1 (*left*), cytoplasmic (and mitotic) patterns are considered ANA positive, while in proposal 2 (*right*), cytoplasmic (and mitotic) patterns are considered ANA negative. The examples shown illustrate alternate possibilities according to the rules in each proposal for reporting ANA test results. If the test result is negative (**a**), this is reported as such in both proposals. If only a cytoplasmic staining is observed (**b**), the result is reported as ANA positive in proposal 1 and as ANA negative in proposal 2. The items positive and negative are highlighted to emphasize the difference in the proposals. In both proposals this result is followed by the statement of the cytoplasmic pattern and antibody level (titer). If a combination of nuclear and cytoplasmic patterns is observed (**c**), the result is reported as positive in both proposals because of the nuclear staining. According to the rule that patterns are reported in the sequence nuclear—cytoplasmic—mitotic, irrespective of the antibody level, the nuclear pattern is mentioned first even when the antibody level of the cytoplasmic pattern is higher. If a combination of different nuclear patterns is observed (**d**), the nuclear pattern with the highest antibody level is to be reported first. The advice to the clinician may be similar for the respective situations in both proposals. The lay-out of the report can be adjusted to be compatible with the local hospital information system

The item "type of assay" should specify the method used, i.e., IIF on HEp-2 cells or alternative HEp-2 substrates, addressable-laser bead immuno-assay (ALBIA), enzyme-linked immunosorbent assay (ELISA), etc. Providing also the name of the assay kit manufacturer is considered important because it is known that some autoantibodies and their respective patterns show-up preferentially in some HEp-2 slide brands, such as anti-SS-A/Ro60 on HEp-2000 substrates and the anti-rods and rings pattern [11, 24]. It was felt, however, that such information is not relevant for the majority of patterns and autoantibodies.

The second item of the report should contain information on the ANA test being positive or negative, the IIF pattern, and the autoantibody titer. The results should be reported in the sequence: positive/negative—pattern—titer. Evidently, reporting an ANA pattern as a test result only applies to ANA IIF results on HEp-2 cells. The nomenclature for ANA patterns should be according the consensus reached in the first ICAP workshop [11]. The autoantibody level can be expressed as titer, fluorescence intensity as being generated by automated digital ANA reading devices, or arbitrary units obtained by alternative ANA assays. The two proposals differ in the assignment of positive/negative because, in contrast to the first proposal, in the second proposal, cytoplasmic and mitotic ANA patterns are considered ANA negative. In case of mixed patterns, all nuclear patterns are reported first and next cytoplasmic, and then mitotic patterns. Each pattern is directly followed with the respective titer. Within each category, i.e., nuclear, cytoplasmic, and mitotic, the pattern with the highest titer is mentioned first. In the second proposal a sample with an exclusively cytoplasmic pattern is reported as negative (with statement of the cytoplasmic pattern and titer), whereas a sample with mixed nuclear and cytoplasmic pattern is reported as ANA positive. Examples for reporting distinct test results are provided in Fig. 1.

The third item of the report, addressing advice for reflex testing in the clinical context, is similar in both proposals. It was strongly advised against providing clinical associations. It was concluded in a separate ICAP discussion, prepared by Minoru Satoh (Japan) and Jan Damoiseaux (The Netherlands), that the clinical associations with ANA patterns are poorly defined. At best, the patterns hint at the antigens recognized and merely the cognate autoantibodies are associated with certain diseases or manifestations of diseases. There is no consensus yet regarding the use of general remarks, like "ANA must be interpreted according to the clinical picture" or "10 % of normal persons may have a positive result" to enable better interpretation of the test results. Further information on interpretation of the test results obtained, however, is considered to be important. The clearly desired goal is to inevitably develop consensus on the content of this information.

Pros and cons for considering cytoplasmic and mitotic patterns as ANA positive

First of all, it is important to recognize that the ICAP discussion on how to report cytoplasmic and mitotic patterns was not just a semantic discussion. As already mentioned, it is widely accepted that the IIF test on HEp-2 cells enables the detection of both nuclear and non-nuclear reactivity. Changing the name of the test to something other than ANA might, for instance, impact on reimbursement policies in several jurisdictions and, therefore, was considered not feasible at this point of time. Interestingly, both the ACR and EASI/IUIS recommendations allow the use of alternative techniques for ANA detection [6, 8]. When applying these alternative immunoassays, however, there appears to be no discussion on the fact that these tests also may reveal a positive result based on the reactivity towards (clinically relevant) non-nuclear autoantigens, for instance Jo-1 or ribosomal P protein. To be consistent with these alternative techniques, this may be used as an argument to also consider cytoplasmic and mitotic patterns as ANA positive. The most important argument for reporting cytoplasmic patterns as ANA positive is the overall acceptance by the ICAP participants as well as the literature that cytoplasmic patterns are clinically relevant [5, 8, 11, 26]. If such a pattern is reported as negative, the additional information in the report on pattern and titer may go unnoticed because clinicians tend to pay less attention to negative results. In Brazil, cytoplasmic patterns have been reported as ANA positive for more than a decade [9]. This has not raised any complaints from either the laboratory or the clinical perspective. An important adjustment that has been implemented in the report of ANA results is the use of a subtitle of the ANA test indicating that the results include more than just the nuclear ANA patterns.

On the other hand, there were also strong arguments against reporting cytoplasmic and/or mitotic patterns as ANA positive. First, from the scientific point of view ANA are defined as autoantibodies directed against nuclear antigens. Second, ANA are included in diagnostic and/or classification criteria for systemic lupus erythematosus (SLE), Sjögren's syndrome, mixed connective tissue disease (MCTD), systemic sclerosis (SSc), and autoimmune hepatitis (AIH) [27–35]. In case of SLE, both in the ACR as well as in the more recent SLICC criteria, a positive ANA test is an important hallmark of the disease [27, 28]. While in the 1982 ACR criteria it is explicitly stated that ANA are to be determined by IIF, no details are provided either in the revised ACR or the SLICC criteria about the interpretation of cytoplasmic and mitotic patterns. Similarly, in the criteria for the Sjögren's syndrome and MCTD, critical results are defined on the ANA titer, indicating that

IIF is the method of choice [29, 30]. Although it is anticipated that the ANA mentioned in the criteria only concerns true nuclear reactivity, none of the AARD criteria, except for the SSc criteria that entail the centromere pattern, has defined which IIF patterns are to be considered positive. Obviously, by defining cytoplasmic and mitotic patterns as ANA positive, test characteristics will change and influence the validity of the criteria. It should be noted, though, that the AARD criteria mentioned are all classification criteria, which are distinct from diagnostic criteria [36]. The criteria for AIH, however, are diagnostic criteria [33–35]. These criteria are linked to a consensus on the detection of autoantibodies in the autoimmune liver diseases [37]. Although it is stated that for AIH an ANA test is to be performed by IIF, it is speculated that in the future more refined techniques using other immuno-assay formats may enable identification of reactants and assessment of their use for diagnosis of AIH [37]. It is evident from these criteria, however, that only nuclear patterns are to be interpreted as ANA positive. This is best illustrated by the extended AIH criteria [33, 35] in which a diagnosis of AIH is based on a scoring system. Parameters positively associated with AIH reveal positive scores, while parameters that direct towards other diagnoses reveal negative scores. As such, a positive ANA may give, depending on the titer, up to 3 points. On the other hand, the presence of anti-mitochondrial antibodies gives a negative score of 4 points. Obviously, this scoring system goes wrong if the cytoplasmic reactivity of anti-mitochondrial antibodies is reported as ANA positive. A third argument against reporting cytoplasmic patterns as ANA positive concerns external quality control (EQC) programs. Indeed, some EQC providers, like UK-NEQAS, require that a cytoplasmic pattern is reported as ANA negative in order to have a full score in the EQC assessment. Obviously, if worldwide consensus on ANA reporting can be achieved, this will inevitably result in adjustment of the EQC scoring.

When considering these arguments, however, one should keep in mind that a long history of technical and scientific evolution regularly reshapes and optimizes regulations and classifications. Therefore, concerns with reimbursement, classification criteria and EQC programs might be considered as relative arguments, since recommendations from an international board of specialists, such as the ICAP, should be able to foster sound and relevant update regarding the interests of all involved stakeholders.

Altogether, the discussion on cytoplasmic and mitotic patterns has not yet reached consensus. Members of the ICAP acknowledged that further discussion is needed for reflection and maturation of this issue. It is anticipated that summarizing the current status of the discussion will be of help to come to a widely accepted consensus.

Conclusions

The clinical value of ANA testing for AARD and autoimmune liver diseases is beyond doubt, but harmonization of reporting ANA test results still needs a few hurdles to take. The current report on the ICAP discussion on reporting of ANA test results, and in particular the issue of cytoplasmic and mitotic patterns being ANA positive or negative, illustrates the intertwining of diagnostic/classification criteria for distinct diseases associated with ANA, methodologic consensus statements, semantic issues, as well as political issues like reimbursement policies and hedging for legal claims. What is needed is a paradigm shift to get out of a vicious circle. The question is, though, where to start? Obviously, the name of the test appears not to be the biggest hurdle. For the time being, there is agreement that the community can live with a misnomer, eventually extended by a subtitle that gives credit to the fact that also non-nuclear autoantibodies can be detected. For alignment of methodological consensus statements, like ICAP and diagnostic/classification criteria, there is a need for close collaboration with the clinical parties that are responsible for the respective criteria. This will create mutual understanding of, and eventually a solution to, the current dichotomy with respect to the interpretation of cytoplasmic and mitotic patterns. If a consensus between all stakeholders is achieved and consistently applied in the relevant documents, there will be a strong motive to also solve the jurisdictional issues and eventually the nomenclature. Full integration of this paradigm shift in the (medical) community will require a transition period in order to facilitate the requested adaptations.

With respect to the interpretation of the test results obtained and the possible advice provided to the clinician, there was consensus that information on the antigens possibly recognized as based on the ANA pattern was more relevant than information on the possible disease association. In the end, a laboratory test is only useful if requested in the appropriate clinical context. It was suggested that for standardization purposes it would be helpful to prepare examples of these advices for each distinct ANA pattern, preferentially in relation to the clinical information provided alongside the ANA request. These advices should be made available on the ICAP website (http://www.anapatterns.org). If pattern and antigen-specificity are concordant, this will also increase the clinical value of the combined laboratory result.

Altogether, the activity of the ICAP initiative has been very successful in bringing up and disseminating the importance of the correct identification and denomination of the various IIF patterns observable in the ANA-HEp-2 test. The discussion on reporting of ANA results upheld

during the second ICAP has pinpointed additional issues that need to be addressed in forthcoming ICAP workshops.

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Compliance with ethical standards

Conflict of interest MF has honoraria and consultation (INOVA Diagnostics Inc and Euroimmun GmbH). Other authors have none.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Research paper

Testing for myositis specific autoantibodies: Comparison between line blot and immunoprecipitation assays in 57 myositis sera

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ABSTRACT

Objective: To analyze the performance of a line blot assay for the identification of autoantibodies in sera of patients affected by myositis, compared with immunoprecipitation (IP) as gold standard.
Methods: 66 sera of patients with myositis (23 polymyositis, 8 anti-synthetase syndromes, 29 dermatomyositis and 6 overlap syndromes) were tested by commercial LB (Euroimmun, Lubeck, Germany); 57 sera were analyzed also by IP of K562 cell extract radiolabeled with ^{35}S-methionine. Inter-rater agreement was calculated with Cohen's k coefficient.
Results: Myositis-specific antibodies (MSA) were detected in 36/57 sera (63%) by IP and in 39/66 sera (59%) by LB. The most frequent MSA found by LB were anti-Jo1 and anti-Mi2 found in 15% (10/66) of sera, followed by anti-NXP2 and anti-SRP detected in 106% (7/66) of sera. Anti-TIF1gamma and anti-MDA5 were found in 6 (9%) and 5 sera (7.6%), respectively.
A good agreement between methods was found only for anti-TIF1γ, anti-MDA5 and anti-NXP-2 antibodies, while a moderate agreement was estimated for anti-Mi2 and anti-EJ. By contrast, a high discordance rate for the detection of anti-Jo1 antibodies was evident (k: 0.3).
Multiple positivity for MSA were found in 11/66 (17%) by LB and 0/57 by IP (p: 0001). Comparing the clinical features of these 11 sera, we found total discrepancies between assays in 3 sera (27.3%), a relative discrepancy due to the occurrence of one discordant autoantibody (not confirmed by IP) in 5 cases (45.5%) and a total discrepancy between LB and IP results, but with a relative concordance with clinical features were found in other 3 sera (27.3%). The semiquantitative results do not support the interpretation of the data.
Conclusions: The use of LB assay allowed the detection of new MSA, such as anti-MDA5, anti-MJ and anti-TIF1gamma antibodies, previously not found with routine methods. However, the high prevalence of multiple positivities and the high discordant rate of anti-Jo1 antibodies could create some misinterpretation of the results from the clinical point of view. These data should be confirmed by enlarging the number of myositis cases.

1. Introduction

Autoantibodies are considered an essential tool for a correct diagnosis of different systemic autoimmune diseases, including idiopathic inflammatory myositides (IIM), in which they are usually divided in myositis-specific (MSA) and myositis associated antibodies (MAA). MAA, namely anti-Ro/SSA, anti-U1RNP, anti-Ku and anti-PM/Scl, are defined as autoantibodies found not only in IIMs but also in other connective tissue diseases, such as Systemic sclerosis (Ssc) or systemic lupus erythematosus (SLE) overlapped with myositis (Targoff, 1992, 2000; Nakashima and Mimori, 2010). On the contrary, MSAs are found almost exclusively in IIMs and allow identifying different subtypes of spectrum of IIMs with specific clinical features and prognosis (Targoff, 1992, 2000; Love et al., 1991). The clinical associations originally described between different MSAs and IIMs were based on immunoprecipitation (IP) or double immunodiffusion (DID) assays (Mathews and Bernstein, 1983; Nishikai and Reichlin, 1980; Meyer et al., 1987). The detection of protein and/or RNA components of autoantigens by IP, using human cultured cells, allows the screening of most of IIMs, based on their known molecular weight and a unique set of proteins for certain MSA. Most anti-synthetase antibodies (antibodies to aminoacyl tRNA synthetases, ARS), anti-Mi2, anti-SRP and newly identified autoantibodies, such as anti-TIF1γ (transcription intermediary factor-1γ), anti-SAE (small ubiquitin-like molecule activating enzyme), anti-MDA-5 (melanoma differentiation associated gene 5), anti-NXP-2/MJ (nuclear matrix protein 2), are well recognized by IP, though some may require additional tests for confirmation (Satoh et al., 2015). However, IP could

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have some limitations in the interpretations of the precipitation bands: many MSAs immunoprecipitate in the same narrow area of gel electrophoresis (i.e. 100–200 kDa) and some antigens migrate very close in SDS-PAGE (such as MDA5 and NXP2) (Muro et al., 2013).

Many commercial kits including enzyme-linked immunosorbent assay (ELISA), line blot (LB) or dot blot assays using recombinant antigens are available on the market. Some studies showed a good performance and good concordance of the data obtained by these assays and by original IP, when single autoantibody reactivity was considered. The widespread use of these assays has allowed non-research laboratories to detect the majority of the MSAs. However, the clinical associations between different subtypes of IIMs and MSA, detected by commercial assays, are not completely investigated, so far.

The aim of the present study is to analyze the performance of a commercial LB assay on sera of patients affected by well-known IIMs, previously characterized by protein and RNA IP, in order to evaluate the correlations between clinical features and MSA and concordance or discrepancy between the two methods.

2. Material and methods

2.1. Patients

Sixty-six adult European Caucasian patients with myositis followed-up in the Rheumatology Unit in Brescia (Spedali Civili, Brescia, Italy) between 2010 and 2012 were enrolled in this retrospective study. Polymyositis (PM) and dermatomyositis (DM) were diagnosed according to Bohan and Peter's criteria (Bohan and Peter, 1975); the anti-synthetase syndrome was defined as the triad of arthritis, myositis and interstitial lung disease associated with ARS (Imbert-Maseau et al., 2003); overlap syndromes included patients affected by PM and Sjogren's syndrome (SS) (Vitali et al., 2002), systemic sclerosis (SSc) (Masi et al., 1980; LeRoy et al., 1988), systemic lupus erythematosus (SLE) (Tan et al., 1982), or rheumatoid arthritis (RA) (Arnett et al., 1988). Patients' clinical data were independently re-viewed by two authors (M.F. and F.F.), in order to confirm or change the diagnoses previously made. Serum samples, taken from each patient during 2012, were tested by IP and LB.

The study was approved by the Institutional Review Board of the Hospital. This study meets, and is in compliance with, all ethical standards of medicine, and informed consent was obtained from all patients in accordance with the Helsinki Declaration of 1975/83/2013.

2.2. Methods

Antinuclear antibodies (ANA) were detected by indirect immunofluorescence (IIF) using HEp-2 cells (BioRad, Hercules, CA, USA) with starting dilution of 1:160. Anti-ENA antibodies were tested by counterimmunoelectrophoresis (CIE) using calf thymus and bovine spleen extracts as antigen sources as described (Bernstein et al., 1982; Clark et al., 1969; Venables et al., 1983). Fifty-seven sera were analyzed by protein-IP of K562 cell extract radiolabeled with ^{35}S-methionine, as previously described, and autoantibodies were determined using reference sera (Yamasaki et al., 2006). Sixty-six sera were tested also by commercial line blot assay (Euroline Autoimmune Inflammatory Myopathies 15 Ag (IgG) Euroimmun, Lubeck, Germany). The results were arbitrarily defined as negative (0), borderline ((+)), positive (+ or ++), and strong positive (+++) as indicated by the manufacturer. Every strip included recombinant human proteins for Mi-2 alpha, TIF1γ, MDA5, NXP-2, SAE, Ku, PM-Scl75/100, SRP, PL-7, PL-12, EJ, OJ and native purified antigen for Jo-1.

2.3. Statistical analysis

Statistical analysis for categorical variables was performed with Chi-square or Fisher's exact tests. Inter-rater agreement was calculated with Cohen's kappa coefficient: values of 0–0.2 are considered as slight agreement, 0.21–0.4 as fair, 0.41–0.6 as moderate, 0.61–0.8 as substantial, 0.81–1 as almost perfect agreement.

2.4. Results

Among 66 enrolled IIMs patients, 23 were affected by PM (34.8%), 8 had anti-synthetase syndrome (12.1%), 29 DM (44%, including 1 amyopathic DM and 1 cancer-related DM), and 6 overlap syndromes: 2 PM-SS, 2 PM-SSc; 1 PM-SLE; 1 PM/RA. The female to male ratio was 3.7:1; the mean age at onset was 45.5 years (SD: 17.3 years), median 46 years (range 6–73 years). Patients were followed-up for a mean of 9 years (SD: 5.9). 3.1 Autoantibodies' analysis IP was performed in 57 sera: ANA were positive in 74% (37/50) cases, with a prevalent speckled pattern detected in 28/37 (75.7%). CIE detected at least one positivity in 36.8% of sera (21/57), represented by anti-Jo1 in 10 cases, anti-Ro in 5 anti-Ro + La in 3, anti-Ku in 2, anti-PM/Scl and anti-Ki in one serum each MSA/MAA positivity was found in 48 cases (84.2%) and MSA in 36 sera (63.1%). The most frequent autoantibody specificity detected by IP was anti-Jo1 (14%) and anti-NXP2 (14%). Autoantibodies' positivity by LB was detected in 44/66 sera (66.7%) and MSA in 39 cases (59%): anti-Jo1 and anti-Mi2 were found in 15% (10/66) of sera, followed by anti-NXP2 and anti-SRP detected in 10.6% (7/66) of sera. Anti-TIF1gamma were found in 6/66 (9%), and anti-MDA5 in 5/66 (7.6%). On these 66 sera ANA were positive in 71.2% (42/59) cases, with a prevalent speckled pattern detected in 36/42 (85.7%). CIE detected at least one positivity in 34.8% of sera (23/66), represented by anti-Jo1 in 11 cases, anti-Ro in 7, anti-Ro + La in 2, anti-Ku in 3, anti-PM/Scl and anti-Ki in one serum each.

2.5. Comparison between LB and IP

Comparing the results of 57 sera analyzed by the two methods, we found an overall concordance rate of 77% with a fair agreement (Cohen's k: 0.30). When we analyzed the concordance rate for the single specificity, a good agreement was found only for anti-TIF1 γ (k: 0.78), anti-MDA5 (k: 0.63) and anti-NXP-2 antibodies (k: 0.61) as shown in Table 1. The two assays showed a moderate agreement for anti-Mi2 (k: 0.5) and anti-EJ (k:0.48) and a fair agreement for anti-Jo1 (k: 0.3).

2.5.1. Anti-Jo-analysis

LB and IP showed a high discordance rate for the detection of anti-Jo1 antibodies (80%: 12 discordant on 15 positive sera), as shown in Table 2. Among 11 LB anti-Jo1 positive sera, 3 sera were IP+, 1 was not examined by IP (excluded from Table 2), 7 resulted discordant for IP (5 positive and 2 negative for other antibodies): 4 discordant sera showed multiple specificities by LB, 3 sera showed a single anti-Jo1 reactivity by LB.

Table 1
Comparison between IP (immunoprecipitation) and line blot (LB) assays in 57 sera of patients affected by IIMs (idiopathic inflammatory myopathies).

MSA	IP	LB	Cohen's k
	n. 57 (%)	n. 57 (%)	
Anti-Jo-1	8 (14)	10 (17.5)	0.3
Anti-PL-7	0	2 (3.5)	0.00
Anti-PL-12	0	3 (5.2)	0.00
Anti-EJ	3 (5.2)	1 (1.7)	0.48
Anti-OJ	2 (3.5)	0	0.00
Anti-SRP	3 (5.2)	4 (7%)	0.00
Anti-Mi-2α/β	3 (5.2)	8 (15.7)	0.50
Anti-NXP-2	8 (14)	7 (12.3)	0.61
Anti-TIF1gamma	4 (7)	6 (10.5)	0.7
Anti-MDA5	4 (7)	5 (8.7)	0.63
Anti-SAE	2 (3.5)	1 (1.7)	0.00
Anti-SMN	1 (1.8)	NA	NA

Table 2
Anti-Jo1 antibodies detected by IP and LB assays.

anti-Jo1 +	IP +	IP-
LB +	37	
LB −	5	42

A clinical diagnosis for classical anti-synthetases syndrome was achieved for 6 patients: 2 IP +/LB +, 3 IP +/LB- and 1 IP −/LB +. Eight anti-Jo1 LB + cases had other diagnoses: PM in 3, DM in 4 and PM/SS in 1 case, without any features of anti-synthetases syndrome, such as lung involvement, Raynaud's phenomenon, mechanic hands or arthritis. One patient anti-Jo1 + by IP and LB showed PM, without other extra-muscular features.

Anti-Jo1 was detected in 16 sera, by at least one of the methods used (CIE, IP and LB), as shown in Table 3. The concordance rate between CIE and IP was high (Cohen's k: 0.87), while moderate concordance was established between CIE and LB (k: 0.50) in fact CIE confirmed only the 6/11 Jo1 + sera identified by LB (54.5%). When we considered clinical diagnoses, we found consistency between clinical features and 10/16 results obtained by CIE (62.5%), 9/16 results by IP (56.5%) and 6/16 data obtained by LB (37.5%).

2.5.2. Other anti-synthetases antibodies' analysis

Anti-PL7 and PL12 antibodies were detected only by LB; however, these cases did not show any feature of anti-synthetase syndrome, so far. Anti-EJ antibodies were detected in 3 cases by IP (1 with anti-synthetase syndrome and 2 with DM): 1/3 was positive also by LB.

2.5.3. Anti-SRP analysis

Anti-SRP antibodies were detected in 3 sera by IP: 1 case with necrotizing myopathy, 1 with PM, 1 with overlap PM/RA. LB detected anti-SRP in 7 other sera (Cohen's k: 0.0): 2 showed ARS, 3 DM, 1 PM and 1 PM/SSc without any feature of necrotizing myopathy.

2.6. Multiple specificities' analysis

Multiple positivities for MSAs were detected in 11/66 cases by LB and 0/57 by IP (p: 0001). Details of 11 sera were shown in Table 4: anti-Jo1 antibody is the most frequent specificity detected (5/11: 45.5%), while anti-Mi2 and anti-SRP antibodies were found in 4/11 sera (36.3%).

Comparing the clinical features of 11 sera with multiple positivities and the results obtained by LB and IP, we found consistency between IP and clinical features in 3 sera (group 1) (27.3%) (serum n. 3, 10, 11), in which IP results were consistent with clinical diagnoses (anti-EJ, SAE and SRP, respectively). Patient n. 3 showed positivity for anti-NXP2 +++, Mi2 ++, SRP ++, not consistent with the diagnosis of anti-synthetase; patient n. 10 with anti-Jo1 + PL7 had DM and patient n. 11 with anti-Mi2 + PL7 was affected by necrotizing myopathy.

A relative discrepancy between IP and LB due to the occurrence of one discordant autoantibody (not confirmed by IP) was found in 5 cases (45.5%) (group 2: sera n. 1, 2, 5, 8, 9) in which the detection of anti-Mi2 (sera 1 and 2), anti-Jo1 (serum 5), anti-SRP (serum 8), anti-PL12 + SRP (serum 9) did not fit with clinical features and these positivities were not confirmed by IP. Clinical features are consistent with IP, and partially with LB.

Finally, a total discrepancy between LB and IP results, but with a relative consistency between LB and clinical features were found in 3 sera (group 3: n. 4, 6, 7): serum 4 showed an unknown IP result with amyopathic DM consistent with anti-MDA5, but not with anti-TIF1gamma and SAE; serum 6 showed a negative IP, but affected by antisynthetase syndrome consistent with anti-Jo1 and not with MDA5; serum 7 anti-EJ + by IP showed a DM diagnosis that is consistent with anti-NXP2 but not with SRP antibodies.

Moreover, the semiquantitative results do not support the interpretation of the results: in fact a highly positive result by LB is frequently inconsistent with IP results or with patients' clinical features.

3. Discussion

The detection of myositis-specific and myositis-associated autoantibodies was originally based on the study of different immunofluorescence ANA patterns or precipiting lines in immunodiffusion techniques such as double immunodiffusion or CIE. These assays were actually useful only for the detection of MAA, while among MSAs, they detected only anti-Jo1 and, more rarely, anti-Mi2 antibodies. ANA by IIF resulted positive in about 50% of IIMs cases, with specific staining patterns such as fine nuclear speckled, consistent with anti-Mi2 or anti-TIF1gamma, or cytoplasmic fine speckled found in anti-Jo1, anti-PL7 or anti-PL12 positive sera (Tansley et al., 2013). The use of protein and RNA IP allowed the recognition of many different MSAs based on the immunoprecipitation of proteins or RNAs on specific gels, given their known molecular weight. However, the use of IP is restricted to few research laboratories, because it is labor and time-consuming and it requires a highly specific training in interpretation of the results in addition to the complication of using radioisotopes. Commercially available ELISA included only anti-synthetases antibodies (Tansley et al., 2013), while multiparametric assays (i.e. line blot or dot blot) show the

Table 3
Anti-Jo1 antibodies detected at least with one assay, including CIE, IP and LB.

n.	CIE (n. 66)	IP (n. 57)	LB (n. 66)	Clinical diagnosis	Concordance between assays
1	Jo1	Jo1	Jo1 + Mi2	Anti-synthetase syndrome	All
2	Jo1	Jo1	Jo1 + Mi2	PM	CIE/IP
3	Jo1	Jo1 + U1RNP	Jo1	Anti-synthetase syndrome	All
4	Neg	MJ	Jo1 + MJ	DM	IP/LB (partial)
5	Ro + Jo1	Neg	Jo1 + MDA5	Anti-synthetase syndrome	CIE/LB (partial)
6	Jo1	Neg	Jo1	DM	No
7	neg	Unknown	Jo1	PM	LB
8	Ro + Ku	Ro + Ku	Jo1 + PM/Scl	PM	LB
9	Jo1	Not examined	Jo1 + PM/Scl	DM	No
10	Ro + La	Ro	Jo1	PM/SS	CIE/IP
11	neg	Ro + Su	Jo1 + Pl7	DM	No
12	Jo1	Jo1	Neg	PM	CIE/IP
13	Jo1	Jo1	Ku + Mi2	Anti-synthetase syndrome	CIE/IP
14	Jo1	Jo1	Neg	PM	CIE/IP
15	Ro + Jo1	Jo1	Neg	Anti-synthetase syndrome	CIE/IP
16	Jo1	Jo1 + U1RNP	PM/Scl + SRP	Anti-synthetase syndrome	CIE/IP (partial)

DM: dermatomyositis, PM: polymyositis, SS: Sjogren's syndrome.

Table 4
Comparison between line blot, IP and clinical features on 11 sera with multiple MSAs obtained by line blot analysis.

group	Serum #	Line blot	IP	Clinical features	Concordance between assays	Consistency with clinical features
1	3	NXP2 +++, Mi2 ++, SRP ++	EJ	Anti-synthetase syndrome	No	Complete with IP
	10	Jo1 +, PL7 +	SAE	DM		
	11	Mi2 +, PL7 +	SRP	Necrotizing myopathy		
2	1	Jo1 +++, Mi2 ++	Jo-1	Anti-synthetase syndrome	IP confirms one LB positivity	Complete with IP, partial with LB
	2	Jo1 +++, Mi2 ++	Jo-1	PM		
	5	Jo1 ++, NXP-2 +	NXP-2	JDM		
	8	EJ +++, SRP +	EJ	DM + ILD		
	9	Mi-2 +++, PL12 +, SRP +	Mi-2	DM		
3	4	TIF1g ++, MDA5 +, SAE ++	Unknown	Amyopathic DM	No	Partial with LB
	6	Jo1 +++, MDA5 +	Neg	Anti-synthetase syndrome		
	7	NXP2 +++, SRP +	EJ	DM		

DM: dermatomyositis, PM: polymyositis, JDM: juvenile dermatomyositis; ILD: interstitial lung disease.

advantage of testing multiple autoantibodies reacting with different purified or recombinant antigens. They are considered tests with high specificity and reliability (Tansley et al., 2013), but inconsistent data between different multiplex assays for important autoantibody specificities, such as anti-dsDNA and -Sm also has been reported.

In our series of 66 patients with IIMs, LB detected MSAs in 59% of cases, including newly reported MSAs (such as anti-TIF1gamma, MDA5, NXP2), while CIE detected only anti-Jo1 as MSAs in a lower rate (16.7%) ($p < 0.0001$). The routine use of LB has given the opportunity to better define the diagnosis and prognosis, predicting organ involvement of myositis patients within the clinical spectrum of IIMs, but in our hands the performance of LB shows some caveats.

First, we found a low concordance rate between LB and IP for anti-Jo1, that is considered the most frequent antibody of IIMs in general. CIE confirmed only 54% (6/11) of anti-Jo1 + sera by LB, and 100% (8/8) of anti Jo1 + by IP.

When we tried to consider clinical diagnoses, from patients' charts and "ad hoc" reviewed, we found a more frequent consistency between clinical data and CIE or IP results than LB. A direct comparison between laboratory test results and clinical features may be potentially problematic, due to the possibility that a DM or a PM or an overlap PM/SS could evolve in an anti-synthetase syndrome during the follow up. In fact, the occurrence of different features of anti-synthetase syndrome could emerge at different time-points during follow up (Cavagna et al., 2015). LB has been used more and more as an option for the detection of anti-synthetase antibodies, however, in our hands this technique showed a low sensitivity for anti-Jo1 antibodies, consistent with previous publications reporting a lower sensitivity of line immunoassays for PM/DM and SS (Jeon et al., 2013).

Second, we found a high frequency of concomitant occurrence of different MSAs (16.7%) in 66 sera tested by LB. MSA are known to be mutually exclusive in most cases, and each of them are often associated with specific clinical subtypes of IIMs. A recent meta-analysis on IIMs confirms that the occurrence of more than one MSAs in each patient is extremely rare, estimated in 0.11‰ of cases (Lega et al., 2014). Within sera with coexisting MSAs, we found consistency between clinical features and LB only in 3/11 (27.3%), and with IP in 8/11 sera (72.7%). In fact, three type of discrepancies were identified when LB, IP and clinical diagnoses were considered: 1) complete discrepancies that do not fit with clinical diagnoses or with IP results in about one third of cases (group 1); 2) relative discordant data found in 45% of cases (group 2: same antibody detected by IP and LB with the addition of a second discordant antibody with LB); 3) a complete discrepancy between LB and IP results, with a relative concordance between LB and clinical features found in 1/3 of sera, not confirmed by IP (group 3).

MSAs have previously been identified in conjunction with less specific autoantibodies, such as anti-Jo1 and anti-Ro52, or anti-MDA5 with anti-Ro52 (Fiorentino et al., 2011; Hall et al., 2013) or anti-Ro60 + Ro52 and anti-PL7 (Labirua-Iturburu et al., 2012). Anti-Ro/SSA were not detected in our series, because the LB used did not include the Ro antigen in the strips while only the newer version of LB contained the, Ro 52 kDa antigen. The co-occurrence of MSA and MAA was detected in our series in 7/66 (10.6%) by LB and 7/57 (12.3%) by IP: anti PM/Scl were found in 6/7 sera added to Jo1 (2 cases), TIF1gamma (1 case), SRP (2 case) and MDA5 (1 case). Only one patient with PM/Scl + SRP showed a diagnosis of overlap PM/SSc, without clinical features of necrotizing myositis: this serum had positive anti-PM/Scl only by IP. The other positivities were not confirmed by IP, and did not show clinical features consistent with the given specificities by LB.

A third caveat concerns the autoantibodies' titer. Line blot assays give a semiquantitative result, corresponding to low, medium or high titer, according to the manufacturer. The autoantibody titer is important for predicting the relapse of some autoimmune disease, such as SLE. Recently the titer of some MSAs detected by ELISA, has been reported as a predicting factor of severe disease or disease activity only in Japanese patients. High titer of anti-MDA5 antibodies could be a predictive factor of death in adult patients with DM (Koga et al., 2012); in addition, the disappearance of anti-MDA5 antibodies has been found in adult patients in stable clinical remission (Muro et al., 2012). When anti-MDA-5 titer was analyzed in the US Caucasian population, data were not as significant as predictive factor (Hall et al., 2013). Other reports described some correlations between antibodies titer and disease severity for anti-Jo1 (Stone et al., 2007), anti-TIF1gamma (Aggarwal et al., 2012), and anti-SRP antibodies (Beneviste et al., 2011). In our series the semiquantitative data are not helpful in the interpretation of the co-existence of two or more MSAs: in some cases the highest titer corresponds to the "correct" autoantibody, but in other sera the MSA fitting with clinical data or IP results showed a low titer positivity. Sometimes the antibody with higher titer is not concordant with the clinical diagnosis.

In conclusion, the use of line blot assay, dedicated to MSAs, allowed the detection of new myositis-specific autoantibodies, previously not found with routine methods, such as anti-MDA5, anti-MJ and anti-TIF1gamma antibodies. However, the high prevalence of multiple positivities and the high discordant rate of anti-Jo1 antibodies could create some misinterpretation of the results from the clinical point of view. These data should be confirmed re-testing the discordant sera and enlarging the number of cases.

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Periodontal bacterial colonization in synovial tissues exacerbates collagen-induced arthritis in B10.RIII mice

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Abstract

Background: It has been previously hypothesized that oral microbes may be an etiological link between rheumatoid arthritis (RA) and periodontal disease. However, the mechanistic basis of this association is incompletely understood. Here, we investigated the role of periodontal bacteria in induction of joint inflammation in collagen-induced arthritis (CIA) in B10.RIII mice.

Methods: CIA-prone B10.RIII mice were infected orally with a polybacterial mixture of *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* for 24 weeks before induction of CIA. The ability of polybacterial mixture to colonize the periodontium and induce systemic response, horizontal alveolar bone resorption in infected B10.RIII mice was investigated. Arthritis incidence, severity of joint inflammation, pannus formation, skeletal damage, hematogenous dissemination of the infection, matrix metalloproteinase 3 (MMP3) levels, and interleukin-17 expression levels were evaluated.

Results: B10.RIII mice had gingival colonization with all three bacteria, higher levels of anti-bacterial immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies, significant alveolar bone resorption, and hematogenous dissemination of *P. gingivalis* to synovial joints. Infected B10.RIII mice had more severe arthritis, and higher serum matrix metalloproteinase 3 levels and activity. Histopathological analysis showed increased inflammatory cell infiltration, destruction of articular cartilage, erosions, and pannus formation. Additionally, involved joints showed had expression levels of interleukin-17.

Conclusion: These findings demonstrate that physical presence of periodontal bacteria in synovial joints of B10.RIII mice with collagen-induced arthritis is associated with arthritis exacerbation, and support the hypothesis that oral bacteria, specifically *P. gingivalis*, play a significant role in augmenting autoimmune arthritis due to their intravascular dissemination to the joints.

Keywords: Rheumatoid arthritis, Periodontal disease, Periodontal bacteria, *Porphyromonas gingivalis*, B10.RIII mice, Collagen-induced arthritis

Background

Rheumatoid arthritis (RA) and periodontal disease (PD) are epidemiologically associated with each other [1–3], but the mechanistic basis of this association is unclear. It is known, however, that the pathogeneses of both conditions involve TNFα, T helper (Th)17 cells, and osteoclast-mediated bone damage [4, 5]. Similar to RA, the susceptibility to PD and its severity depend on overlapping environmental and genetic factors, as both RA and PD have been shown to be associated with the human leukocyte antigen (HLA) shared epitope [6–8] and cigarette smoking has been shown to increase disease risk in both conditions [9].

It has long been hypothesized that oral microbes may be an etiological link between PD and RA in humans [10, 11]. For example, in Native American patients with RA and their relatives, antibodies to the periodontal bacteria *Porphyromonas gingivalis* (*P. gingivalis*) were found

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to be associated with anti-citrullinated protein antibodies, suggesting that *P. gingivalis* may be breaking the immune tolerance towards citrullinated antigens [12]. However, there is no direct causative evidence to support this or other proposed mechanisms for the long-observed association between human RA and PD.

It is worth noting that heat-killed *P. gingivalis* have been previously proposed as a "priming" inflammatory agent linking experimental PD and arthritis in a rat model [13]. That study demonstrated that the presence of extra-synovial chronic inflammatory lesions, induced by heat-killed *P. gingivalis*, promoted the induction and severity of experimental arthritis. Further, the same group [14] tried to assess the influence of preexisting PD on induction and severity of collagen antibody-induced arthritis (CIA) in mice. Using pristine-induced arthritis (PIA) Trombone et al. [15] described the clinical association between RA and PD in the acute inflammatory reactivity maximum (AIR-max) mice. More recently, Marchesan and associates have demonstrated strain-specific immune system divergence following infection with different strains of *P. gingivalis* [16]. Moreover, *P. gingivalis* orally-infected DBA/1 mice with CIA had more severe arthritis associated with activation of Th17-related pathways [16], suggesting that this T cell subset may directly contribute to the observed association between arthritis and PD. However, given the generalized nature of the Th17 activation, the mechanistic basis of the predilection of *P. gingivalis*-infected mice to trigger a tissue-specific inflammatory process in the joints is still unclear.

To better elucidate the mechanistic basis of the association between RA and PD, here we have taken a different experimental approach by focusing on CIA in B10.RIII mice, which are genetically susceptible to collagen type II (CII)-induced arthritis. As periodontal disease always results from the dysbiotic interaction between the oral microbiota and host immunity we have chosen the most representative microorganisms that have been established as periodontal pathogens. We induced periodontal disease in these B10.RIII mice by chronic gingival infection with a combination of *P. gingivalis*, *T. denticola* and *T. forsythia* for 24 weeks [17, 18]. Our data demonstrate that mice chronically infected with these PD-causing bacteria experienced aggravated clinical signs of CIA with increased metalloproteinase activity, intense immune-based inflammatory cellular infiltration, and enhanced destruction of articular cartilage and bone. Importantly, a fluorescence *in situ* hybridization technique revealed dissemination of the periodontal bacteria to the synovial tissues.

These findings substantiate a previously unappreciated mechanism of cause-effect relationship between periodontal infection and arthritis, and support the hypothesis that PD-causing bacteria, specifically *P. gingivalis*, may contribute to the susceptibility and severity of inflammatory arthritis due to their tropism to synovial tissues, where they may contribute to intensifying the inflammatory process.

Methods

Microbial strains and inocula

P. gingivalis FDC 381, *T. denticola* ATCC 35404, and *T. forsythia* ATCC 43037 were used in this study and were routinely cultured anaerobically at 37 °C as described previously [17–19]. Bacterial inocula were prepared and used for gingival infection of mice by oral lavage as described previously [17, 18, 20, 21].

Mouse infection and oral plaque sampling

The polybacterial oral infection and sampling methodology were done as described previously [17, 18]. Briefly, six-week-old male B10.RIII mice (The Jackson Laboratories, Bar Harbor, ME, USA) were kept in groups and housed in microisolator plastic cages. Mice were randomly distributed into four groups; polybacterial infection alone (group I; $n = 10$), polybacterial infection and immunization with complete Freund's adjuvant (CFA)/CII and incomplete Freund's adjuvant (IFA)/CII (group II; $n = 10$), immunization with CFA/CII and IFA/CII alone (group III; $n = 10$), and sham-infection control (group IV; $n = 10$) (Table 1). B10.RIII mice were administered kanamycin (500 μg/ml) daily for 3 days in the drinking water and the mouse oral cavity was rinsed with 0.12 % chlorhexidine gluconate (Peridex: 3M

Table 1 Distribution of B10.RIII mice groups, polybacterial infection and collagen administration in the induction of collagen-induced arthritis

Group	Bacterial infection (24 weeks)	Collagen-induced arthritis (25–28 weeks)	Number of mice
I	*Pg/Td/Tf*	————————————————————	10
II	*Pg/Td/Tf*	CII + CFA primary	10
		CII + IFA booster	
III	Sham infection	CII + CFA primary	10
		CII + IFA booster	
IV	Sham infection	————————————————————	10

Pg indicates *P. gingivalis*; *Td* indicates *T. denticola*; *Tf* indicates *T. forsythia*. *CII* collagen type II, CFA complete Freund's adjuvant, *IFA* incomplete Freund's adjuvant

ESPE Dental Products, St. Paul, MN, USA) mouth rinse to reduce endogenous murine microorganisms and to enhance subsequent colonization of human periodontal bacteria [17]. The concentration of each bacterium used for infection was determined quantitatively, and the organisms were resuspended in reduced transport fluid at 1×10^{10} bacteria per ml. Bacteria were then mixed with an equal volume of sterile 4 % (wt/vol) low-viscosity carboxymethylcellulose (CMC; Sigma-Aldrich, St. Louis, MO, USA) and polybacterial inocula used for gingival infection were administered (10^9 cells in 0.2 ml) for 4 consecutive days per week on 8 alternate weeks to mimic chronic exposure during 24 weeks of the infection period (Fig. 1a). Mice swallowed the bacterial inoculum, which ends up in the gut where there is a chance of gut infection-induced inflammation and possible systemic entry or a potential route for the induction of experimental arthritis. Sham-infected control mice received sterile 4 % carboxymethylcellulose (CMC) only. Gingival plaque samples were collected at 3 days post-infection by swabbing the gingival surface of the mice, especially the teeth and surrounding gingival tissue, using a sterile veterinary cotton swab with a head width of 2.6 mm.

Induction and clinical evaluation of collagen-induced arthritis

For induction of CIA, pre-dissolved liquid bovine type II collagen (bCII; 2 mg/ml, Chondrex LLC, Redmond, WA, USA) was emulsified with an equal volume of CFA or IFA (Chondrex LLC). After 24 weeks of infection, mice were immunized intradermally at the base of the tail with 0.1 ml of emulsion containing 100 μg of CII and CFA. Three weeks after priming (day 21), the mice were boosted with 0.1 ml of bovine CII (100 μg) emulsified in an equal volume of IFA. All mice were monitored three times a week by the same person blinded to the groups and arthritis

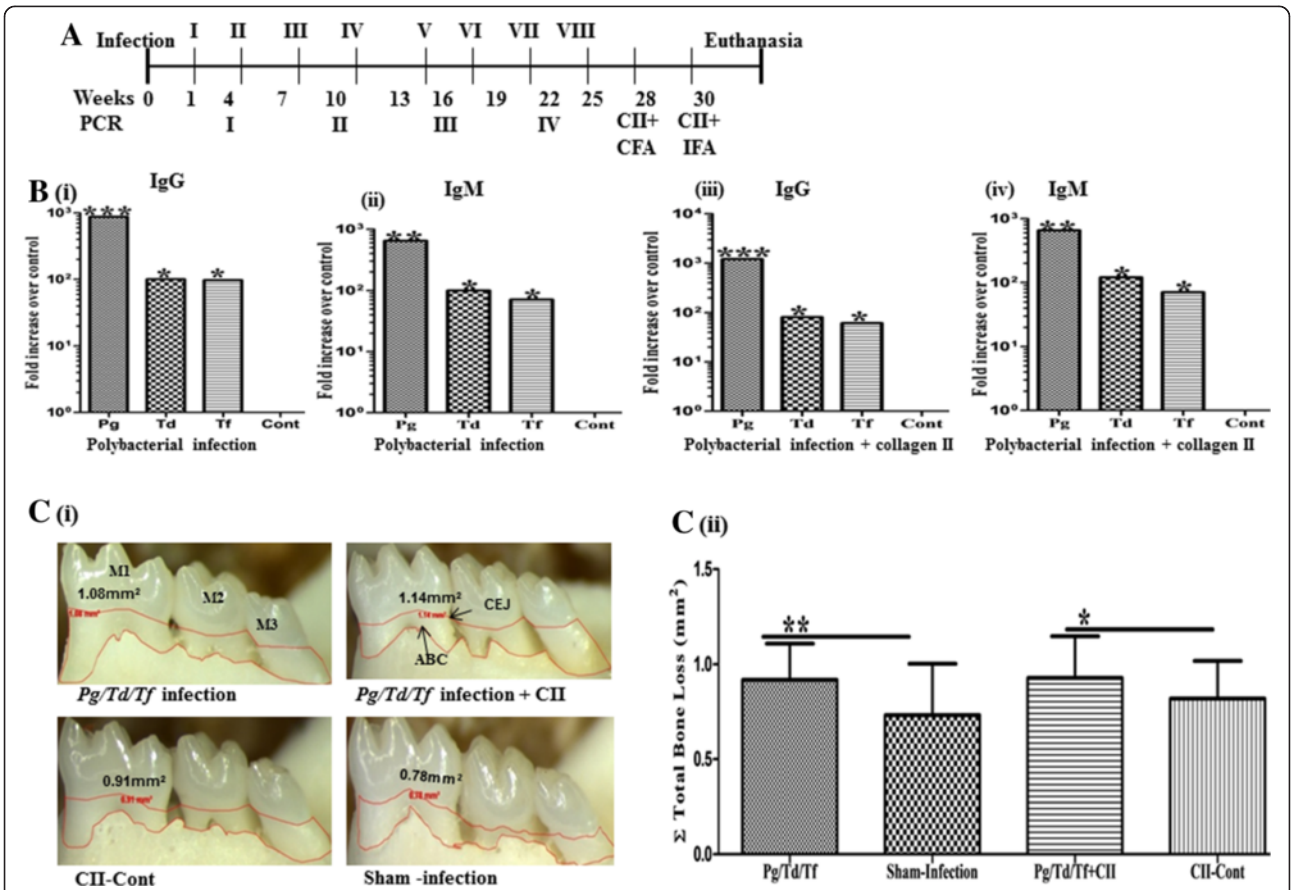

Fig. 1 Establishment of periodontal disease in B10.RIII mice. **a** Schematic diagram of the experimental design. **b (i)**, **(ii)** Polybacterial-infected mice IgG, IgM systemic immune response against *P. gingivalis* (*Pg*), *T. denticola* (*Td*), and *T. forsythia* (*Tf*), respectively; **(iii)**, **(iv)** polybacterial-infected + collagen type II (CII)-immunized mice IgG, IgM systemic immune response against *Pg, Td*, and *Tf*, respectively. **c (i)** Representative images of horizontal alveolar bone resorption area in mandibular surfaces of B10.RIII mice; **(ii)** total alveolar bone resorption in B10.RIII mice. Each *bar* indicates the mean alveolar bone resorption for three molars in each quadrant. Three bacteria were used in oral infection for 24 weeks. $N = 10$ in each group. $^*P < 0.05$, $^{***}P < 0.001$. *Cont* control

severity was assessed using criteria as follows: 0 = no swelling or redness (normal); 1 = mild erythema or swelling of the wrist or ankle or erythema and swelling of one digit; 2 = moderate erythema and swelling of the wrist or ankle or more than three inflamed digits; 3 = severe erythema and swelling of the wrist or ankle; and 4 = complete erythema and swelling of the wrist and ankle including all digits [22–24]. A paw was considered arthritic when the individual paw score was >1 and severe arthritis was defined as an arthritis score >3 for the purpose of comparing data between groups.

Detection of *P. gingivalis*, *T. denticola,* and *T. forsythia* genomic DNA in oral plaque

Colony PCR was performed with gingival plaque samples obtained after every infection in a Bio-Rad thermal cycler using 16S rRNA gene species-specific oligonucleotide primers. *P. gingivalis*: 5′-TGTAGATGACTGATGGTGAAAACC-3′ (forward), 5′-ACGTCATCCCCACCTTCCTC-3′ (reverse); *T. denticola* 5′-TAATACCGAATGTGCTCATTTACAT-3′ (forward), 5′-CTGCCATATCTCTATGTCATTGCTCTT-3′ (reverse); and *T. forsythia* 5′-AAAACAGGGGTTCCGCATGG-3′ (forward), 5′-TTCACCGCGGACTTAACAGC-3′ (reverse). Genomic DNA extracted from these three strains served as positive controls and PCR performed with no template DNA served as negative control. PCR was performed following the conditions described previously [17], PCR products were separated by 1.5 % agarose gel electrophoresis and the bands were visualized using the UVP BioDoc-It Imaging System (UVP, Upland, CA, USA).

Pathogen-specific immune response in mice

Sera from infected and control B10.RIII mice were used to determine immunoglobulin G (IgG) and IgM antibody concentrations against whole cells (formalin-killed) of *P. gingivalis*, *T. denticola*, and *T. forsythia* by ELISA [18, 20].

Morphometric analysis of alveolar bone resorption

The horizontal alveolar bone resorption (ABR) area and the presence of periodontal intra-bony defects were measured by histomorphometry as described previously [17, 21].

Detection of bacterial genomic DNA in internal organs

Heart, aorta, liver, pancreas, spleen, kidney, lung and joints/synovial tissues were harvested after euthanasia of infected/sham-infected mice. Tissues were transferred to the laboratory in vials containing reduced transport fluid and were stored at −80 °C until further use. Later they were thawed, homogenized using a mechanical tissue disruptor (TissueRuptor®, QIAGEN, Valencia, CA, USA) and genomic DNA extracted using QIAGEN DNeasy blood and tissue kit (QIAGEN) per the protocol described in the kit. Subsequently PCR was performed using 16S rRNA primers specific for each of the infection bacteria as described previously [19].

Detection of bacteria by fluorescence *in situ* hybridization (FISH)

FISH was performed on formalin-fixed paraffin-embedded ankle tissue sections using oligonucleotide probes labeled with Alexa Fluor 568 (Invitrogen, Carlsbad, CA, USA) that are specific for 16S rRNA of *P. gingivalis*, (5′-CAATACTCGTATCGCCCGTTATTC-3′), ’*T. denticola* 5′- CATGACTACCGTCATCAAAGAAGC-3′), or *T. forsythia* (5′-CGTATCTCATTTTATTCCCCTGTA-3′) [24–26] 16S rRNA. The protocol was performed as previously described [24].

In vivo molecular imaging/tomography of mice

An intravenous injection of fluorescent imaging agent (MMP Sense 750 FAST, PerkinElmer, Waltham, MA, USA) specific for MMP3 was given at a recommended dose of 2 nmol/100 μl per mouse (as per the manufacturer's instruction) to measure the progression of arthritis [27]. MMP Sense FAST is an activatable fluorescent imaging compound that is optically silent upon injection but produces fluorescent signal after cleavage by disease-related MMPs. The signals emitted were detected using the IVIS system (Caliper Life Sciences, MA, USA) which is an optimized set of high-efficiency filters and spectral un-mixing algorithms to measure the light emission across the blue to near infrared wavelength region.

Histological examination

Hind limbs together with the overlying skin from mice were excised at the termination of the experiment, and fixed in 10 % neutral buffered formalin. Later they were decalcified in 10 % ethylenediaminetetraacetic acid (EDTA) and embedded in paraffin. Serial sections (5 μm) were made and stained with hematoxylin/eosin and evaluated for synovial inflammation, pannus formation, and bone erosion [27]. Histopathological analysis was conducted by a pathologist who was blinded to the experimental study groups. Pannus formation was quantified based on a scoring criterion as follows: a score of 0 was given to no pannus formation; 1 for minimal formation; and 2 for definitive formation. Skeletal damage was quantified as 0 for none; 1 for one or two foci seen; 2 for multiple areas seen; and 3 for extensive damage among all surface areas. Synovial inflammation was scored based on scoring criteria [28] as follows, 0: no hyperplasia or inflammation; 1: slight hyperplasia with scattered acute inflammation; 2: multiple foci of inflammation predominantly with neutrophils; and 3: strong inflammation with inflammatory cell infiltration.

Matrix metalloproteinase 3 levels in serum from infected mice

Serum from B10.RIII mice (four groups; $n = 6$) was used to detect levels of MMP3 using the commercial serum MMP3 ELISA kit (Sigma-Aldrich Co, USA).

Immunohistochemical analysis

Tissues were fixed in 10 % buffered formalin, decalcified, embedded in paraffin wax and cut to 5 μm thickness, mounted on slides and air-dried at room temperature. Sections were deparaffinized, rehydrated and incubated with 3 % hydrogen peroxide in methanol for 15 minutes at room temperature to eliminate endogenous peroxidase activity. Antigen retrieval was carried out at 95 °C for 30 minutes by placing the slides in 0.01 M sodium citrate buffer (pH 6.0). The slides were then incubated with a primary rabbit polyclonal antibody for IL-17 (Abcam #ab79056) at 4 °C overnight. For immune detection, the avidin-biotin complex method was performed according to the manufacturer's instructions. Color development was achieved with 3, 3′-diaminobenzidine, which renders positive cells brown. Photographs were taken using an Olymbus BX-60 upright microscope (Center for live-cell imaging, UM-Michigan, USA).

Immunofluorescence

For immunofluorescence quantification, slides were stained with a primary rabbit polyclonal antibody for IL-17 as above, followed by secondary donkey anti-rabbit IgG antibody conjugated to Alexa Fluor® 594 (Abcam #ab150076) and stored at 4 °C until use. 4′ 6-diamidino-2-phenylindole (DAPI) was used for nuclei staining. Quantification was done by a Biotek Cytation 5 instrument, using the Gen5Image + software (Biotek), which allows for calculation of the percentage of nucleated (DAPI-positive) cells with cytoplasmic IL-17.

Statistical analysis

Group measures are expressed as mean plus SEM. Statistical analyses were performed using the two-tailed Student's t test with GraphPad Prism 5 (GraphPad, San Diego, CA, USA), and $P < 0.05$ was considered statistically significant ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). For multiple-group comparisons, one-way analysis of variance ($P < 0.05$) with Bonferroni's multiple-comparison was performed posttest.

Results

Gingival colonization and antibody response to periodontal-disease-associated polybacteria in mice

To investigate the cause-effect relationship between PD and inflammatory arthritis, we sought to determine the effects of known human PD-associated polybacteria on the severity of CIA. Because DBA/1, the most commonly used mouse strain for the arthritis model, were resistant to oral colonization despite prolonged attempts to infect them over a 24-week period (data not shown), we chose to focus on B10.RIII mice. The B10.RIII mice were susceptible to such colonization. As shown in Table 2, B10.RIII infected mice had colonization (second and sixth infection cycle) with three periodontal pathogens, *P. gingivalis*, *T. denticola,* and *T. forsythia*. All B10.RIII mice in the polybacteria-infected group (Fig. 1b (i) and (iii)) developed significantly elevated IgG antibody to *P. gingivalis* ($P < 0.001$), *T. denticola* ($P < 0.05$), and *T. forsythia* ($P < 0.05$) compared to the levels in sham-infected mice. However, anti-*P. gingivalis* IgG antibody titers were higher than anti-*T. denticola* and anti-*T. forsythia* IgG antibody levels (Fig. 1b (i) and (iii)). Similarly, all B10.RIII mice in the polybacteria-infected group (Fig. 1b (ii) and (iv)) developed significantly elevated IgM antibodies to *P. gingivalis* ($P < 0.01$), *T. denticola* ($P < 0.05$), and *T. forsythia* ($P < 0.05$) compared to the levels in sham-infected mice and uninfected CIA mice. Thus, B10.RIII mice are susceptible to infection with PD-associated polymicrobial pathogens, and develop antibody response to these pathogens.

Table 2 Gingival plaque samples positive for bacterial gDNA identified by PCR

Group	Polybacterial infection	Positive gingival plaque samples ($n = 10$)				
		1 week[a]	2 weeks	4 weeks	6 weeks	8 weeks
I	*Pg/Td/Tf*	NC	7/0/6	NC	7/7/7	NC
II	*Pg/Td/Tf* + collagen	NC	8/0/8	NC	9/7/7	NC
III	Collagen-control	NC	NC	0/0/0	NC	0/0/0
IV	Sham-infected	NC	NC	0/0/0	NC	0/0/0

Total numbers of gingival plaque samples that were collected after infections (1, 2, 4, 6 and 8 weeks) following polymicrobial (*P. gingivalis/T. denticola/T. forsythia* (*Pg/Td/Tf*)) infection and were positive as determined by PCR analysis. [a]Time points at which gingival plaque samples were collected. The first value corresponds to the number of mice that tested positive for *Pg* genomic DNA, the second value to the number of mice that tested positive for *Td* genomic DNA, and the third value to the number of mice that tested positive for *Tf* genomic DNA at each time point. *NC* not collected (to allow bacterial biofilm to adhere to the gingival surface, invade epithelial cells, and multiply), *Pg/Td/Tf* polybacterial-infected mice, *Pg/Td/Tf + collagen* polybacterial-infected mice administered collagen II, *Collagen-control* mice administered collagen II

Periodontal-disease-associated bacteria increase periodontal disease and severity of collagen-induced arthritis

The impact of these three bacteria on PD disease severity was investigated next. To this end we determined alveolar bone resorption, the hallmark characteristic of PD, using a morphometric approach. Among the B10.RIII mice the polybacterial-infected mice had a significantly larger ($P < 0.01$) mandibular horizontal ABR area when compared to sham-infected mice (Fig. 1c (i) and (ii)).

We then evaluated the effect of chronic gingival infection on CIA, which was induced in B10.RIII mice after chronic polybacterial infection. As shown in Fig. 2a (i), all (polybacterial-infected and CII-immunized) B10.RIII mice developed greater clinical signs of arthritis in the ankle joints and paws (100 %, 10 out of 10 mice) compared to mice that were polybacterial-infected only (0 %, 0 out of 10 mice). Collagen control B10.RIII mice also developed clinical signs of arthritis in the hind paws (100 %, 10 out of 10 mice). Without CII immunization, none of the polybacterial-infected mice or sham-infected control mice developed clinical signs of arthritis (0 %).

There were robust differences in the polybacteria-infected mice with CIA compared to mice with CIA but without such bacterial infection, in terms of day of arthritis onset, disease incidence, and joint swelling (Fig. 2a (i) and (ii)). In vivo tomography demonstrated the rapid progression of arthritis in polybacterial-infected mice with CIA compared to uninfected mice with CIA (Fig. 2b). There were no signs of arthritis development in polybacterial-infected non-CII immunized mice or in sham-infected mice. While we observed the progression of arthritis with the emission of fluorescence signals specific for arthritis in both the polybacterial-infected plus CII-immunized mice and mice that were CII-immunized only, the degree of intensity and severity was five times greater (measured by emission of radiance) in polybacterial-infected plus CII-immunized mice than in the CII-immunized mice (Fig. 2b). Evaluation of mice for progression of severe arthritis by in vivo tomography correlated with the observed clinical signs of arthritis in polybacterial-infected plus CII-immunized mice (Fig. 2b).

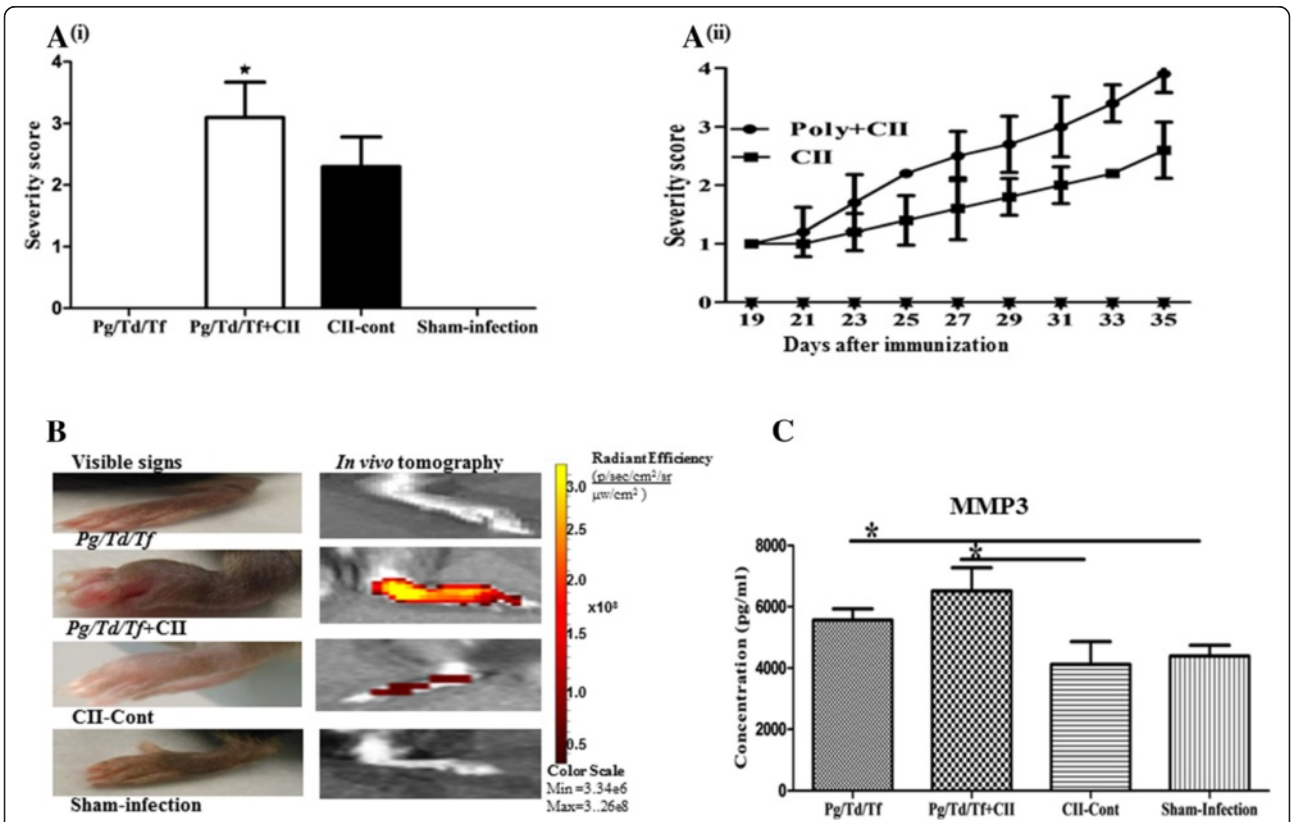

Fig. 2 Periodontal infection exacerbates CIA in B10.RIII mice. **a (i)** Visual clinical severity score in B10.RIII mice. Poly + CII group mice were infected with polybacterial infection first followed by primary immunization with collagen type II (*CII*) + complete Freund's adjuvant followed by booster after 3 weeks with CII + incomplete Freund's adjuvant. ($n = 10$); **(ii)** increased clinical arthritis scores in the hind paws upon concomitant periodontitis and collagen immunization. **b** Exacerbation of arthritic signs detected by in vivo tomography in B10.RIII mice. Photographs show visible inflammation in hind paws of mice (*left*). IVIS spectrum images show emission of fluorescence signals in mice (*right*). **c** Elevation of serum matrix metalloproteinase 3 (*MMP3*) in a mouse infected with *P. gingivalis/T. denticola/T. forsythia* (*Pg/Td/Tf*). *Bars* show mean ± SD. $n = 6$; *$P < 0.05$. *Cont* control

Effects of polybacterial infection on arthritis biomarkers

We determined the levels of MMP3, an enzyme capable of degrading cartilage and connective tissue in joint tissues in the sera, by ELISA. A significant ($P < 0.05$) elevation in serum MMP3 levels was detected in mice with polybacterial infection with or without CIA compared to sham-infected controls and mice with CIA but without polymicrobial infection (Fig. 2c). In addition, polybacterial-infected mice with CIA had higher MMP3 levels than mice with polybacterial infection without CIA.

Impact of coincidental periodontal disease and collagen type II immunization on the histopathology of arthritis

When we determined that the specific histopathological features of arthritis are affected by periodontal bacterial infection and CII immunization microscopically, we observed a significant increase in synovial inflammation, pannus formation, and skeletal damage when there is coincidental PD and CII immunization in comparison to CII immunization alone, PD alone, or sham-infection (Fig. 3a-c). Further, polymicrobial-infected CIA mice had characteristic inflammatory cell infiltrates and pannus formation in the ankle and paw joints (Fig. 3d (ii); Additional file 1: Figure S1A (i-iii)). These mice also had destruction of cartilage and bone in the joints (Fig. 3d (vi)). In contrast, although uninfected mice with CIA developed minimal inflammatory cell infiltration, pannus and joint destruction could be seen in these mice (Fig. 3d (iii) and (vii); Additional file 1: Figure S1B (i-iii)). There were no observable histopathological signs of arthritis in polybacterial-infected mice that were not immunized with CII (Fig. 3d (i) and (v)), or in sham-infected mice (Fig. 3D (iv) and (viii)).

Expression of IL-17 in paw tissue

There are increasing reports suggesting that Th17 plays a dominant role in the progression of periodontal disease [29–31] and IL-17A, produced by Th17 and other cells, has previously been described as an inflammatory cytokine, which induces additional cytokines, chemokines, and

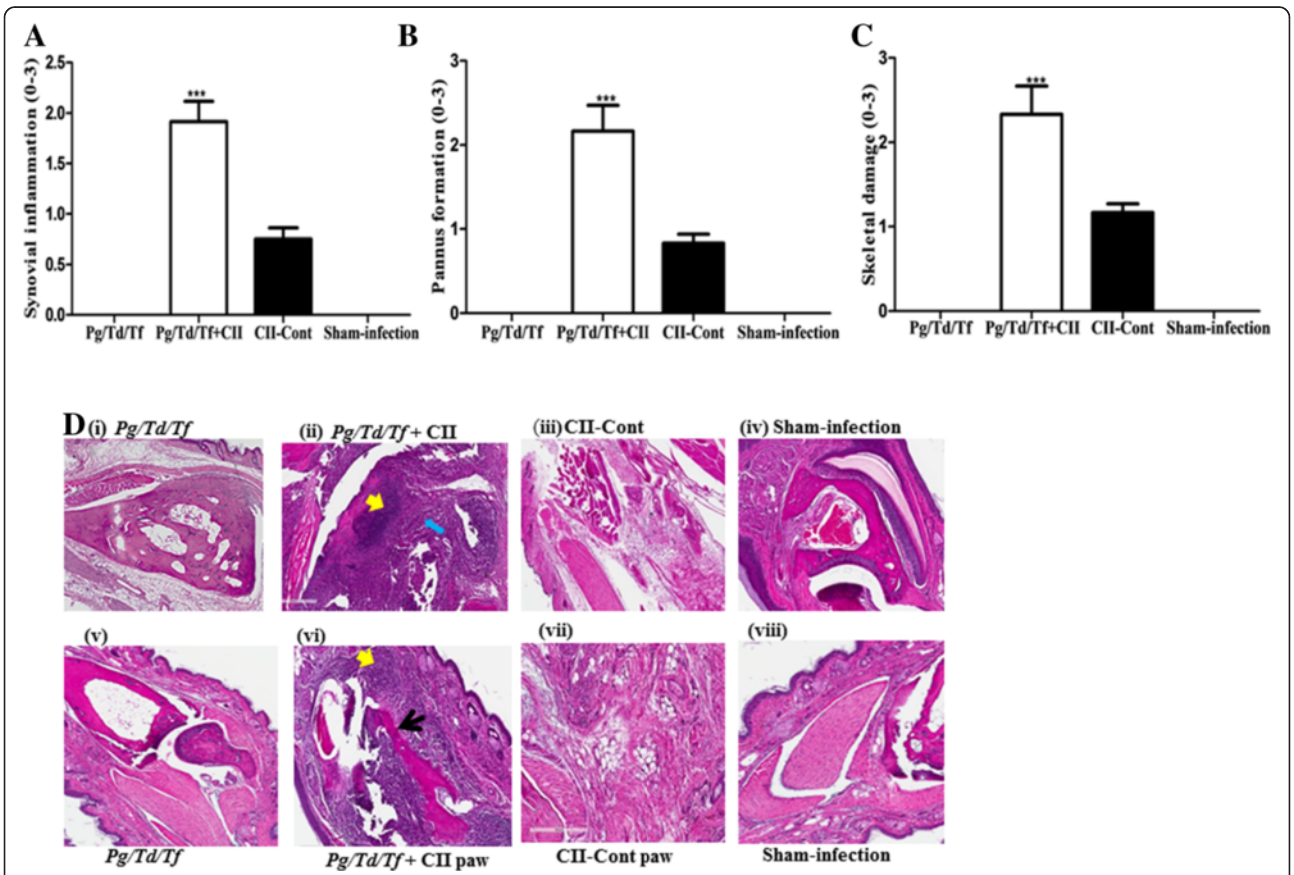

Fig. 3 Chronic periodontal infection and concomitant collagen immunization aggravates experimental arthritic pathological change. Histological assessment of ankle joints after immunization with collagen type II (*CII*). **a** Synovial joint inflammation, **b** pannus formation and **c** skeletal damage scores on H&E-stained tissue sections. Data are mean + SEM (scale 0–3) of six mice per group. **d** Histopathological evidence of development of arthritis in B10.RIII mice. H&E staining of ankle joint tissue from B10.RIII mice (*top row*). H&E staining of paw tissue from B10.RIII mice (*bottom row*). *Yellow arrowheads* indicate inflammatory infiltration; *thick blue arrows* indicate pannus formation and *thick black arrows* indicates cartilage destruction. Original magnification ×20. $N = 6$ in each group. *Cont* control, *Pg/Td/Tf*, *P. gingivalis/T. denticola/T. forsythia*

metalloproteinases that contribute to joint destruction in arthritis [32]. We therefore sought to determine whether expression of IL-17 in tissue could distinguish between the different mouse groups. To this end, we performed immunohistochemical (IHC) staining for IL-17. As can be seen in Fig. 4a, high expression of IL-17 (brown-colored tissue staining) was observed in group II (mice with polybacterial infection and CII-induced arthritis). In contrast, in group IV (sham infection), group I (polybacterial infection only), and group III (sham infection and mice with collagen II-induced arthritis) there was much lower abundance of this cytokine in joint tissue. Immunofluorescence-based quantification (Fig. 4b) indicated that in group II 16 % of joint tissue cells were positive for IL-17 (red staining), compared to only 2.2 % in group I, and 1.3 % in group IV. As could be expected, mice with active CIA (group III) had a higher percentage of IL-17-expressing cells (8 %); this figure however, was much lower than in group II (16 %).

Identification of oral bacteria in remote tissues

To investigate whether bacterial tissue dissemination takes place in this model, gingivae, heart, aorta, liver, pancreas, spleen, kidney, and lung were harvested after 24 weeks of infection and were examined for the presence of *P. gingivalis*, *T. denticola*, and *T. forsythia* genomic DNA by PCR using 16S rRNA gene species-specific PCR primers [17, 18, 20, 21]. As can be seen in Table 3, genomic bacterial DNA was identified in the heart, liver, kidney, and lungs of polybacterial-infected mice without CIA, and in mice infected with CIA. Among the three periodontal pathogens used for infection, there was more systemic spread of *P. gingivalis* and *T. denticola* than of *T. forsythia*

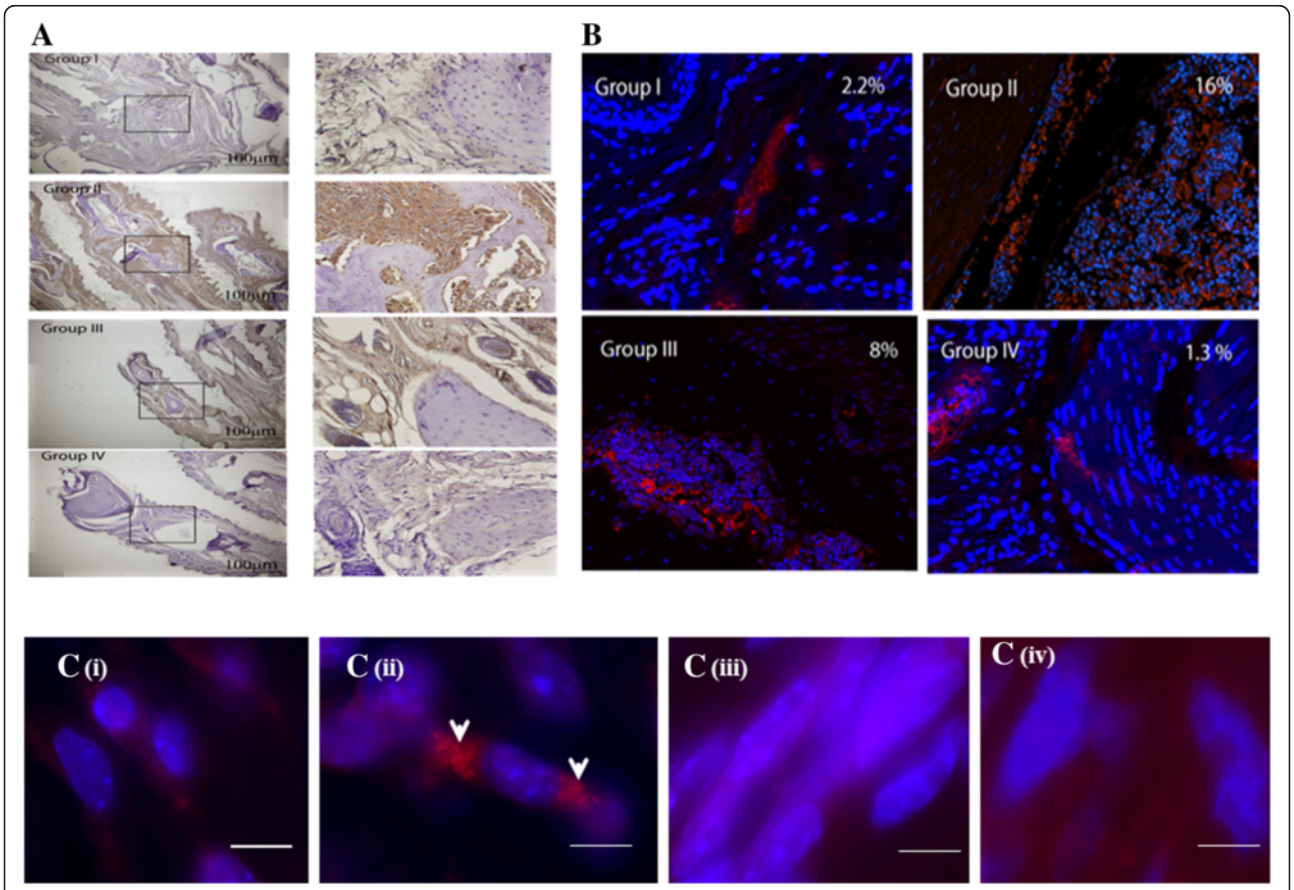

Fig. 4 IL-17 expression in synovial tissues. **a** Histological sections of representative B10.RIII mice displaying IL-17 expression in the four mouse groups. *Left column* shows low magnification (×4). *Right column* shows a higher magnification (×40) of the respective *boxed areas* shown in the *left column*. Group II paws had severe joint tissue-invading pannus tissue, which was heavily infiltrated by IL-17-expresscsing cells. **b** Immunofluorescence. Representative images of the joint tissues from different treatment groups. *Blue staining* represents 4′ 6-diamidino-2-phenylindole; *red fluorescence* represents IL-17. The calculated percentage of IL-17-positive cells is shown in the *right upper corner* of each image. Group II paws had much higher abundance of IL-17-expressing cells compared to the three other groups. Group III had an intermediate level of IL-17 abundance**. c (i)**, **(ii)**, **(iii)**, **(iv)** Representative fluorescence *in situ* hybridization images of **c (i)** *P. gingivalis/T. denticola/T. forsythia* (*Pg/Td/Tf*)-infected mice with no presence of bacteria, **c (ii)** the presence of *P. gingivalis* (*bright red fluorescence* denoted by *white arrowheads*) in ankle joint tissue of mice infected with *Pg/Td/Tf* along with collagen immunized in B10.RIII mice, **c (iii)** collagen control infected mice with no presence of bacteria, **c (iv)** sham-infected mice with no presence of bacteria. *Scale bar* represents 10 μm

Table 3 PCR detection of *P. gingivalis, T. denticola* and *T. forsythia* genomic DNA in infected B10.RIII mice tissues

Group	Polybacterial infection	Positive systemic tissue samples					
		Heart	Liver	Spleen	Kidney	Lung	Knee joint
		$n = 10$	$n = 10$	$n = 5$	$n = 10$	$n = 10$	$n = 10$
I	*Pg/Td/Tf*	4/3/1	2/3/1	0/0/0	5/4/2	2/0/0	0/0/0
II	*Pg/Td/Tf* + collagen	5/2/0	3/2/1	0/0/0	4/5/2	3/1/0	2/0/0
III	Collagen control	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0
IV	Sham infection	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0

After 24 weeks of gingival infection, heart, liver, spleen, kidney, lung, and hind limb ankle joint tissues were harvested from B10.RIII mice and extracted genomic DNA were subjected to 40 cycles of PCR analysis using species-specific primers for the three oral bacteria *P. gingivalis (Pg), T. denticola (Td), and T. forsythia (Tf)*. The numbers indicated with forward slash correspond to the number of mice positive for *Pg/Td/Tf* genomic DNA, respectively

(Table 3). Importantly, on FISH analysis we observed *P. gingivalis* in the perinuclear area of cells in infected ankle joint tissue (Fig. 4c (ii)). Taken together, our data demonstrate that oral infection with a PD-associated polybacterial consortium aggravates PD and CIA, and that *P. gingivalis* can be found in the inflamed joints of mice with bacterial infection-aggravated CIA, suggesting that *in situ* presence of such bacterial antigens might contribute to PD-associated arthritis.

Discussion

It has long been observed that patients with PD are at a higher risk of developing RA [3, 33], and patients with RA have increased likelihood of suffering from PD [34, 35]. However, the mechanistic basis of this association remains unclear. PD is triggered by bacterial gingival infection. Therefore, the realization that PD and RA share similar risk factors and pathological pathways, and the fact that effective PD treatment is associated with reduced severity of RA [36], prompted us to investigate the role of bacteria as a potential link between the two diseases. In contrast to other in vivo models of PD-associated arthritis, which are induced by infection with a single bacterial species, we studied the effect of polymicrobial dysbiotic bacterial interactions, involving a synergistic polybacterial infection. Our data show that this infection protocol required a shorter PD induction period and resulted in conclusive evidence of aggravation of CIA in B10.RIII mice. The protocol used here, involving chronic recurrent gingival infection with major periodontal bacteria, was highly efficient in adherence and colonization of infected mice with all three periodontal pathogens. This enhanced colonization was associated with a strong humoral immune response with production of IgG and IgM antibodies against the periodontal pathogens. Further, we observed significant alveolar bone resorption in polybacterial-infected mice, with or without concomitant CIA. Thus, the polybacterial colonization protocol described here produced synergistic induction of PD in B10.RIII mice.

Importantly, induction of PD by polybacterial infection facilitated CIA, as evidenced by earlier arthritis onset and a more severe arthritic process, including increased inflammatory cell infiltration and pannus formation. In addition, in vivo tomographic analysis using an MMP3 probe corroborated the induction of inflammation and enhanced severity of CIA in mice infected with polybacterial inocula compared to uninfected mice with CIA. Interestingly, the in vivo imaging data were corroborated by correspondingly increased MMP3 serum levels, consistent with our observation that polybacterial-infected mice with CIA developed more severe clinical arthritis.

Cytokines play a crucial role in the pathophysiology of RA as pro-inflammatory cytokines such as TNFα, IL-1, and IL-17 stimulate inflammation and degradation of bone and cartilage. Th17 lymphocytes and IL-17 have been recognized as essential mediators of cartilage and bone destruction. The number of Th17 cells is increased in the early stages of the disease and in active RA [37–39]. The mechanisms behind the factors promoting the Th17 differentiation in RA are poorly understood. With the significant role of pathogen-mediated TLR activation in shaping the T cell response, we reasoned that the interaction between PD and RA may in part be a direct result of skewing the Th17 cell balance. The aim of the current study was to investigate the influence of periodontitis on clinical severity and specific histopathologic features of T cell-dependent experimental arthritis. IL-17, in turn, is critical in stimulating the release of TNF-α and chemokines by joint tissues in arthritis [32].

Our IHC and immunofluorescence data supports the increased expression of IL-17 after infection with polymicrobial periodontal microflora followed by collagen administration. These observations support the hypothesis that IL-17 induces receptor activator of NF-*k*B ligand (RANKL) expression that is vital for osteoclastogenesis and bone resorption. The observations in the current study further authenticate the notion postulated by previous groups about the promotion of inflammation and the catabolic effects of IL-17 on cartilage and bone leading to the propagation of arthritis.

Finally, the identification of *P. gingivalis* in joint tissues of mice with CIA challenged with polybacterial inocula suggests that target-tissue-disseminated bacteria may contribute to the local inflammatory process, thereby augmenting

the severity of arthritis in these mice. As the degree of severity, incidence, and levels of inflammatory biomarkers were all increased in polybacterial-infected mice with CIA, it is tempting to speculate that these target-tissue-seeded bacteria may be at least partly responsible for the observed association between PD and arthritis. Tissue dissemination of oral pathogens has been implicated in atherosclerotic vascular disease [40], another PD-associated condition. In that condition, it has been proposed that periodontal bacteria invade the bloodstream after various manipulations such as dental procedures or tooth brushing. According to this proposed mechanism, such bacteria are deposited in the atheromatous plaque and contribute to disease pathogenesis. It is worth noting, however, that recovery of viable bacteria from atheroma tissue cultures has been difficult, suggesting that active plaque infection by live bacteria is unlikely to be the causative mechanism. Instead, bacterial antigen-triggered stimulation of biochemical pathways or the immune system seems a more likely explanation.

Analogous to atherosclerotic vascular disease, in RA it has long been speculated that the disease may be triggered by indolent joint tissue infection. These hypotheses have been largely refuted based on the failure to consistently recover viable microorganisms from synovial tissues; however the possibility that non-viable microbial antigens might contribute to the inflammatory process in arthritis has remained a plausible hypothesis [41, 42]. For example, based on close similarities between their respective signaling pathways, we have previously proposed that the known arthritogenic effect of the bacterial antigen muramyl dipeptide (MDP) might be due to its ability to functionally mimic the shared epitope ligand [43]. We have pointed out that MDP, a building block of the bacterial cell wall and a potent immune adjuvant, displays many functional similarities to the SE ligand, including production of IL-6, TNFα, nitric oxide, reactive oxygen species, activation of osteoclasts, Th17 polarization and, importantly, facilitation of inflammatory arthritis in rodents [6, 43–46]. Obviously, the definitive molecular mechanism by which synovial tissue-seeded *P. gingivalis* contribute to the severity of arthritis needs to be examined experimentally.

Conclusions

In summary, the findings reported herein suggest that oral bacteria play a significant role in augmenting autoimmune arthritis. Our observations support the two-hit model [47] whereby periodontopathic subgingival bacteria provide the first hit on the host immune system, leading to periodontal disease, while a second hit generated as a result of chronic inflammatory conditions in the host will eventually produce irreversible tissue damage leading to arthritis in the human host. As this model simulates the natural course of infection of oral pathogens with the naturally occurring periodontal disease in humans, it provides relevant insights into the pathogenesis of RA and could conceivably open the door to identification of new therapeutic strategies.

Additional file

Additional file 1: Figure S1. Periodontal infection induces active inflammation in polymicrobial infected + CII immunized mice. Polymicrobial-infected + CII immunized mice, *left* metatarsal tissue H&E staining showing active inflammation with infiltration of neutrophils and macrophages. **A** × 1 magnification, **B** × 2 magnification, **C** × 10 magnification (*top panel*). *I*, *II*, *III*, *IV*, and *V* are digits. CII-immunized mice metatarsal tissue H&E staining showing minimal inflammation (**A** × 1, B × 2, and **C** × 10 magnification (*bottom panel*)). *N* = 6 in each group. (TIF 2050 kb)

Abbreviations

ABR, alveolar bone resorption; CII, collagen type II; CFA, complete Freund's adjuvant; CIA, collagen-induced arthritis; CMC, carboxymethylcellulose; DAPI, 4′ 6-diamidino-2-phenylindole; ELISA, enzyme linked immunosorbent assay; FISH, fluorescence *in situ* hybridization; H&E, hematoxylin and eosin; IFA, incomplete Freund's adjuvant; IgG, immunoglobulin G; IgM, immunoglobulin M; IHC, immunohistochemistry; IL17, interleukin-17; MDP, muramyl dipeptide; MMP3, matrix metalloproteinase 3; PCR, polymerase chain reaction; PD, periodontal disease; *Pg*, *P. gingivalis*; RA, rheumatoid arthritis; RANKL, receptor activator of NF-kB ligand; SEM, standard error of the mean; *Td*, *T. denticola*; *Tf*, *T. forsythia*; Th, T helper; TNF-α, tumor necrosis factor-alpha

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Authors' contributions

SC, IB, MS, EKLC, JH, and LK were involved in drafting the article, or revising it critically (LK, SC, JH) for important intellectual content. All authors approved the final version to be published. Dr. LK had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design: LK, SC, MS, and EKLC. Acquisition of data: SC, Rivera-Kweh, Gehlot, Velsko, IB, Calise, MS, EKLC, and LK. Analysis and interpretation of data: SC, Rivera-Kweh, Gehlot, Velsko, IB, MS, EKLC, JH, and LK.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All mouse procedures were performed in accordance with the guidelines and the protocol (# 5673) approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Florida. The University of Florida has an assurance with the Office of Laboratory Animal Welfare (OLAW) and adheres to U.S. Public Health Services (PHS) policy, the Animal Welfare Act and Animal Welfare Regulations, and the Guide for the Care and Use of Laboratory Animals. The University of Florida is accredited by the AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care).

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Ethnic Differences in Autoantibody Diversity and Hierarchy: More Clues from a US Cohort of Patients with Systemic Sclerosis

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Ethnic Differences in Autoantibody Diversity and Hierarchy: More Clues from a US Cohort of Patients with Systemic Sclerosis

Sarada L. Nandiwada, Lisa K. Peterson, Maureen D. Mayes, Troy D. Jaskowski, Elisabeth Malmberg, Shervin Assassi, Minoru Satoh, and Anne E. Tebo

ABSTRACT. ***Objective.*** To determine the autoantibody repertoire and clinical associations in a multiethnic cohort of American patients with systemic sclerosis (SSc).

Methods. There were 1000 patients with SSc (196 Hispanic, 228 African American, 555 white, and 21 other) who were screened for antinuclear antibodies (ANA), including anticentromere antibodies (ACA) by indirect immunofluorescence assay, antitopoisomerase-1 (topo-1/Scl-70) by immunodiffusion, and anti-RNA polymerase III (RNAP III) by ELISA. Sera from 160 patients with mainly nucleolar and/or speckled ANA pattern, but negative for ACA, Scl-70, and RNAP III, were further characterized by immunoprecipitation for SSc-specific antibodies.

Results. The prevalence of antibodies against RNAP III, Th/To, and PM/Scl did not differ significantly among the ethnic groups. The frequency of anti-Scl-70 was lowest in whites (18.0%) compared with 24.0% and 26.8% in Hispanics and African Americans ($p = 0.01$), respectively. Compared with African American patients, Hispanic and white subjects had a higher frequency of ACA ($p < 0.0001$) and lower frequency of U3-RNP ($p < 0.0001$). U3-RNP antibodies were uniquely higher in African American patients, independent of clinical subset, while Th/To autoantibodies were associated with limited cutaneous SSc in white subjects. Overall, Hispanic and African American patients had an earlier age of onset and a predominance of diffuse cutaneous SSc compared with their white counterparts.

Conclusion. SSc-specific antibodies may predict disease subset; however, the hierarchy of their prevalence differs across ethnic groups. This study provides the most extensive analysis to date on the relevance of autoantibodies in the diagnosis and clinical manifestations of SSc in Hispanic American patients. (First Release August 1 2016; J Rheumatol 2016;43:1816–24; doi:10.3899/jrheum.160106)

Key Indexing Terms:
SYSTEMIC SCLEROSIS AUTOANTIBODIES ANTINUCLEAR ANTIBODIES

Systemic sclerosis (SSc) is characterized by fibrosis of skin and diverse internal organs, vascular damage, and altered immune functions. It is a chronic and potentially life-threatening disease. Based on the extent of skin involvement, the disease can be classified into diffuse cutaneous SSc (dcSSc) and limited cutaneous SSc (lcSSc)[1]. lcSSc is characterized by fibrosis of the skin confined to the distal extremities, a lower incidence of renal involvement, and restrictive pulmonary disease with a better prognosis[1]. In contrast, dcSSc is characterized by skin fibrosis involving the proximal extremities and/or the trunk, in addition to acral skin thickening, and has a poorer prognosis[1,2,3].

The immunopathology associated with SSc involves multiple compartments of the immune system, including T

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cells, B cells, and cytokines/chemokines, with fibroblasts and endothelial cells as both effectors and targets[4]. Abnormal activation of the immune system is reflected by the presence of circulating, essentially mutually exclusive SSc-specific antinuclear antibodies (ANA) with striking associations between autoantibody specificities and clinical phenotypes[4,5,6,7,8]. SSc autoantibodies target both nuclear and nucleolar proteins, and certain specificities have diagnostic and predictive relevance[7,8,9,10]. Of the ANA, centromere (ACA), topoisomerase-I (topo-I/Scl-70), RNA polymerase III (RNAP III), U3-RNP (fibrillarin), PM/Scl, and Th/To constitute ~80% of SSc-specific autoantibodies[5,6,7]. Autoantibodies less specific for SSc, targeting the nucleolar organizing region 90/human upstream-binding factor (NOR90/hUBF), U1RNP, Ro52, and Su/Argonaute2 (Ago2) antigens, have been described as well[5,7,9]. Currently, only ACA, anti-Scl-70, and anti-RNAP III are included in the 2013 classification guidelines for SSc[10].

The presence of specific antibodies has been associated with lcSSc and dcSSc, as well as certain SSc clinical manifestations. ACA are mainly associated with lcSSc with more favorable prognosis[11,12,13,14]. Antinucleolar antibodies (ANoA) such as anti-Th/To and anti-PM/Scl are also associated with lcSSc[15,16], whereas anti-Scl-70 and RNAP III and ANoA that react with U3-RNP are associated with dcSSc and poorer prognosis[7,11,12,16,17,18]. Patients with anti-RNAP III usually have dcSSc, with an increased risk of renal crisis and a shorter survival time[16,17,18]. At disease onset, dcSSc is more prevalent with poorer outcomes in African Americans than white Americans[19,20,21]. These associations correlate with higher incidences of anti-Scl-70, anti-U3-RNP, and anti-RNAP III compared with ACA in individuals of African American descent relative to their white and Hispanic counterparts[16,20,21,22]. Thus, specific ANA have diagnostic, prognostic, and predictive relevance in the evaluation and management of SSc and tend to cluster within particular ethnic and/or racial groups[16,23,24].

Our study examined the presence of SSc-associated autoantibodies in a large US multiethnic cohort of patients with SSc and associations with clinical subsets and/or certain manifestations in Hispanic subjects compared with their white and African American counterparts.

MATERIALS AND METHODS

Patient population. Patients enrolled in the Scleroderma Family Registry and DNA Repository were characterized clinically based on the extent of skin involvement, age at disease onset, disease duration at blood draw, sex, and ethnicity. All patients with SSc either fulfilled the 1980 American College of Rheumatology classification criteria for SSc[25] or had at least 3 of the 5 features of CREST syndrome (calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, telangiectasias)[26]. Patients with SSc were further characterized as having lcSSc or dcSSc based on their skin involvement[1]. Disease duration was measured from the onset of the first non-Raynaud phenomenon symptom attributed to SSc. Clinical information regarding renal crisis was available for 183 of the patients. Ethnic categories were determined by self-report and defined as follows: African American (non-Hispanic African American), white (non-Hispanic white), Hispanic, and other (Hispanic African American, American Indian/Alaskan Native, more than 1 race, and other). The institutional review boards at the University of Utah School of Medicine, Salt Lake City, and the University of Texas Health Science Center at Houston approved our study. Written informed consent was obtained from all subjects at the University of Texas Health Science Center at Houston according to the Declaration of Helsinki.

Detection of autoantibodies. Sera from a multiracial cohort of 1000 patients with SSc (Scleroderma Family Registry and DNA Repository) were screened for immunoglobulin ANA by indirect fluorescent assay (IFA; Inova Diagnostics Inc.) with specific patterns identified if present [centromere (ACA), nuclear speckled, and nucleolar]. All ANA-positive and a subset of ANA-negative samples were screened for anti-Scl-70 by immunodiffusion (Inova Diagnostics Inc.) and anti-RNAP III by ELISA (MBL Co. Ltd.; Figure 1). From 346 ANA-positive patients negative for ACA, anti-Scl-70, and anti-RNAP III, 160 sera [ANoA alone (n = 142) or ANoA and nuclear speckled patterns (n = 18)] were tested for autoantibody characterization by radioimmunoprecipitation (IP) assay. Autoantibodies detected by IP included anti-Scl-70, anti-RNAP I/III, anti-U3-RNP, anti-Th/To, anti-PM/Scl, anti-NOR90, and anti-Su/Argonaute2. Unlike the anti-RNAP III ELISA, the IP assay allows classification of RNAP antibody types (I, II, III) based on the presence of characteristic bands allowing for differentiation. Antibodies to RNAP I and III coexist in most cases and are specific for SSc; specimens with these autoantibodies are reported as RNAP III.

Statistical analysis. Significance of associations between sets of categorical data was determined using a chi-square test or a Fisher's exact test when expected values were low. The ANOVA test was used to determine the difference between continuous variables (mean ages or mean disease duration in yrs) in the different subset classifications for the markers with pairwise comparisons performed posthoc on groups that showed significance with a Tukey test. To determine the predictive power of specific markers, a logistic regression analysis was performed in the model using the presence or absence of specific autoantibodies. All statistical analyses were performed using SAS 9.4 (SAS Institute) or Prism 5.0 (GraphPad Software). Differences were considered statistically significant for p values < 0.05.

RESULTS

Demographics and characteristics of the study cohort. Table 1 summarizes the demographic and clinical information for the study cohort. The mean age at disease onset differed significantly among ethnicities: Hispanic and African American patients had a similar age of onset (40.2 ± 14.0 yrs vs 40.6 ± 13.0 yrs) that was significantly earlier than that observed in the white subjects (44.4 ± 14.1 yrs, $p < 0.01$). There was no difference in disease duration at the time of blood draw between the Hispanic and African American patient groups (6.7 ± 6.9 yrs vs 6.6 ± 6.2 yrs). Disease duration at the time of blood draw was significantly longer in whites (9.2 ± 9.0 yrs) compared with Hispanic ($p < 0.01$) and African American patients ($p < 0.05$; Table 1). Frequencies of female and male patients were comparable in all ethnic groups with more women than men, irrespective of ethnicity.

Hispanic (57.2%) or African American patients (66.2%) had a significantly higher prevalence of dcSSc ($p < 0.0001$) compared with whites (38.0%). Conversely, the prevalence of lcSSc was significantly higher in the white group (61.4%) compared with the Hispanic (41.3%) or African American cohort (32.0%, $p < 0.0001$; Table 1). Of the documented patients with renal crisis (n = 180), African Americans had the highest frequency (13/53, 24.5%), followed by Hispanics (9/43, 20.9%) and whites (13/84, 15.5%).

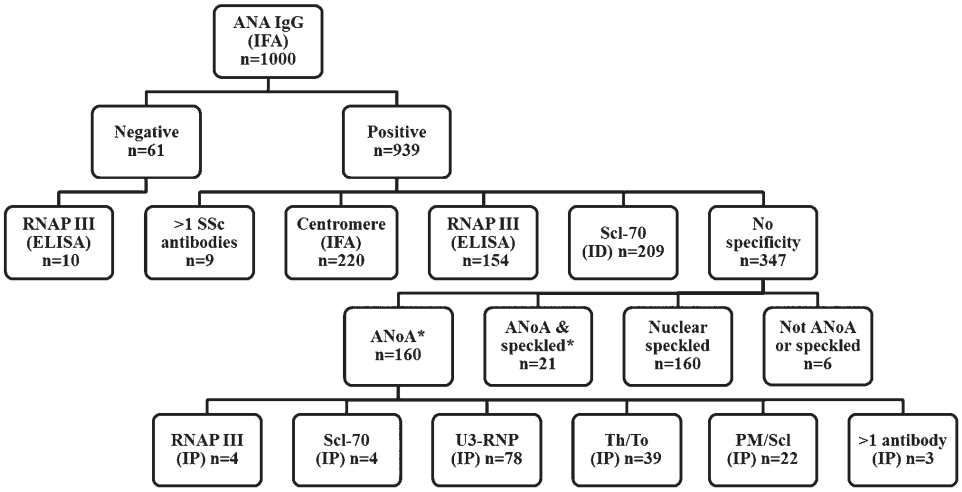

Figure 1. Algorithm for identifying autoantibodies in patients with SSc. Sera from 1000 patients with SSc were screened for ANA by IFA with specific patterns identified if present (centromere, nuclear speckled, and nucleolar). All ANA+ and a subset of ANA– samples were screened for anti-Scl-70 by ID and RNAP by ELISA. From 346 ANA+ patients negative for ACA, anti-Scl-70, and anti-RNAP III, 160 sera were selected for autoantibody characterization by IP assay. * The 142 patients with an ANoA IFA pattern and 18 patients with an ANoA and nuclear speckled IFA pattern were tested by IP. ANA: antinuclear antibodies; IgG: immunoglobulin G; RNAP III: RNA polymerase III antibodies; SSc: systemic sclerosis; IFA: indirect fluorescent assay; ID: immunodiffusion; ANoA: antinucleolar antibodies; IP: radioimmunoprecipitation.

Table 1. Demographic characteristics of study cohort (n = 1000). Values are % (n) unless otherwise specified.

Characteristics	African American, n = 228	White, n = 555	Hispanic, n = 196	Other, n = 21	p
Age at disease onset, yrs, mean ± SD	40.6 ± 13.0[1]	44.4 ± 14.1[1,4]	40.2 ± 14.0[4]	40.9 ± 13.6	0.0002
Disease duration, yrs, mean ± SD	6.6 ± 6.2[2]	9.2 ± 9.0[2,4]	6.7 ± 6.9[4]	6.6 ± 5.6	< 0.0001
Female, n = 828	84.7 (193)	83.1 (461)	85.7 (168)	76.2 (16)	NS
Male, n = 162	15.3 (35)	16.9 (94)	14.3 (28)	23.8 (5)	NS
dcSSc, n = 483	66.2 (151)[3]	38.0 (211)[3,5]	57.2 (112)[5]	42.9 (9)	< 0.0001
lcSSc, n = 507	32.0 (73)[3]	61.4 (341)[3,5]	41.3 (81)[5]	57.1 (12)	< 0.0001
Unspecified, n = 10	1.8 (4)	0.5 (3)	1.5 (3)	0.0 (0)	NS

African American versus white: [1] $p < 0.01$ (age at disease onset), [2] $p < 0.05$ (disease duration), and [3] $p < 0.0001$ (diffuse, limited). African American versus Hispanic: NS for all categories. White versus Hispanic: [4] $p < 0.01$ (age at disease onset, disease duration) and [5] $p < 0.0001$ (diffuse, limited). SSc: systemic sclerosis; dcSSc: diffuse cutaneous SSc; lcSSc: limited cutaneous SSc; NS: not significant.

Autoantibody profiling in patients with SSc: an extended approach. The majority of the patients in our study were ANA-positive (93.9%); 60.3% (603/1000) of the patients were identified as ACA-, Scl-70–, or RNAP III antibody-positive (Figure 1). There were 593 patients who had a single SSc-specific antibody, 10 of which had more than 1 autoantibody specificity (1 with ACA and anti-Scl-70, 4 with ACA and anti-RNAP III, and 5 with anti-Scl-70 and anti-RNAP III). Interestingly, 10 samples that were ANA-negative by IFA were found to be anti-RNAP III-positive by ELISA. Of the remaining 346 ANA-positive samples with no defined autoantibody specificity (346/939, 37.1%), 160 had an ANoA pattern, 158 had a nuclear speckled pattern, 22 were positive for both patterns, and 6 were negative for both patterns. Of the patients with no defined autoantibody specificities, 142 cases with ANoA only and 18 with ANoA and speckled patterns were further characterized by IP.

IP identified autoantibodies in 152 of 160 patients with SSc with an ANoA IFA pattern (Figure 1); 149 had SSc-specific antibodies and 3 had non-SSc–specific autoantibodies. SSc-specific antibodies identified by IP included U3-RNP (78/160, 48.8%), Th/To (39/160, 24.4%), PM/Scl (22/160, 13.8%), Scl-70 (4/160, 2.5%), and RNAP III (4/160, 2.5%; Figure 1). Two patients with dcSSc had dual-positive SSc-specific antibodies [an African American patient (U3-RNP and Th/To) and a Hispanic patient (U3-RNP and PM/Scl)]. Of the 3 patients with autoantibodies not specific for SSc, 1 had anti-NOR90, another anti-NOR90 and anti-Su, and the third only had anti-Su (data not shown). Interestingly, in the patients with both an ANoA and a speckled pattern, there was a higher frequency of antibodies against Th/To

(6/18, 33.3%), PM/Scl (4/18, 22.2%), Scl-70 (3/18, 16.7%), and NOR90 (1/18, 5.6%) detected by IP compared with patients with a purely nucleolar pattern. Eight of the ANoA-positive patients had no identifiable autoantibodies (4 had only an ANoA pattern and 4 had an ANoA and speckled pattern).

For all of the autoantibodies detected, women represented the majority of the positive patients, which may be skewed owing to the composition of the cohort (data not shown). The mean age at disease onset differed significantly among autoantibodies, with anti-U3-RNP associated with the earliest age at onset. Anti-U3-RNP (35.6 ± 14.6 yrs) and anti-Scl-70 (40.3 ± 14.4 yrs) were associated with a significantly earlier age of onset compared with ACA (44.7 ± 14.9 yrs; $p < 0.0001$ and $p = 0.0009$, respectively) and RNAP III (45.8 ± 12.6 yrs; $p < 0.0001$ and $p = 0.0001$, respectively). Patients with ACA (12.5 ± 10.7 yrs) had a significantly longer duration of disease compared with patients with anti-Scl-70 (7.6 ± 8.7 yrs, $p < 0.0001$), anti-RNAP III (6.3 ± 7.5 yrs, $p < 0.0001$), and anti-U3-RNP (5.9 ± 6.4 yrs, $p < 0.0001$). ACA (88.1%) and anti-Th/To (84.6%) were found at higher frequencies in patients with lcSSc compared with those with dcSSc. In contrast, anti-Scl-70 (63.4%), anti-RNAP III (78.6%), and anti-U3-RNP (78.2%) were more prevalent in patients with dcSSc than those with lcSSc, which was consistent with published findings[7,11,12,13,14,15,16,17,18]. Information about kidney involvement was only available for 18.3% of the cohort. However, anti-RNAP III (37.5%) was detected at a significantly higher frequency than ACA (3.2%, $p = 0.0003$) and anti-Scl-70 (8.8%, $p = 0.0042$) in patients with renal crisis. Anti-U3-RNP (33.3%) was also present at a higher frequency compared with anti-Scl-70 (8.8%, $p = 0.0466$) in patients with renal crisis.

Autoantibody hierarchy is dependent on ethnicity. Of the SSc-associated antibodies, ACA, Scl-70, and RNAP III have been shown to be the most common, are routinely available for testing, and are included in the 2013 classification criteria for SSc[9,10]. In our cohort, ACA, Scl-70, and RNAP III made up 79.9% of the antibodies detected (Table 2). These 3 antibodies represented significantly less of the autoantibodies detected in African American (63.6%) compared with both white (86.0%, $p = 0.0003$) and Hispanic patients (82.8%, $p = 0.0023$). ACA was significantly more prevalent in individuals of white and Hispanic descent, while anti-Scl-70 had the highest frequency in African American patients (Table 2). There was no significant difference in the prevalence of anti-RNAP III among ethnic groups. Although these 3 specificities represented the majority of SSc-specific autoantibodies in all ethnic groups, the hierarchies of these antibodies differ among racial groups (Table 2, Figure 2).

In the Hispanic group, anti-Scl-70 (24.0%), anti-RNAP III (20.9%), and ACA (19.4%) were the most prevalent autoantibodies and were detected at similar frequencies (Table 2). Anti-U3-RNP, anti-Th/To, and anti-PM/Scl were uncommon in Hispanic patients. However, the prevalence of anti-U3-RNP was significantly higher in Hispanic patients compared with white patients (6.1% vs 1.8%, $p < 0.0001$). In African American patients, anti-Scl-70 (26.8%) was the most prevalent autoantibody specificity included in the current SSc diagnostic criteria, followed by anti-RNAP III (14.0%) and ACA (6.6%), with each of these comparisons achieving statistical significance (ACA vs Scl-70, $p < 0.0001$; ACA vs RNAP III, $p = 0.0112$; Scl-70 vs RNAP III, $p = 0.0006$; Table 2). Interestingly, anti-U3-RNP (22.8%) was the second most prevalent autoantibody overall in African American patients. Anti-Scl-70, anti-U3-RNP, and anti-RNAP III made up 88% of the identified SSc-specific autoantibody repertoire in African American individuals. Compared with white and Hispanic patients with SSc, ACA, anti-Th/To, and anti-PM/Scl were uncommon in African American patients.

Table 2. Frequency of SSc-specific autoantibodies in the different ethnic groups. Values are % (n) unless otherwise specified.

Autoantibody	Entire Cohort, n = 1000	African American, n = 228	White, n = 555	Hispanic, n = 196	Other*, n = 21	p
ACA	22.0 (220)	6.6 (15)[1,3]	29.2 (162)[1,6]	19.4 (38)[3,6]	23.8 (5)	< 0.0001
Scl-70**	21.3 (213)	26.8 (61)[2]	18.0 (100)[2]	24.0 (47)	23.8 (5)	0.04
RNAP III**	16.8 (168)	14.0 (32)	16.6 (92)	20.9 (41)	14.3 (3)	NS
U3-RNP	7.8 (78)	22.8 (52)[1,4]	1.8 (10)[1,5]	6.1 (12) [4,5]	19.1 (4)	< 0.0001
Th/To	3.9 (39)	1.8 (4)	4.5 (25)	4.0 (8)	9.5 (2)	NS
PM/Scl	2.2 (22)	0.9 (2)	2.9 (16)	2.0 (4)	0 (0)	NS
More than 1	1.2 (12)	0.9 (2)	1.1 (6)	2.0 (4)	0 (0)	NS
Undefined	19.7 (197)	21.9 (50)	20.0 (111)	17.9 (35)	4.8 (1)	NS
ANA–	6.1 (61)***	5.7 (13)***	6.7 (37)***	5.1 (10)***	4.8 (1)	NS

* Excluded in statistical analyses. ** Includes 4 samples that were negative by immunodiffusion or ELISA, but positive by immunoprecipitation. *** There are 10 overlapping RNAP III and ANA– samples (3 African American, 4 white, and 3 Hispanic). African American versus white: [1] $p < 0.0001$ (ACA and U3-RNP), [2] $p = 0.008$ (Scl-70). African American versus Hispanic: [3] $p < 0.0001$ (ACA), [4] $p = 0.033$ (U3-RNP). White versus Hispanic: [5] $p < 0.0001$ (U3-RNP), [6] $p = 0.007$ (ACA). No other significant differences in antibody prevalence were detected between any 2 ethnic groups. ANA+ samples negative for ACA, Scl-70, RNAP III, U3-RNP, Th/To, and PM/Scl were classified as undefined. SSc: systemic sclerosis; ACA: anticentromere antibodies; RNAP III: RNA polymerase III antibodies; ANA: antinuclear antibodies; NS: not significant.

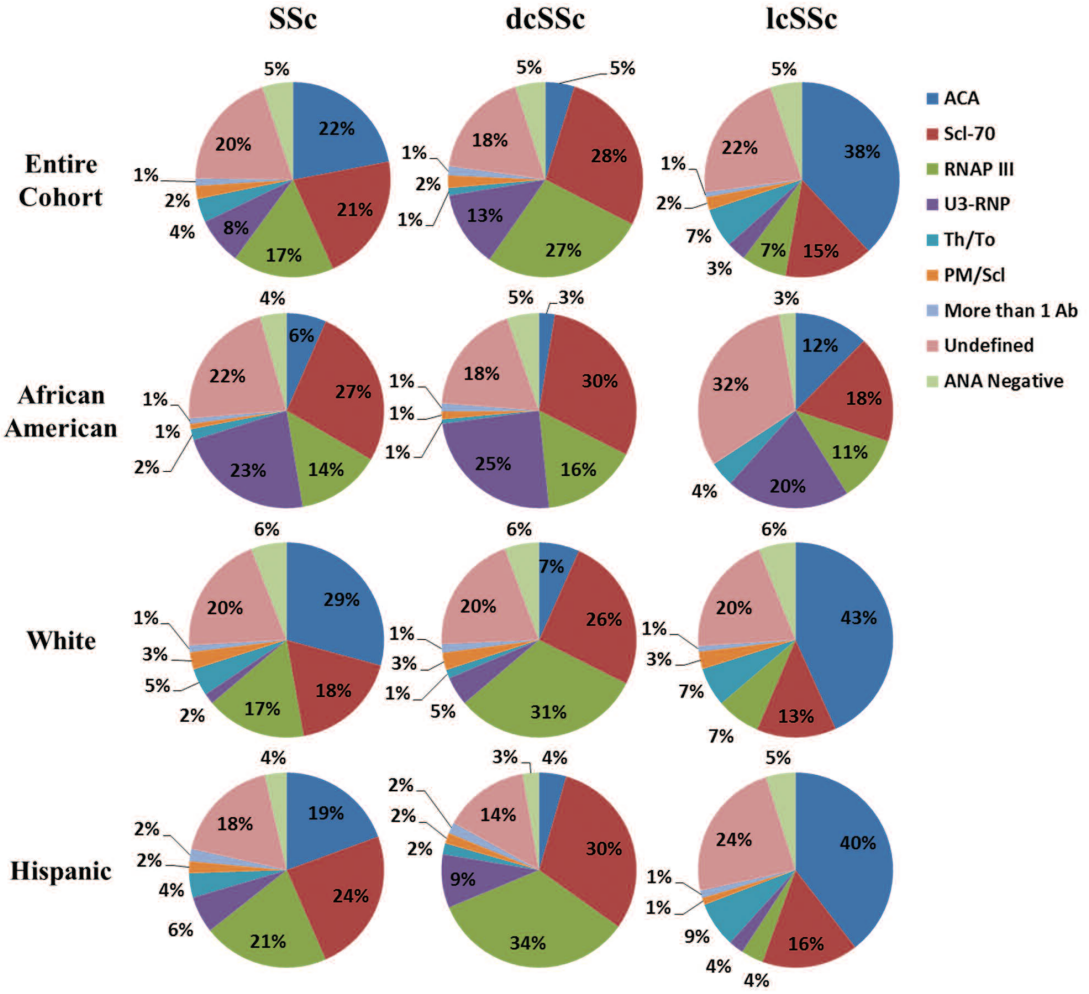

Figure 2. Autoantibody hierarchy in SSc is dependent on ethnicity. Pie charts show positivity rates for defined autoantibodies, undefined ANA specificities, or ANA– results in the entire SSc cohort, patients with dcSSc, or patients with lcSSc based on ethnicity. SSc: systemic sclerosis; dcSSc: diffuse cutaneous SSc; lcSSc: limited cutaneous SSc; ACA: anticentromere antibodies; RNAP III: RNA polymerase III antibodies; Ab: antibody; ANA: antinuclear antibodies.

In white patients, ACA (29.2%) was present at a significantly higher frequency than anti-Scl-70 (18.0%) and anti-RNAP III (16.6%, both $p < 0.0001$; Table 2), whereas anti-Scl-70 and anti-RNAP III did not differ in prevalence. Relative to all other ethnic groups, anti-U3-RNP was uncommon in white patients. Although significantly less common than the SSc antibodies included in the criteria, anti-Th/To and anti-PM/Scl were detected in white (4.5% and 2.9%, respectively), Hispanic (4.0% and 2.0%, respectively), and African American patients with SSc (1.8% and 0.9%, respectively). The prevalence of ANA-negative or -positive cases with no identifiable SSc-specific autoantibodies using standard assays as well as the IP method was similar (~20%) across the 3 ethnic groups.

Associations between autoantibodies, clinical subset, and ethnicity in patients with SSc. To determine the involvement of antibodies in SSc stratification by ethnicity, the prevalence of all identified autoantibodies in the 2 main clinical subsets were analyzed. Comparison of antibody frequencies between ethnic groups for patients with dcSSc demonstrated that anti-RNAP III and anti-U3-RNP had significantly different prevalences based on ethnicity (Table 3). Anti-RNAP III occurred in higher frequencies in Hispanic (33.9%) and white patients with dcSSc (31.4%) compared with individuals of African American descent (15.9%, $p = 0.0002$). While the Hispanic and white cohorts had comparable prevalence of anti-RNAP III, there were significant differences between the African American and Hispanic groups, as well as African American and white ($p = 0.0011$ and $p = 0.0008$, respectively). Anti-U3-RNP was unique in that it was present in a higher frequency in African American (24.5%) relative to Hispanic (8.9%, $p = 0.0011$) and white patients with dcSSc (4.8%, $p < 0.0001$). No significant difference in prevalence was noted for the other autoantibodies based on ethnicity in our investigation.

In patients with lcSSc, only ACA and anti-U3-RNP had

Table 3. Correlation between specific SSc autoantibodies and clinical subsets within race. Results for patients with a defined subset of SSc are shown. Of the 1000 patients with SSc, 10 were unspecified for dsSSc or lcSSc (4 African American, 3 white, and 3 Hispanic patients). ANA+ samples negative for ACA, Scl-70, RNAP III, U3-RNP, Th/To, and PM/Scl were classified as undefined. Values are % (n) unless otherwise specified.

Race	Autoantibody	Disease Subsets		p
African American, n = 224		dcSSc, n = 151	lcSSc, n = 73	
	ACA, n = 13	2.6 (4)	12.3 (9)	0.011
	Scl-70, n = 58	29.8 (45)	17.8 (13)	NS
	RNAP III, n = 32	15.9 (24)	11.0 (8)	NS
	U3-RNP, n = 52	24.5 (37)	20.5 (15)	NS
	Th/To, n = 4	0.7 (1)	4.1 (3)	NS
	PM/Scl, n = 2	1.3 (2)	0.0 (0)	NS
	More than 1, n = 2	1.3 (2)	0.0 (0)	NS
	Undefined, n = 51	18.5 (28)	31.5 (23)	0.041
	ANA–, n = 13*	7.3 (11)*	2.7 (2)	NS
White, n = 552		dcSSc, n = 210	lcSSc, n = 342	
	ACA, n = 162	6.7 (14)	43.2 (148)	< 0.0001
	Scl-70, n = 99	25.7 (54)	13.2 (45)	0.0002
	RNAP III, n = 91	31.4 (66)	7.3 (25)	< 0.0001
	U3-RNP, n = 10	4.8 (10)	0.0 (0)	< 0.0001
	Th/To, n = 25	1.4 (3)	6.4 (22)	0.005
	PM/Scl, n = 16	2.9 (6)	2.9 (10)	NS
	More than 1, n = 6	1.4 (3)	0.9 (3)	NS
	Undefined, n = 110	20.0 (42)	19.9 (68)	NS
	ANA–, n = 37*	6.7 (14)*	6.7 (23)*	NS
Hispanic, n = 193		dcSSc, n = 112	lcSSc, n = 81	
	ACA, n = 37	4.5 (5)	39.5 (32)	< 0.0001
	Scl-70, n = 47	30.4 (34)	16.0 (13)	0.0271
	RNAP III, n = 41	33.9 (38)	3.7 (3)	< 0.0001
	U3-RNP, n = 12	8.9 (10)	2.5 (2)	NS
	Th/To, n = 8	1.8 (2)	7.5 (6)	NS
	PM/Scl, n = 3	1.8 (2)	1.2 (1)	NS
	More than 1, n = 3	1.8 (2)	1.2 (1)	NS
	Undefined, n = 35	14.3 (16)	23.5 (19)	NS
	ANA–, n = 10*	5.4 (6)*	4.9 (4)	NS
Other, n = 21		dcSSc, n = 9	lcSSc, n = 12	
	ACA, n = 5	0.0 (0)	41.7 (5)	0.045
	Scl-70, n = 5	11.1 (1)	33.3 (4)	NS
	RNAP III, n = 3	33.3 (3)	0 (0)	NS
	U3-RNP, n = 4	44.4 (4)	0 (0)	0.021
	Th/To, n = 2	0.0 (0)	16.7 (2)	NS
	PM/Scl, n = 0	0.0 (0)	0 (0)	NA
	More than 1, n = 0	0.0 (0)	0 (0)	NA
	Undefined, n = 1	0.0 (0)	8.33 (1)	NS
	ANA–, n = 1	11.1 (1)	0 (0)	NS

* There are 10 overlapping RNAP III and ANA– samples (3 African Americans with dcSSc, 2 whites with dcSSc, 2 whites with lcSSc, and 3 Hispanics with dcSSc). SSc: systemic sclerosis; dcSSc: diffuse cutaneous SSc; lcSSc: limited cutaneous SSc; ANA: antinuclear antibodies; ACA: anticentromere antibodies; RNAP III: RNA polymerase III antibodies; NS: not significant; NA: not applicable.

significant differences in frequency based on ethnicity. African American patients (12.3%) had a significantly lower prevalence of ACA compared with their Hispanic (39.5%, p = 0.0002) or white counterparts (43.2%, p = 0.0002). Similar to what was observed for dcSSc, the prevalence of anti-U3-RNP was higher in African American (20.5%) compared with Hispanic (2.5%, p = 0.0004) and white patients with lcSSc (0%, p < 0.0001). Although limited by sample size, more Hispanic patients with lcSSc were positive for anti-U3-RNP relative to white patients (p = 0.0363). In addition to the prevalence of specific autoantibodies based on ethnicity, the associations of these antibodies between clinical subsets in each ethnic cohort were also evaluated and compared (Table 3). Anti-Scl-70 and anti-RNAP III were more prevalent in patients with dcSSc for all ethnic groups, but this association only reached statistical significance for Hispanic (p = 0.0271 and p < 0.0001, respectively) and white patients (p = 0.0002 and p < 0.0001, respectively). In the white group, anti-U3-RNP was detected exclusively in patients with dcSSc (p < 0.0001), while anti-Th/To was

significantly more frequent in the lcSSc subset (p = 0.005). Similar trends were observed in the Hispanic group, but were not statistically significant. Anti-U3-RNP was more prevalent in African American than in Hispanic or white patients, and was associated with patients with dcSSc in general. In African American patients, the frequency of anti-U3-RNP was similar in dcSSc and lcSSc. No significant difference in prevalence of anti-PM/Scl was found between disease subsets for any ethnic group. Thus, in addition to the antibody hierarchies differing between the 3 ethnic groups, they also differed based on disease type within each ethnic group (Figure 2).

Regression analysis of the data showed that the presence of ACA, anti-Scl-70, anti-RNAP III, and anti-U3-RNP were significant for predicting disease type ($p < 0.0001$ for each autoantibody) and age of onset ($p = 0.0033$, $p = 0.0394$, $p = 0.0003$, and $p < 0.0001$, respectively) in patients with SSc, independent of ethnicity (Table 4). Anti-Th/To was significant for predicting disease type ($p = 0.0002$), but not age of onset. In addition, anti-RNAP III was found to be predictive of renal crisis ($p = 0.0012$). When the interaction between antibody and race was factored into the analysis, only ACA ($p < 0.0001$) and anti-Th/To ($p = 0.0068$) retained their significance for predicting disease type (data not shown).

DISCUSSION

The presence of specific autoantibodies associated with ANA is of diagnostic relevance in SSc, and may help predict the likelihood of certain clinical manifestations, allowing for patient risk stratification and optimal disease management. Further, certain SSc autoantibodies tend to be associated with ethnicity, and correlate with manifestations involving frequency and severity of lung and renal involvement, as well as dcSSc or lcSSc. Most of these observations are based on studies composed primarily of white, African American, or Japanese patients, with limited information on Hispanic American SSc populations. In our study, we report unique differences in the frequencies, hierarchies, and associations of specific autoantibodies in the 3 US ethnic groups, including a significant number of Hispanic American patients with SSc.

Table 4. Predictive power of the antibodies to determine disease status, renal crisis, or age at disease onset in cohort by regression analysis.

Autoantibody	Disease Subset	Renal Crisis	Age at Disease Onset, Yrs
ACA	< 0.0001	NS	0.0033
Scl-70	< 0.0001	NS	0.0394
RNAP III	< 0.0001	0.0012	0.0003
U3-RNP	< 0.0001	NS	< 0.0001
Th/To	0.0002	NS	NS
PM/Scl	NS	NS	NS
More than 1	NS	NS	NS
Undefined	NS	NS	NS
ANA–	NS	NS	NS

ACA: anticentromere antibodies; RNAP III: RNA polymerase III antibodies; ANA: antinuclear antibodies; NS: not significant.

Studies that have included a sufficient number of Hispanic American patients with SSc have not presented an analysis of autoantibody profiles regarding race or ethnicity[27,28,29]. The Genes versus Environment in Scleroderma Outcomes Study (GENISOS) included 77 Hispanic patients (29% of cohort), but evaluated only 3 antibody markers (ACA, Scl-70, and U3-RNP)[19]. In a study of Mexican Mestizo patients with SSc, a higher frequency of ACA, anti-PM/Scl, anti-Ku, and anti-Scl-70 were observed compared with African American and/or white subjects with a lower prevalence of anti-RNAP III relative to the other ethnicities investigated[30]. Differences between the results from the Mexican Mestizo SSc cohort and data presented in our study can likely be explained by the heterogeneity within the Hispanic populations, as well as environmental factors[31]. Arnett, *et al* reported that the HLA class II alleles and haplotypes that showed the strongest association with SSc in Hispanic patients were also associated with SSc in white patients[27], which could explain some of the similarity in autoantibody profiles between these groups.

The relevance of autoantibody testing in the evaluation of SSc cannot be overemphasized. Indeed, the majority of the patients in our cohort were ANA-positive, with a large proportion identified as ACA, Scl-70, or RNAP III antibody-positive. Although significant differences in the prevalence of ACA were observed among ethnic groups, the high frequency in white subjects and overall association with lcSSc within the different categories in our study is consistent with previous reports[16,19,20]. No ethnic differences were observed in the prevalence of anti-Scl-70 in the lcSSc or dcSSc clinical subset. However, the frequency of anti-Scl-70 appears to be highest in African American patients with SSc, irrespective of clinical subset relative to other ethnic groups investigated. Previous studies have shown that a significant proportion of anti-Scl-70–positive African American patients had dcSSc (71% and 87%)[19,23]. In our cohort, there was a trend toward a higher prevalence of anti-Scl-70 in African American patients with dcSSc, but these associations did not reach statistical significance. This may be due to the sample size of antibody-positive patients (n = 61) or to other characteristics of our cohort. In the case of anti-RNAP III, white and Hispanic patients with dcSSc had significantly higher frequencies compared with their African American counterparts. A very limited number of African American patients were positive for anti-RNAP III, likely contributing to the lack of association with dcSSc compared with lcSSc. Increased occurrence of anti-U3-RNP in African American relative to white or Hispanic patients was previously reported in our cohort and is similar to findings by other investigators in the United States[16,19,20,23,32]. In both white and Hispanic patients with SSc, the prevalence of anti-U3-RNP was

relatively higher in dcSSc than in lcSSc. However, unlike the white cohort where anti-U3-RNP was associated with dcSSc, the presence of anti-U3-RNP was not associated with dcSSc or lcSSc in African American patients. The lack of association between anti-RNAP III or anti-U3-RNP (and possibly anti-Scl-70) and dcSSc in the African American patients differs from published reports[7,11,12,16,17,18,19,23], which were mainly based on data on white and Japanese patients. Last, the frequency of anti-Th/To did not differ significantly among ethnic groups, and was only associated with lcSSc in white patients. One European and 2 US studies reported a high prevalence of anti-Th/To and/or its correlation with lcSSc in whites[15,16,33]. However, correlation between anti-Th/To and lcSSc has not always been consistent, which may be attributable to the study size and number of anti-Th/To–positive cases[11,16,19,34]. It also remains to be clarified whether these variations are related to the diagnostic methods used for detecting the antibody marker. For example, to detect Th/To antibodies, Kuwana, *et al* used both RNA and protein immunoprecipitation assays[34] while other investigators have predominantly used the protein immunoprecipitation method[15,16,19,33] or radioactively labeled antisense riboprobes[11]. More studies are needed to further define the association between the presence of Th/To antibodies with clinical subsets and/or ethnicity in SSc.

Currently, only ACA, anti-Scl-70, and anti-RNAP III are included in the 2013 classification guidelines for SSc[8] and are widely available for testing in clinical laboratories. The analysis of autoantibody prevalence by ethnicity and/or disease subset in patients with SSc presented in our study provides compelling evidence for testing antibodies to U3-RNP, PM/Scl, and Th/To in patient care. In the absence of specific autoantibody tests, the data also suggest that ANA IFA pattern(s) may be useful in predicting clinical subset. Alternatively, the ANA IFA pattern, clinical presentation, and/or ethnic background may inform the request for specific autoantibody tests. Further, for every subset of SSc in patients, the results indicate that there exist a substantial number (14%–32%) of cases that are ANA-positive but negative for known SSc-specific autoantibodies. Thus, there exists a significant gap in the autoantibody repertoire in SSc and this highlights opportunities for identifying novel diagnostic markers for this disease.

Our study has limitations. Similar to most studies of this design, ours is cross-sectional and limited by the paucity of clinical data for internal organ involvement and survival. Further, IP was not performed on all samples, but limited to those with mainly nucleolar staining. Thus it is likely that the frequency of some autoantibodies may be underestimated or skewed toward specificities associated with ANoA patterns. While the ANoA pattern is strongly associated with a number of antibody specificities in SSc, its association is not always consistent for some autoantibodies, including RNAP III as observed in our investigation and previously reported by others[35]. Another limitation is the likely heterogeneity within each ethnic group, which could affect the interpretation of our results. Despite these limitations, our study further highlights the relevance of specific autoantibody detection in the evaluation and management of SSc, providing the most extensive characterization of the autoantibody profile in Hispanic American patients thus far, to our knowledge. Overall, serologic testing represents a cost-effective and personalized approach for this rare and heterogeneous disease with its diverse clinical outcomes.

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SHORT COMMUNICATION

Anti-rods/rings autoantibody seropositivity does not affect response to telaprevir treatment for chronic hepatitis C infection

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Abstract

Purpose Autoantibodies to intracellular 'rods and rings' structures (anti-rods/rings or anti-RR) are strongly associated with hepatitis C (HCV) patients treated with interferon-α/ribavirin (IFN/RBV) and are linked with non-responsiveness to IFN/RBV or relapse, especially in Italian patients. This is the first study to determine whether there is any correlation of anti-RR with non-responsiveness to IFN/RBV treatment in patients also treated with telaprevir (TPV), one of several new therapies for chronic HCV recently implemented.

Methods From 2013 to 2014, 52 HCV-infected patients were treated with IFN/RBV and TPV at five Italian clinics. Patient sera were collected and analyzed by indirect immunofluorescence for the presence of anti-RR antibodies. Patients were classified as anti-RR positive or anti-RR negative, and then various biological and clinical variables were analyzed to compare the two groups, including gender, age, HCV genotype, previous IFN/RBV treatment, and IFN/RBV/TPV treatment outcome.

Results Of these 52 HCV patients treated with IFN/RBV/TPV, 10/32 (31%) who previously received IFN/RBV were anti-RR positive, compared to 0 of 20 treatment-naïve patients. Anti-RR-positive patients relapsed more than anti-RR-negative patients (3/10, 30% vs. 2/42, 5%; $p < 0.05$). However, zero anti-RR-positive patients were non-responsive, and frequencies of sustained virological response were similar (anti-RR positive: 7/10, 70% vs. anti-RR negative: 33/42, 79%).

Conclusions Overall, the data suggest that anti-RR seropositivity is not associated with resistance to TPV treatment in this patient cohort, but monitoring anti-RR-positive patients for relapse within the first 6 months after treatment may be useful.

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Keywords Direct-acting antivirals · Inosine monophosphate dehydrogenase · Interferon-α · Ribavirin · Rods and rings · Telaprevir

Abbreviations

ANA	Antinuclear antibody
anti-RR	Anti-rods/rings autoantibody
DAAs	Direct-acting antivirals
HCV	Hepatitis C
IFN	Interferon-α
IFN/RBV	Interferon-α and ribavirin therapy
IMPDH	Inosine 5′-monophosphate dehydrogenase
RBV	Ribavirin
RRs	Rods and rings
SVR	Sustained virological response
TPV	Telaprevir

Introduction

Chronic hepatitis C (HCV) infection is associated with the production of autoantibodies, including organ-specific autoantibodies directed against targets in the thyroid, adrenal cortex, pancreatic islet cells, and gastric parietal cells, and non-organ-specific autoantibodies such as antinuclear, anti-smooth muscle, anti-mitochondrial, anti-liver/kidney microsomal, and anti-neutrophil cytoplasmic antibodies [1–8]. Recent studies have also demonstrated a link between HCV and the production of autoantibodies targeting intracellular filamentous structures termed 'rods and rings' (RRs) [9–16]. In most studies, anti-rods/rings (anti-RR) seropositivity appears to be almost exclusive to HCV patients treated with interferon-α and ribavirin combination therapy (IFN/RBV) and is rarely seen in treatment-naïve HCV patients or other disease groups. However, anti-RR has been observed in one hepatitis B patient [11], one systemic lupus erythematosus patient [17], and healthy individuals with no previous IFN/RBV treatment [17]. In cultured cells, RRs are composed of inosine 5′-monophosphate dehydrogenase (IMPDH), and/or cytidine 5′-triphosphate synthase under certain conditions [9, 18, 19]. RRs tend to assemble when de novo purine biosynthesis is inhibited and guanine nucleotide levels become depleted [20–25]. Many patients with anti-RR react with IMPDH, which is inhibited by direct binding to RBV and appears to be the major autoantigen in RRs [9, 26, 27]. Although no mechanistic evidence suggesting that anti-RR autoantibody contributes to resistance to IFN/RBV therapy has yet been reported, previous studies showed that anti-RR antibodies were more prevalent in patients who did not respond to therapy or relapsed, when compared to sustained responders [10, 15]. Additionally, non-responsive or relapsing patients had higher anti-RR titers, suggesting that anti-RR positivity may be indicative of poor treatment outcomes [28]. In recent years, direct-acting antivirals (DAAs), such as telaprevir (TPV), have been developed for chronic HCV infection in an effort to reduce therapy duration and increase drug tolerability, while also improving patient outcomes. Currently, TPV is included with IFN/RBV as a triple therapy. Here, we examine the relationship between anti-RR and treatment outcomes in a cohort of Italian patients treated with IFN/RBV and telaprevir.

Methods

Patient and treatment information

From 2013 to 2014, 52 HCV-infected patients were treated with IFN/RBV and TPV at five Italian clinics located at the (1) Ospedale San Antonio, Tolmezzo, (2) Ospedale Santa Chiara, Trento, (3) Università degli Studi di Siena, (4) Ospedale Regionale, Treviso, and (5) Università degli Studi di Genova. Dosages depended on patient weight (75 kg discriminating weight) and were typically administered as follows: 80–180 μg weekly pegylated interferon-α, 600–1400 mg daily ribavirin, and 2250 mg daily TPV. Patients were classified as: not responsive to therapy (HCV RNA still detectable at week 24 of therapy), relapsed (HCV RNA detectable after the end of treatment in patients with previous virological response), or responsive to therapy (HCV RNA not detectable in the 24 weeks after the completion of therapy). The study conforms to the Institutional Review Board requirements in all institutions.

Informed consent Informed consent was obtained from all individual participants included in the study. All patients provided written informed consent to receive IFN/RBV/TPV and permission for use of their medical records for this study.

Antinuclear antibody indirect immunofluorescence assay (ANA-IIF)

Anti-rods/rings in patient sera were detected by indirect immunofluorescence, using NOVA Lite HEp-2 ANA substrate (INOVA Diagnostics, San Diego, CA: 508100) as previously described [10]. Staining patterns of test sera were compared to staining of human prototype anti-RR serum It2006 used in previous studies [9, 10]. It2006 and all anti-RR-positive sera described in this study correctly recognize the rods and rings ANA pattern, designated as pattern AC-23 by the International Consensus on ANA Patterns (ICAP) [29]. All sera were tested at a dilution of 1:80 in PBS as previously described [30]. For anti-RR-positive patients who also had serial samples available, anti-RR end point titers were determined using twofold serial dilution of sera in PBS, with a starting dilution of 1:80 and ending dilution of 1:1280. Anti-RR positivity and titers were independently validated by two trained individuals (S.J.C. and T.N.). End point titer was defined by more than 50% of cells containing detectable RR staining. Donkey anti-human IgG conjugated to DyLight 488 (Jackson ImmunoResearch, West Grove, PA) diluted 1:100 in PBS was used to detect autoantibody staining. Fluorescent images were captured with a Zeiss Axiovert 200 M microscope fitted with a Zeiss AxioCam MRm camera using a 40× (0.75 NA) objective (Carl Zeiss Microscopy, Jena, Germany).

Statistical analysis

Biological and clinical variables analyzed for statistical significance include: gender, age, HCV genotype, previous treatment with IFN/RBV (prior to beginning of TPV

regimen), and treatment outcome (see Table 1). Mann–Whitney U test was used to compare different groups containing continuous data, and Fisher's exact test or the Fisher–Freeman–Halton exact test was used for categorical data. Differences were considered statistically significant if $p < 0.05$. Mann–Whitney U test and Fisher's exact test were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). Fisher–Freeman–Halton test was performed using StatXact 10 (Cytel, Cambridge, MA).

Results and discussion

The goal of this study was to probe for an association between the presence of anti-RR autoantibody and treatment outcome in a cohort of 52 Italian HCV patients treated with the new DAA telaprevir. This is the first study to examine anti-RR in patients treated with any of the recently approved DAAs. It must be pointed out that, in general, the availability of patients for studies of anti-RR antibody is naturally limited by the low prevalence of this autoantibody response. Although it has been reported that 20–40% of HCV patients treated with IFN/RBV produce anti-RR autoantibodies, this response is very rarely observed in treatment-naïve HCV patients or other disease groups [10, 11, 14–16, 31].

A total of 52 HCV-infected patients were treated with IFN/RBV and TPV. Thirty-six patients (69%) were male and 16 (31%) were female, with a mean age of 54 ± 9 years. Twelve patients (23%) had genotype 1a, 39 patients (75%) had genotype 1b, and one patient (2%) had genotype 3b. Thirty-two patients (62%) had been previously treated with IFN/RBV prior to being put on TPV. Patient demographics are included in Table 1. All 52 patients were assayed for the presence of anti-RR according to standard antinuclear antibody indirect immunofluorescence (ANA-IIF) protocols using HEp-2 cells as a substrate. ANA-IIF analysis revealed that 10 out of 52 patients (19%) were positive for anti-RR at a dilution of 1:80. Figure 1 displays the images of the 'rods and rings' staining pattern from all ten anti-RR-positive patients (patient codes C1TN, FV1S, TBN1S, VA1S, TG1TO, SL1G, VG1G, MS1G, RC1G, and CB1G). Anti-RR-negative patients CD2T and E1TN are also shown for comparison. Twenty-one out of 52 patients had serial collections of sera available (104 total sera for 52 patients), representing multiple visits to the clinic over time periods ranging from 2 weeks to 13 months. Anti-RR status did not change over time in any of these 21 patients (i.e., anti-RR-positive patients remained positive and negative patients remained negative) and anti-RR titers did not significantly change in positive patients. Accordingly, for purposes of statistical analysis, patients were simply considered either positive or negative, and serial collections were not taken into consideration.

Patients were divided into two main groups, anti-RR-positive ($n = 10$) and anti-RR-negative ($n = 42$), for subsequent statistical analysis. These groups were

Table 1 Summary of anti-RR autoantibody reactivity in HCV patients treated with interferon-α/ribavirin and telaprevir

Parameters	Total patients ($n = 52$)	Anti-RR-positive patients ($n = 10$)	Anti-RR-negative patients ($n = 42$)	p value
Male	36 (69%)	7 (70%)	29 (69%)	NS
Female	16 (31%)	3 (30%)	13 (31%)	NS
Age (years) ± SD	54 ± 9	53 ± 14	54 ± 8	NS
Genotype 1a[#]	12 (23%)	3 (30%)	9 (21%)	NS
Genotype 1b[#]	39 (75%)	7 (70%)	32 (76%)	NS
Previous IFN/RBV	32 (62%)	10 (100%)	22 (52%)	<0.01
Treatment outcome				
SVR, no side effects	35 (67%)	6 (60%)	29 (69%)	NS
SVR, but side effects	5 (10%)	1 (10%)	4 (10%)	NS
SVR, combined	40 (77%)	7 (70%)	33 (79%)	NS
Relapse	5 (10%)	3 (30%)	2 (5%)	<0.05
No response	7 (13%)	0 (0%)	7 (17%)	NS
Fisher–Freeman–Halton exact test				
Anti-RR positive vs. anti-RR negative, with SVR separated				NS
Anti-RR positive vs. anti-RR negative, with SVR combined				<0.05

Values presented as n (%) unless otherwise indicated

Anti-RR anti-rods/rings autoantibody, *IFN/RBV* interferon-α/ribavirin therapy, *NS* not statistically significant ($p > 0.05$), *SD* standard deviation, *SVR* sustained virological response

[#] One patient without anti-RR was genotype 3b

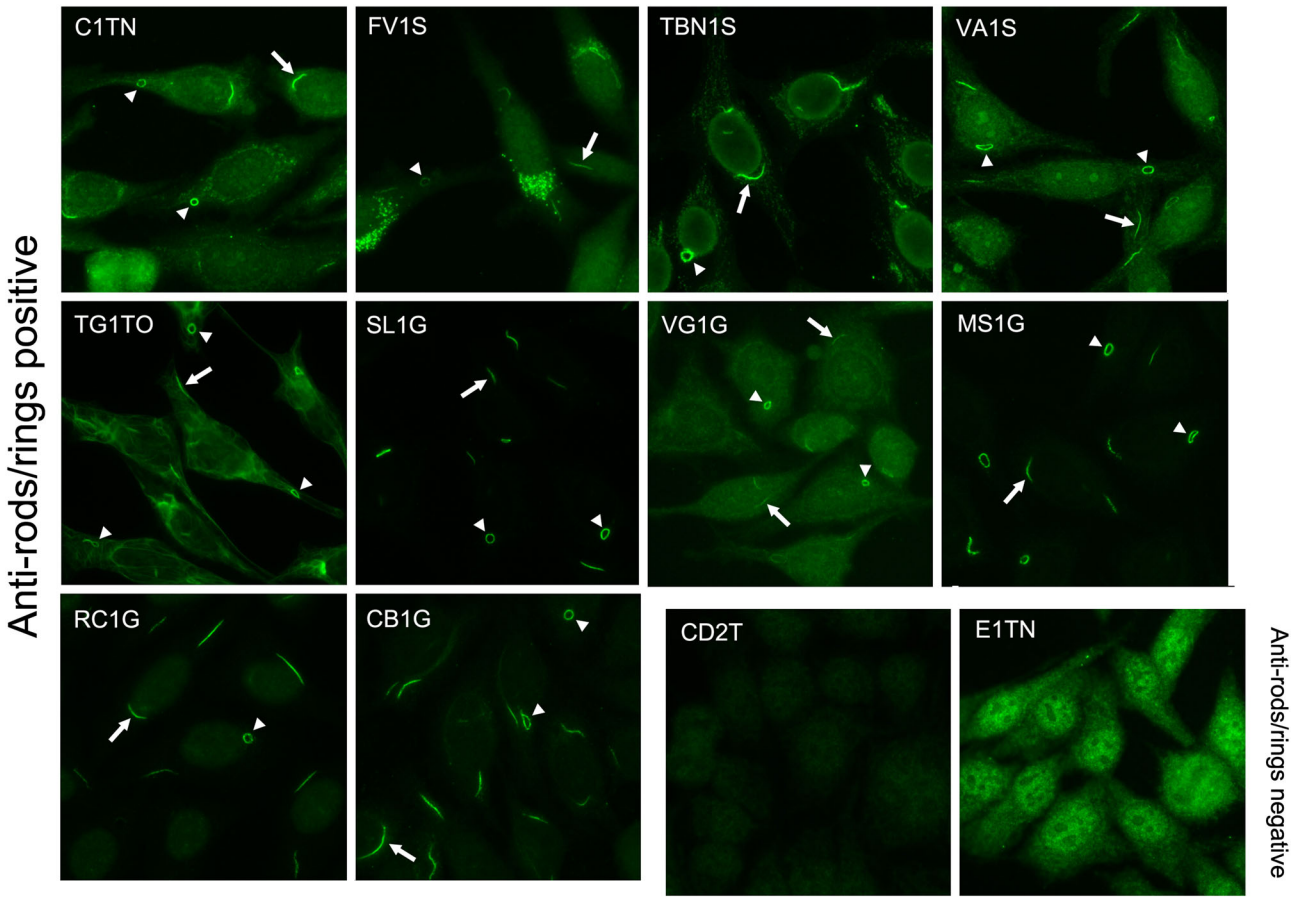

Fig. 1 Anti-rods/rings seropositivity detected by the ANA-IIF assay. Fifty-two Italian HCV patients were subjected to the antinuclear antibody indirect immunofluorescence (ANA-IIF) assay. Anti-rods/rings autoantibody was detected in 10 of 52 patients (19%), shown by the 'rods and rings' ANA pattern in patient codes C1TN, FV1S, TBN1S, VA1S, TG1TO, SL1G, VG1G, MS1G, RC1G, and CB1G. *Arrows* point to examples of rods, which are most often observed in cytoplasmic and perinuclear regions (see C1TN, RC1G, or CB1G for examples of perinuclear rods). *Arrowheads* point to rings, which may sometimes appear twisted into a "hairpin" shape, as in the *bottom left corner* of the *panel* TG1TO. CD2T and E1TN are negative for anti-rods/rings and are included for comparison. CD2T contains no detectable autoantibody reactivity, while E1TN displays the nuclear speckled ANA pattern. All *panels* shown are of sera tested at 1:80 dilution and detected by donkey anti-human IgG conjugated to DyLight 488. All images were taken with a 40× objective

compared based on several demographic, clinical, and serological parameters to determine any differences between anti-RR-positive and anti-RR-negative patients (Table 1). There was no significant difference observed between the two groups when comparing gender, age, or HCV genotype. Both patient groups were also categorized into four different treatment outcome groups: (1) sustained virological response (SVR) with no side effects, (2) initial SVR but therapy was discontinued due to side effects, (3) relapse within 6 months of treatment, and (4) no response to treatment. SVR patients with or without side effects were all determined to be SVR at the same time point (after 1 month, according to international guidelines). When the Fisher–Freeman–Halton exact test was performed to compare anti-RR-positive vs. anti-RR-negative patient groups based on all four treatment outcome parameters, no significant difference was observed. However, additional Fisher–Freeman–Halton analysis with all SVR patients combined (regardless of side effects) resulted in a statistically significant p value <0.05 (Table 1, bottom row). Thus, our data indicate that anti-RR-positive and anti-RR-negative patients in this cohort appear to differ with regard to distribution of treatment outcomes. The most notable difference is that anti-RR-positive patients were more likely to relapse than anti-RR-negative patients ($p < 0.05$). Despite the increase in relapse, 0 of the 10 anti-RR-positive patients were non-responsive to therapy, compared to 7 of the 42 (17%) anti-RR-negative patients ($p = 0.32$). Additionally, there was no significant difference in SVR rates between both groups. SVR rates were compared with patients who experienced side effects and those who did not separated as different outcomes ("SVR, but side effects" and "SVR, no side effects") or with all SVR patients combined ("SVR, combined"), but both

analyses showed no significant difference between anti-RR-positive and anti-RR-negative patients.

We also discovered that anti-RR can be detected years after treatment with IFN/RBV. Two of the ten anti-RR-positive patients had serum collected both prior to and after IFN/RBV/TPV therapy was initiated. All samples from both patients were positive for anti-RR, initially suggesting that these patients may have been positive with no previous IFN/RBV treatment. However, after careful examination of medical records, it was determined that both patients had received IFN/RBV more than a decade prior to treatment with TPV. Patient TBN1S was diagnosed with liver cirrhosis in 2000 and treated with IFN/RBV from 2000 to 2002, but treatment was eventually discontinued due to side effects. In 2014, TBN1S began receiving IFN/RBV again with addition of TPV, but relapsed within 6 months. Patient VA1S previously received IFN/RBV for 6 months in 2003. Eleven years later, in 2014, the patient began receiving IFN/RBV again with addition of TPV, but therapy was discontinued due to side effects. Despite the lack of exposure to IFN/RBV for more than 10 years, both patients remained positive for anti-RR autoantibody, and VA1S even remained positive down to 1:1280 dilution. We speculate that long-lived plasma cells might be responsible for the long-term presence of anti-RR antibody in these patients. Previous studies have suggested that anti-RR titer increases throughout the duration of therapy, but declines upon cessation of treatment [11, 12, 15, 31]. To our knowledge, this is the first report of long-lived anti-RR autoantibody.

Previous studies established a strong association between anti-RR and IFN/RBV therapy [10–12, 14, 15], such that we previously described anti-RR as a drug-induced autoantibody [13]. Additionally, prolonged exposure to IFN/RBV increases the likelihood of anti-RR autoantibody production [11, 12, 15]. Our data support these findings, considering that 10 out of 32 (31%) patients previously treated with IFN/RBV were anti-RR positive, compared to 0 out of 20 patients who had not previously received IFN/RBV. In terms of treatment outcome, previous reports have indicated a link between anti-RR and non-responsiveness or relapse in American and Italian HCV patient cohorts [10, 15, 28]. In the current study with a new cohort of Italian patients, we again found that anti-RR seropositivity was associated with increased frequency of relapse. Interestingly, the frequency of non-responsiveness appeared to be decreased in anti-RR-positive patients (0/10, 0%) compared to anti-RR-negative patients (7/42, 17%), despite the opposite trend in relapse. When patients with no previous IFN/RBV treatment are removed from analysis, the trend remains similar, with non-responsiveness occurring in 0/10 (0%) of anti-RR-positive patients compared to 6/16 (27%) of anti-RR-negative patients ($p = 0.14$). Overall, 19% (10/52) of HCV patients in this study were positive for anti-RR, which is similar to the rates observed in previous studies [10, 11, 14–16]. Importantly, the addition of TPV to the IFN/RBV regimen did not induce a new anti-RR response in any patients; anti-RR-negative patients previously treated with IFN/RBV did not become positive after TPV was included in the regimen. Likewise, anti-RR titers did not significantly change after addition of TPV in anti-RR-positive patients with serial samples available. In all, our data suggest that inclusion of TPV in the IFN/RBV regimen for the treatment of chronic HCV does not alter the production of anti-RR autoantibody. However, based on the statistically significant increase in relapse rate, it may be useful to carefully monitor anti-RR-positive patients during and for 6 months after IFN/RBV/TPV therapy. While our study is limited by the number of available patients, the data indicate that anti-RR seropositivity does not affect the response to TPV treatment for chronic HCV.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Informed consent Informed consent was obtained from all individual participants included in the study. All patients provided written informed consent to receive IFN/RBV/TPV and permission for use of their medical records for this study.

Human and animal rights The study conforms to the Institutional Review Board requirements in all institutions. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Animals were not used in this study.

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Immunodiagnosis of Autoimmune Myopathies

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Certain autoantibodies can be clinically useful biomarkers associated with a particular disease and/or clinical features. Some of them, called disease marker antibodies, are highly specific for a particular diagnosis and have predictive value for the development of the disease, and they are included in classification criteria for systemic autoimmune rheumatic diseases (1, 2). The majority of disease-associated autoantibodies in systemic lupus erythematosus and scleroderma (systemic sclerosis) have been known for decades. However, autoantibody research in polymyositis/dermatomyositis (PM/DM) has been highly active in recent years as several new, clinically important autoantibody specificities with strong clinical impact have been identified, such as antibodies to transcription intermediary factor 1γ/α (TIF1γ/α; p155/140), which are frequently found in cancer-associated DM (3–5); and to melanoma differentiation-associated gene 5 (MDA5), associated with clinically amyopathic DM (CADM) with rapidly progressive interstitial lung disease (ILD) (6–9). Autoantibodies that are found in PM/DM are often classified into myositis-specific autoantibodies (MSAs) and myositis-associated autoantibodies (10, 11). MSAs are found almost exclusively in PM/DM among systemic rheumatic diseases, although some are also found in patients classified into idiopathic ILD, such as anti-PL-12 and anti-KS antibodies (12, 13).

Autoantibodies in PM/DM have been tested by a combination of immunoprecipitation (IP) analysis of proteins and RNA components of the target antigens along with enzyme-linked immunosorbent assay (ELISA) using purified or recombinant antigens, Western blot, and IP-Western blot as necessary (1, 14). In this chapter, we focus on experimental procedures of IP and ELISA using recombinant proteins, revising the previously published chapter of this manual (15). This chapter does not discuss details of molecular targets or the clinical significance of each autoantibody specificity, as many recent review articles on MSAs are available (11, 16–18).

IP ANALYSIS OF PROTEIN COMPONENTS

Radioimmunoprecipitation analysis of protein components of autoantigens using [^{35}S]methionine-labeled cell extract is a very powerful technique that allows screening for almost all known PM/DM autoantibodies in a single assay. Many of them can be interpreted almost conclusively, while a few may require additional techniques for confirmation (1). Advantages of IP include (i) screening of almost all known MSAs by a single assay, (ii) antigens being closer to the native condition compared with those in other immunoassays such as Western blot or ELISA, (iii) detection of multiproteins or multiprotein-nucleic acid complexes, and (iv) confirmation of the specific reactivity to the target antigen of the corresponding molecular weight (versus optical density [OD] in ELISA, which may not reflect the reactivity with the actual target in some cases).

Technology and Instrumentation

Antibodies in sera are affinity-purified onto beads coated with protein A or protein G. Beads with purified antibodies are mixed with radiolabeled cell extract. Antigens recognized by autoantibodies are affinity-purified onto the beads. Radiolabeled proteins are separated by SDS-PAGE and detected by autoradiography. A similar approach can be used to analyze the nucleic acid components of RNA-protein complex autoantigens. Required apparatus includes an electrophoresis power supply, vertical electrophoresis apparatus, tabletop centrifuge, CO_2 incubator, cell sonicator (Branson, Thomas Scientific, Swedesboro, NJ), and microcentrifuge.

Materials and Reagents

The following materials and reagents are needed.

1.5 g protein A-Sepharose CL4B (PAS) (17-0780-01; GE Healthcare, Little Chalfont, United Kingdom)

Prepare PAS stock solution as follows.

1. Add deionized H_2O (dH_2O) to 1.5 g of dry PAS in the vial, transfer to 15-ml centrifuge tubes, and let it swell by leaving at room temperature for 20 to 30 min.
2. Centrifuge for 30 s in a tabletop centrifuge (300 × *g*).
3. Aspirate water, add water to the top of the tube, and mix by inverting tubes. Wash the beads three times by centrifuging and aspirating supernatant.
4. Based on the volume of the beads, add dH_2O to make 50% (vol/vol) solution. Usually 14 to 15 ml of 50% (vol/vol) solution can be made from 1.5 g of PAS.
5. Add 1/100 amount of 2 M Tris-HCl (pH 7.5) to a final concentration of 20 mM and 10% NaN_3 to a final concentration of 0.1%. The beads can be stored at 4°C in this buffer.

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Protein A, protein G, and protein A/G-Sepharose or -agarose beads are sold by many companies with variable binding capacity and nonspecific background. Although we have been using PAS for almost all IP experiments (including mouse IgG1 monoclonal antibodies), some mouse IgG1 and human IgG3 may not bind PAS efficiently; thus protein G or A/G may be more suitable for these.

0.5 M NaCl NET/IGEPAL buffer: 50 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 2 mM EDTA, and 0.3% IGEPAL CA-630 (Sigma I3021; Sigma-Aldrich, St. Louis, MO). Nonionic, nondenaturing detergent Nonidet P-40 was replaced by IGEPAL CA-630, which is chemically indistinguishable from Nonidet P-40.

NET/IGEPAL buffer: 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 2 mM EDTA, and 0.3% IGEPAL CA-630

Phenylmethylsulfonyl fluoride (PMSF) (Sigma P7626): 50 mM in absolute ethanol stored at −80°C

Aprotinin (Sigma A6279): Aprotinin from bovine lung aseptically filled solution in 0.9% NaCl and 0.9% benzyl alcohol. Activity: 3 to 7 trypsin inhibitor units (TIU)/mg protein; 5 to 10 TIU/ml solution.

3× sample buffer: mix 0.5 M Tris-HCl (pH 6.8) (6.0 ml), 1% bromophenol blue (0.6 ml), 2-mercaptoethanol (3.0 ml), 25% SDS (4.8 ml), and glycerol (6.0 ml). When it is used for IP samples, make 1× by mixing 1 volume of 3× sample buffer with 2 volumes of dH_2O.

Sterile phosphate-buffered saline (PBS)

[^{35}S]Methionine EXPRESS Protein Labeling Mix, [35S]-, 14 mCi (518 MBq), 50 mM Tricine (pH 7.4), and 10 mM 2-mercaptoethanol (NEG-072; PerkinElmer, Waltham, MA). Available in 2-, 7-, or 14-mCi vial.

Methionine- and cysteine-free culture medium RPMI 1640 (17-104-CI; Cellgro, ThermoFisher, Waltham, MA)

Penicillin (10,000 IU/ml)-streptomycin (= 100× Pen-Strep; Gibco BRL, ThermoFisher)

L-Glutamine, 200 mM (= 100× Gibco BRL catalog no. 25030-081)

HEPES (100×)

PBS-dialyzed fetal bovine serum (FBS) (SH3007902; HyClone, ThermoFisher)

^{35}S-labeling culture medium: 39 ml methionine- and cysteine-free RPMI, 4.5 ml PBS-dialyzed FBS, 0.45 ml L-glutamine, 0.45 ml HEPES, and 45 μl Pen-Strep

Assays: Cell Culture

Radiolabeled cell extract is prepared using K562 cells (human erythroleukemia cell line; ATCC CCL-243). The cells are thawed for 2 to 3 days before use and grown in RPMI 1640 with 10% FBS, 1:100 L-glutamine, 1:100 Pen-Strep, and 1:100 HEPES in a 5% CO_2 atmosphere at 37°C. Cells should be visually inspected carefully using an inverted microscope at high magnification for viability and contamination before labeling.

Note: K562 cells grow in clusters that can sometimes be quite large (20 to 30 to hundreds of cells per cluster) when they are proliferating rapidly. When clusters are smaller or rarely seen, with most of the cells as a single cell, proliferation may be slow and not optimal for efficient radiolabeling. When K562 cells start adhering, which indicates differentiation, expression of topoisomerase I (Scl-70) is very weak to deficient. When Plasmocin is used as an antibiotic in culture, cells proliferate normally without making any clusters. No clear differences in expression of known autoantigens are noted when K562 cells cultured with Plasmocin are used for radiolabeling and IP.

Radiolabeling

Collect cells by centrifuge (300 × *g* for 5 min in a tabletop centrifuge). Typically, 10^8 cells are cultured in 45 ml of complete methionine- and cysteine-free ^{35}S-labeling culture medium in a 250-ml flask. Add 3 mCi of [^{35}S]methionine (4.2 mCi as total radioactivity) and incubate cells for 12 to 14 h at 37°C in a 5% CO_2 atmosphere. After 12 to 14 h radiolabeling, add clean PBS to the culture and divide into 10 15-ml tubes, centrifuge (300 × *g*, 5 min), aspirate supernatant, and freeze at −80°C. Polypropylene tubes should be used to avoid cracking. Aliquots contain ~2×10^7 cells and can be used immediately or frozen at −80°C until use. After radiolabeling, there should be ~1.8×10^8 cells and the medium turns yellow. It is normal not to observe big clusters, and some apoptotic cells are seen after radiolabeling. However, contamination of bacteria and fungi should be carefully monitored after radiolabeling using high magnification.

Note: Levels of RNA polymerase II phosphorylated form were low after labeling for >16 h. It is better not to label for >14 h, though this also may depend on the condition of cells.

Preparation of PAS Beads with Purified Antibodies

Serum or plasma stored frozen or at 4°C can be used. Usually sera stored at 4°C with NaN_3 maintain their autoantibody reactivity for 10 to 15 years, though reduction of autoantibody reactivity in some sera was noticed. When sera are frozen, avoid repeat freezing and thawing. Clearing of sera by centrifuge is unnecessary. A heat-inactivated serum sample cannot be used because it causes very high background that makes interpretation of IP results impossible. This problem was not solved by microcentrifuging or filtering of sera.

Prepare labeled 1.6-ml microcentrifuge tubes and add 25 μl of 50% (vol/vol) PAS, followed by adding 8 μl of serum (or plasma). When serum is added, place the pipette tip in the PAS and pipette several times, as this will allow most IgG to bind to PAS immediately. Then add 500 μl of 0.5 M NaCl NET/IGEPAL. Microcentrifuge tubes are placed on a rack, which is then wrapped using plastic film and rotated for 1 h at 4°C up to 24 h.

Avoid vortexing the PAS beads at any stage of the procedure.

Preparation of Radiolabeled Cell Extract

When the beads are ready for washing, cell extract is prepared. Beads are washed and buffer aspirated while centrifuging the cell extract. For a standard reaction, radiolabeled cell extract from ~2×10^6 cells/sample is used. Add 2 ml of 0.5 M NaCl NET/IGEPAL to the frozen cell pellet (~2×10^7 cells; final cell concentration, 10^7/ml), then add 1:100 (20 μl) PMSF and aprotinin and leave on ice. Do not try to thaw at 37°C, as once the cells are thawed, they are disrupted and protein degradation commences. Also, avoid excessive heating of the cell suspension during sonication. With the above standard condition, heating is not a concern, but when the condition is changed, it will need to be carefully monitored for possible overheating. Do not put PMSF directly onto the frozen cell pellet because the 100% ethanol will denature the proteins. Sonicate the cells for 45 s (Branson Sonifier, duty cycle 25%, output 2.5), then place the tube on ice for 1 min, then sonicate again for 45 s. Transfer the cell lysate to microcentrifuge tubes and centrifuge at 9,000 × *g* for 30 min at 4°C. Carefully collect the

supernatant; avoid disturbing the small pellet that is usually seen at the bottom toward the outside. Combine all of the lysate in a 50-ml tube on ice. Use a 50-ml tube to avoid contamination of a pipette, as it is virtually impossible not to touch the inside wall with the pipette if a 15-ml tube is used.

Incubation with Cell Extract

Pellet the antibody-coated PAS beads by microcentrifuging for 8 s; aspirate the supernatant using a Pasteur pipette attached to a vacuum. For each aspiration step, carefully remove the bubbles on the surface of the liquid first since they may cause high background. Add 1 ml of 0.5 M NaCl NET/IGEPAL and microcentrifuge for 8 s and aspirate the supernatant. To each tube with PAS beads, add 160 μl of cell extract per sample. It is unnecessary to mix tubes as the beads and cell extract will be mixed completely during incubation. Make sure that caps are closed completely and tightly to prevent leaking of radioactive sample during incubation. Put tubes on a plastic rack, wrap with a plastic film, and rotate end over end to incubate for 1 h at 4°C.

Washing Beads

Microcentrifuge for 8 s, aspirate supernatant, add 1 ml of 0.5 M NaCl NET/IGEPAL, wash the inside of the cap by inverting once, and microcentrifuge for 8 s. Repeat washing three times and then once with NET/IGEPAL buffer. At each step, aspirate as completely as possible to reduce the background. Tubes can be aspirated in a horizontal position (versus vertical) for more complete aspiration. In the presence of proteins or detergent, PAS beads stay together and will not be easily aspirated (versus buffer without proteins or detergent such as PBS).

Add 50 μl of 1× SDS-PAGE sample buffer and freeze at −80°C or −20°C until running gels or proceed to boiling. Boiling could be before freezing or just before running gels. Boil samples for 3 min and absorb water around the cap using a paper towel immediately after boiling as it could get into the tubes when the air inside cools down and the pressure inside becomes negative. Microcentrifuge for 8 s and load 20 to 25 μl in each lane.

SDS-PAGE Gels

Since the smallest proteins that can be fractionated on 8% gels are ~25 kDa, while proteins of >100 kDa are not separated well on 12.5% gels, both 8 and 12.5% gels are run in routine screening of sera with unknown specificities. When running medium-size gels, it takes 3 to 5 h at a constant voltage of 100 to 130 V for the tracking dye front to reach to the bottom of the gels. For 8% gels, it is fine to let the dye front run out of the gels. For 12.5% gels, the thick blue dye band moves slower than the front of the samples; thus they need to be stopped when the thick blue dye band is 3 to 4 mm from the bottom. When a very thin blue line or a clear line at the sample front is seen, stop gels when it reaches the bottom of the gels.

SDS-PAGE Fluorography Reagents

Proteins immunoprecipitated are analyzed by SDS-PAGE, fluorography, and autoradiography after IP. Reagents are as follows.

25% SDS (Fisher, Sigma, or Bio-Rad, Hercules, CA). Filter this solution and all other stock solutions through a 0.45-μm bottle-top filter.

Coomassie blue stain (4 liters): Coomassie brilliant blue R-250 (catalog no. 161-0400; Bio-Rad) (5 g), methanol (1,200 ml), glacial acetic acid (400 ml), and dH_2O (2,400 ml)

Destaining solution (20 liters): 2 liters of glacial acetic acid, 7 liters of methanol, and 11 liters of dH_2O

2,5-Diphenyloxazole (PPO) (catalog no D144-100; Fisher), 20% (wt/vol) in dimethyl sulfoxide (DMSO). *Note:* PPO is carcinogenic.

Glycerol

BioMax MR film (catalog no. 870-1302; Eastman Kodak, Rochester, NY)

30% acrylamide–0.8% bisacrylamide stock: 30% (wt/vol) acrylamide (catalog no. BP 170-500; Fisher) plus 0.8% bisacrylamide (catalog no. 160-0201; Fisher) (wt/vol) in water (ratio, 37.5:1). Filter through a 0.45-μm bottle-top filter. Protein migration and mobility are affected by the ratio of acrylamide/bisacrylamide used. For detection of Ro52 protein, it is necessary to use acrylamide/bis = 166:1. Protein mobility on 12.5% acrylamide/bis = 166:1 gel is similar to 8% acrylamide/bis = 37.5:1 gel. However, 52-kDa Ro can be distinguished only on the former gel.

5× Tris-glycine (SDS-PAGE running buffer): 120 g of Tris base plus 576 g of glycine; add dH_2O to 4 liters (do not adjust pH; store at room temperature)

The procedure for preparing gels is as follows. *Note:* Precast minigels may be substituted for homemade ones. They can be purchased from Bio-Rad and other vendors. Identifying autoantibody specificities characterized by multiprotein complexes such as small nuclear ribonucleoproteins should not be difficult; however, a single protein autoantigen of close molecular weight (such as the ones of ~140 kDa [MDA5 and MJ; Table 1]) (19) will be difficult to differentiate with minigels.

If casting your own gels, keep in mind that acrylamide is a neurotoxin and suspected carcinogen. Gel plates, spacers, and rubber gaskets are washed thoroughly with detergent (e.g., PCC-54; Pierce, Rockford, IL), rinsed carefully, and wiped dry with ethanol. Gel plates should be assembled and clamped together with clips carefully to avoid leaking.

Preparation of SDS-PAGE Gels

We run both 12.5 and 8% SDS-PAGE for routine screening. Mix everything except 10% ammonium persulfate and TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine; Bio-Rad) in 50-ml plastic tubes. Stacking gels are common regardless of the percentage of acrylamide in resolving gels. Add ammonium persulfate and TEMED to resolving gel solutions only to start polymerization. Mix thoroughly, but avoid excess bubbles by inverting the tubes several times. Pour the gel solution between glass plates. Usually bubbles go up to the surface, but when they stick to the glass in the middle, they can be removed by gently tapping the side of the plates. This needs to be done prior to overlaying ethanol. After checking bubbles, gently overlay 70% ethanol on the gel solution. Add from one site and avoid disturbing the gel solution. A spreading of ethanol on the surface with a clear border should be seen. Adding a 3- to 5-mm layer of ethanol is enough. Make sure the plates stand vertically at an ~90° angle to the bench and leave them without disturbing until the gels polymerize. Speed of polymerization depends on temperature but usually takes 30 to 40 min. The line between the overlaid ethanol and gel solution becomes unclear, and when it appears clearly again, it is a sign of polymerization.

After polymerization of resolving gels, pour out ethanol and rinse with dH_2O ~10 times to wash out ethanol, then completely remove water using small pieces of Whatman

TABLE 1 Identification of myositis autoantibodies by IP analysis

Autoantibodies	Target molecule	Function	Protein	RNA
Aminoacyl-tRNA synthetase				
Jo-1	Histidyl-tRNA synthetase	Incorporate histidine into proteins	50 kDa	$tRNA^{His}$
PL-7	Threonyl-tRNA synthetase	Incorporate threonine into proteins	80 kDa	$tRNA^{Thr}$
PL-12	Alanyl-tRNA synthetase	Alanine and aspartate biosynthesis and alanine incorporation into proteins	110 kDa	$tRNA^{Ala}$
EJ	Glycyl-tRNA synthetase	Glycine, serine, and threonine metabolism and aminoacyl-tRNA biosynthesis	75 kDa	$tRNA^{Gly}$
OJ	Isoleucyl-tRNA synthetase	Incorporate isoleucine into proteins	150 kDa (multienzyme complex, 170, 130, and 75 kDa)	$tRNA^{Iso}$
KS	Asparaginyl-tRNA synthetase	Glutamate, alanine, and aspartate metabolism	65 kDa	$tRNA^{Asp}$
ZO	Phenylalanyl-tRNA synthetase	Incorporate phenylalanine into proteins	60/70 kDa	$tRNA^{Phe}$
YRS (HA)	Tyrosyl-tRNA synthetase	Incorporate tyrosine into proteins	59 kDa	$tRNA^{Tyr}$
Anti-SRP	Signal recognition particle	Protein maturation in the ribosome	72, 68, 54, 19, 14, and 9 kDa	7SL RNA
Anti-Mi-2	Helicase protein	Transcriptional regulation	240, 150, 72, 65, 63, 50, and 34 kDa	
Anti-MDA5/ CADM140	MDA5	RNA-specific helicase that mediates the antiviral response	140 kDa	
Anti-TIF1γ/α (p155/140)	TIF1γ/α	Transcription and RNA metabolism	155 and 140 kDa	
Anti-MJ	NXP-2 (MORC3)	Transcriptional regulation and activation of the tumor suppressor p53	140 kDa	
Anti-SAE	SAE	Posttranslational modifications	90 and 40 kDa	
Anti-PMS1	PMS1 (postmeiotic segregation increased 1)	DNA mismatch repair enzyme	120 kDa	

filter paper. In particular, water tends to stay at each corner; thus, it should be removed completely until the sharp 90° angle can be seen. When plates are ready for stacking gels, add ammonium persulfate and TEMED to the stacking gel solution. Mix gently by inverting the tubes and pour the solution between glass plates. Insert combs, avoiding bubbles under the comb. If bubbles are seen under the comb, remove it and insert again. Resolving gels can be polymerized in 30 min. We usually leave gels at 4°C overnight because we feel the protein bands are sharper in 8% gels by this step. The polymerization step should be at room temperature.

SDS-PAGE Procedure

The gels are run as follows.

1. Make 1 liter of running buffer: 1 liter is used for a pair of gels; 200 ml of 5× Tris-glycine plus distilled water to make 1,000 ml; add 4 ml of 25% SDS and mix well by inverting a measuring cylinder.

2. Boil samples, including molecular weight marker if necessary. Prestained molecular weight marker is relatively expensive but convenient, as the quality and progress of running gels can be monitored.

3. Carefully remove the combs and silicone rubber spacer from the gel, and then remove clips.

4. Clamp the gel to the gel apparatus and add running buffer to the top and bottom chambers of the apparatus.

5. Flush out bubbles from the bottom of the gel immersed in the bottom chamber, using a 5- to 10-ml syringe with a bent (90°) 18-gauge needle.

6. Gently flush out each well with running buffer using a Hamilton microsyringe. Pieces of gels in the wells should be carefully removed.

7. Load a 20- to 25-μl sample to each well, using either a Hamilton syringe or pipette.

8. Attach leads to the electrodes of the gel apparatus (proteins treated with SDS are negatively charged and will migrate toward the cathode).

9. Attach the power supply and run at 100 V until the blue tracking dye reaches the resolving gel; the voltage can later be increased to 120 to 130 V.

10. Run the gel until the blue tracking dye reaches the bottom of the gel or to the appropriate level as discussed earlier.

11. Turn off the power, remove the electrodes, and unclamp the gel from the gel apparatus. Carefully pry the plates apart, leaving the gel adherent to one of the plates. Using a pizza cutter, cut off the stacking gel (leave 1 to 2 mm of stacking gel) and avoid cutting resolving gel. Also, diagonally cut off a small piece of the gel from the upper right-hand corner to aid in orienting the gel.

12. Stain the gel for 30 to 60 min with Coomassie blue solution in a box, making sure gels are not stacked together and freely moving on a shaker. Use low-speed orbital shaking. Prolonged staining makes gels fragile and should be avoided.

13. Gels can be destained in the solution overnight. Proteins can still be transferred to other gels at this stage if they stick together. Separating each gel in a small plastic box is ideal.

Fluorography

After staining and destaining, the gels are fluorographed as follows.

1. Place the first DMSO (100 to 150 ml, enough to cover all gels and allow them to move freely) in a Tupperware or similar (polyethylene or polypropylene, but not polystyrene) box and add gels after removing as much excess destaining solution as possible. Incubate in the first DMSO for 30 min on an oribital shaker at low speed.

2. Discard/save the first DMSO (can be reused for up to ~8 gels; keep in a glass bottle with a tight cap) and add 100 to 150 ml of the second DMSO and shake for another 30 min.

3. Discard/save the second DMSO and add 100 to 150 ml of 20% (wt/vol) PPO in DMSO and shake for 1 h. The second DMSO can be reused for up to ~8 gels and then can be used as a first DMSO.

4. Save the PPO-DMSO in brown glass bottles for reuse. It can be used a few times, though the effectiveness gradually diminishes.

5. Wash the gels with many changes of water (tap water is fine) to remove DMSO and make PPO precipitate in the gels. Clear gels will turn white and opaque upon washing with water. Repeat, changing water and putting on a shaker. Minimum washing will be 30 min, but it is important to change water frequently rather than keeping on a shaker with the same water. Removing DMSO is critical to make thinner, nonsticky gels after drying.

6. Add glycerol to a concentration of ~3%. Insert a piece of filter paper under the gel in a box, pick up, and set on a gel dryer (Bio-Rad model 583) at 60°C with the timer set to 3 h. Gels can be dried completely after 2 to 2.5 h; however, minor air leaking in the system or other reasons could delay this process. Thus, check the appearance of the water droplets inside of the vacuum tubing (large water droplets indicate gels are still wet) carefully prior to stopping the vacuum of the gel dryer. *Note:* Higher temperature should be avoided because PPO can be destroyed at >60°C.

7. Remove gels from gel dryer. Tape filter paper with the dried gel on a solid flat support, such as a used piece of X-ray film; avoid taping over the dried gels. If it is sticky, dust the gel lightly with talc powder to prevent sticking. Place the gels in a film cassette and place in direct contact with BioMax MR film in the darkroom. The film is coated on one side, and the coated side must contact the gel directly. Expose films for 3 to 5 days at −70°C before developing. Exposure time can be adjusted based on the decay of ^{35}S (half-life, 89 days) and the signals of the molecules of interest for the experiment. Wash the box used for PPO using 100% ethanol (PPO is insoluble to water) a few times to remove PPO.

Interpretation

As a basic rule in interpretation of IP, observation of any protein bands that are present in a particular lane but not in other lanes means that they are specifically recognized by autoantibodies to these proteins. Interpretation of specificity of autoantibodies by IP is based primarily on the migration of immunoprecipitated proteins, ideally by comparing with the size and the pattern of proteins immunoprecipitated by reference sera. Representative IP analysis of autoantigenic proteins is shown in Fig. 1. Molecular weight and other characteristics of each MSA are summarized (Tables 1 and 2). Identifying a multiprotein or multiprotein-nucleic acid complex characterized by a set of proteins, such as anti-OJ, anti-SRP (signal recognition particle), anti-Mi-2, anti-TIF1γ/α (p155/140), and anti-SAE (small ubiquitin-like modifier-1 [SUMO-1] activating enzyme), is usually not so difficult. Nevertheless, occasionally patterns that look similar to OJ or TIF1γ/α are seen. For identification of SAE, there are many other proteins recognized by sera close to 90 kDa and 40 kDa where components of SAE migrate; thus, the mobility of the candidate proteins needs to be carefully assessed. Conclusively identifying SRP72/68 and SRP54 is not always easy, as there are many proteins that are recognized by patients' sera in this range of molecular weight. Detecting a set of the smaller subunits of 19, 14, and 9 kDa appears more unique, though a high-percentage (e.g., 12.5%) gel is necessary to separate these components. Jo-1 is seen as a thin band of ~50 kDa that is shifted due to interference of IgG heavy chain (not shown; this effect is unclear in Fig. 1 due to a small amount of anti-Jo-1 serum used). The presence of Jo-1 can be suspected in most cases based on IP; however, confirmation by other tests such as ELISA or Western blot is necessary for anti-Jo-1. IP of an ~50-kDa thin band and confirmation of tRNA (it cannot be confirmed specifically as $tRNA^{His}$) is practically considered anti-Jo-1. Although interpretation of a single protein autoantigen needs to be done carefully, strong anti-PL-7 or -EJ can be easily interpreted by IP in most cases, though weak bands need additional confirmation. PL-12 also shows a strong band; however, there are several autoantigenic ~120-kDa proteins that migrate close to PL-12. Mobility of TIF1β is identical to that of PL-12; however, a good-quality gel can show a characteristic appearance of the band: a sharp line in the middle and diffuse band around it. MJ/NXP-2 (nuclear matrix protein 2) is a sharp thin band, and interpretation needs to be done carefully as MDA5 migrates very close and there are many other proteins of ~140 kDa recognized by some sera. IP-Western blot or ELISA will be necessary to confirm (19). Identification of MDA5 in IP is not easy, as the expression of MDA5 in culture cells varies and there are MJ and other proteins of ~140 kDa to be differentiated (7).

Quality Assurance, Quality Control, and Test Validation

Having positive controls is ideal, but running all references each time is not practical. As far as the cells in good condition are radiolabeled, expression of all autoantibodies discussed in this chapter appears consistent except MDA5 (7, 19). As there are proteins that are nonspecifically immunoprecipitated by virtually all sera, including negative controls is necessary.

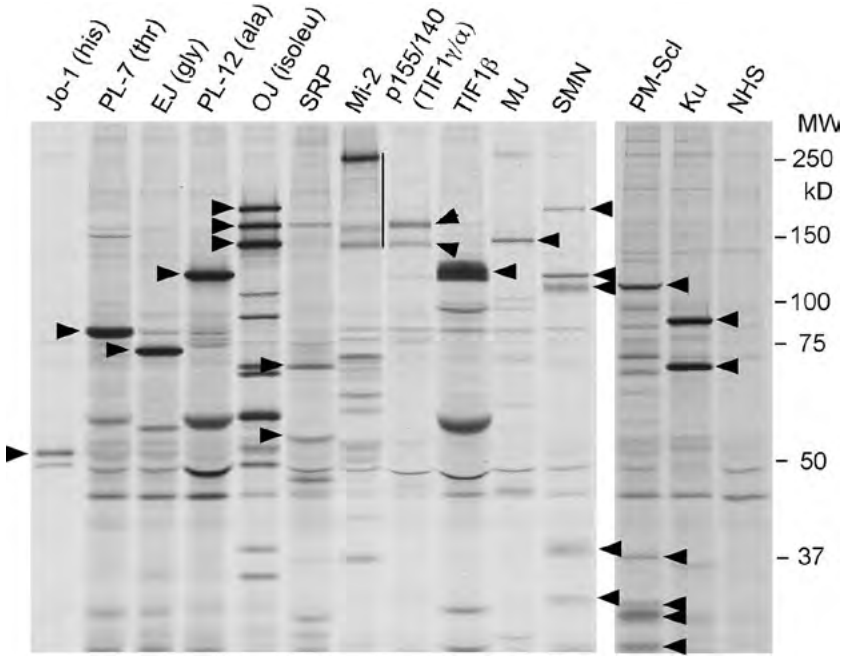

FIGURE 1 IP analysis of protein components of autoantigens recognized by autoantibodies in PM/DM. [^{35}S]Methionine-labeled K562 cell extract was immunoprecipitated by sera from patients with PM/DM. The main components of each autoantigen are indicated by arrowheads or a line drawn on the right (Mi-2). Jo-1, histidyl-tRNA synthetase; PL-7, threonyl-tRNA synthetase; EJ, glycyl-tRNA synthetase; PL-12, alanyl-tRNA synthetase; OJ, isoleucyl-tRNA synthetase multienzyme complex; SMN, survival of motor neuron; NHS, normal human serum; MW, molecular weight.

IP ANALYSIS OF SMALL RNAs

Small RNAs, components of autoantigens recognized by sera from patients, can be identified based on IP analysis of RNAs, similar to the IP procedure described above for proteins using radiolabeled K562 cells. The main differences are that (i) instead of [^{35}S]methionine-labeled cells, either unlabeled cells with silver staining of RNAs or [^{32}P]orthophosphate-labeled cells with autoradiography are used; and (ii) RNAs are extracted at the end of IP for analysis by urea-PAGE gels.

Due to concerns about strong β radiation produced by [^{32}P]orthophosphate-labeled cells, most laboratories working on autoantibody analysis switched from ^{32}P-radioimmunoprecipitation to silver staining analysis of RNAs. Thus, the method for the nonradioactive procedure is described in the following section.

In general, try to use autoclaved reagents and high-quality deionized water to avoid RNA degradation and reduce background in silver staining. Avoid any possible contamination of metal throughout the step because it will cause background in silver staining.

Steps for preparing PAS beads, stock, and incubation with sera are the same as the method for protein analysis by IP. Beads after incubation with sera can be refrigerated for several days in NET/IGEPAL buffer. Aspirate buffer before adding cell extract.

Cell Lysate

Unlabeled K562 cells (either frozen pellet or fresh cells; 5×10^6 cells/sample) are used. The number of cells necessary depends on specificities, but this number of cells is enough to detect RNA components for all MSAs that recognize RNA-protein complexes.

1. Add 2 ml of 0.5 M NaCl NET/IGEPAL plus PMSF (1:100) and aprotinin (1:100) to 50×10^6 cell pellets (20 μl each to 2 ml of the buffer). Final concentration: PMSF, 0.5 mM; aprotinin, 0.24 TIU/ml (Sigma). Do not put PMSF directly on cell pellets since ethanol will denature proteins. When frozen cell pellets are used, *do not* try to thaw cell pellets. Once the cells are thawed, cells will be disrupted and proteases and nucleases will be active. Also, it is unnecessary to mix and resuspend cells as they will be resuspended soon after sonication is started.

2. Sonicate cells for 45 s at duty cycle 25%, output control 2.5 (Branson Sonifier). Put on ice for 1 min. Sonicate again for 45 s. Keep samples on ice when it is possible. Transfer cell lysate to microfuge tubes. Microfuge for 30 min at 9,000 × *g* in a cold room (not at room temperature).

3. Wash PAS beads incubated with sera while clearing the cell lysate.

4. Carefully collect supernatant, avoiding the pellet. If there is more than one tube, pool all supernatant together in a tube before adding to samples. Add cell extract on beads (usually 200 μl/sample, but this can be adjusted depending on the experiment).

5. Put tubes on a plastic rack, wrap with a plastic film, and rotate at 4°C for 1 h.

Preparation of a Total RNA Sample as a Standard

1. Take 20 μl (extract from ~5×10^5 cells) and add 400 μl of NET/IGEPAL, 16 μl of 25% SDS, and 40 μl of 3 M Na acetate (pH 5.2). Add 400 μl of phenol/chloroform/isoamyl alcohol (25:24:1, pH 5.2) (Fisher). *Note:* Phenol/chloroform/isoamyl alcohol is available at pH 5.2 and pH 8.0. The pH 8.0 solution is supposed to allow extraction of both DNA and

TABLE 2 Prevalence and clinical association of myositis autoantibodies

Autoantibodies	Prevalence (%)	Clinical association/significance
Aminoacyl-tRNA		
Jo-1	15–30	Anti-synthetase syndrome (myositis, ILD, polyarthritis, Raynaud's phenomenon, mechanic's hands)
PL-7	<5	Myositis, ILD, DM-specific skin manifestations (heliotrope rash, Gottron's sign)
PL-12	<5	ILD, CADM, DM-specific skin manifestations (heliotrope rash, Gottron's sign)
EJ	<5	Myositis, ILD, DM-specific skin manifestations (heliotrope rash, Gottron's sign)
OJ	<5%	Anti-synthetase syndrome
KS	<5 (rare)	ILD
ZO	<1 (rare)	Myositis
YRS (HA)	<1 (rare)	Myositis
Anti-SRP	5	Myositis (necrotizing)
Anti-Mi-2	10	DM with typical skin lesions and mild myositis
Anti-MDA5/CADM140	15–20	CADM, rapidly progressive ILD, severe skin manifestations
Anti-TIFγ/α (p155/140)	10–15	Malignancy-associated DM
Anti-MJ/NXP-2	1–5	Adult and juvenile DM with severe skin disease
Anti-SAE	1	DM

RNA, whereas the pH 5.2 stock is designed mainly for RNA extraction.

2. Vortex vigorously for 1 min.

3. Spin for 1 min at room temperature at 9,000 × *g*.

4. Handle tubes very carefully and transfer supernatant to a clean new tube, avoiding the protein layer at the interphase between supernatant and phenol phase. Use a P200 pipette and harvest ~150 μl × 2 = ~300 μl.

5. Add 900 μl of 100% ethanol (200 proof) and *mix well by inverting tubes* (IMPORTANT).

6. Leave at −80°C overnight (>1 h is probably enough).

Washing Beads

1. After incubation of beads with cell extract, spin for 8 s and aspirate cell extract.

2. Add 1 ml of 0.5 M NaCl NET/IGEPAL. Wash the inside of the cap by inverting tubes.

3. Wash four times with 0.5 M NaCl NET/IGEPAL. Spin for 8 s.

4. Add 1 ml of NET/IGEPAL (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, and 0.3% IGEPAL CA-630). Spin for 8 s and aspirate supernatant completely.

Extraction of RNAs

1. Mix 400 μl of NET/IGEPAL, 16 μl of 25% SDS, and 40 μl of 3M Na acetate (pH 5.2) and add 456 μl of the mixture to the beads.

2. Add 400 μl of phenol/chloroform/isoamyl alcohol (25:24:1). Vortex vigorously for 1 min.

3. Purify RNA as described above in "Preparation of a Total RNA Sample as a Standard."

Preparation of RNA Samples for Urea-PAGE

RNA samples in microcentrifuge tubes are spun at 9,000 × *g* for 15 min in a cold room. The supernatant can be discarded by gently pouring, and then carefully aspirate liquid using a thin Pasteur pipette (heat the thin part of the Pasteur pipette over a gas burner and pull to make a thin glass pipette).

The RNA pellet is usually not visible, but the RNA pellet will stay on the surface of the tubes if aspiration is along the opposite side of the tube, where the pellet is supposed to be. Leave tubes under a biological safety hood to dry.

Preparation of Urea-PAGE Gel

1. Wash a 100-ml glass beaker and stirrer using detergent and rinse very well with Milli-Q water.
2. Weigh 21 g of urea, add 20 ml of 30% acrylamide/bis and 5 ml of 10× Tris-borate-EDTA (TBE), and stir until urea is completely dissolved.
3. Wash a measuring cylinder, and rinse with Milli-Q water and transfer the above solution.
4. Adjust the volume to 50 ml using Milli-Q water. Make fresh 10% ammonium persulfate using Milli-Q water.

5. Add 300 μl of ammonium persulfate and 30 μl of TEMED and mix completely.
6. Pour between a pair of glass plates and a spacer, assembled using clips, and insert comb.

Urea-PAGE

1. Start running gel at a constant voltage of 480 to 500 V (current and watts at maximum position; adjust output by voltage). Check the temperature of the gel using a paper thermometer and try to keep the temperature between 55 and 60°C. This range of temperature can be reached with voltage between 480 and 500 V, but the necessary voltage could be different in different systems.
2. Add 50 μl of urea-PAGE sample buffer to each sample tube and vortex to dissolve RNA. Turn the power off. Load 20 μl of sample using thin plastic pipette tips with a P20 pipette, quickly before the gel cools down.
3. Before prerun and before loading samples, quickly flush each well using a Hamilton microsyringe. Urea emerges from the gel and may collect at the bottom of the wells, disturbing the sample.
4. Two dye bands are seen while running gels, a blue dye band of bromphenol blue and a purple dye band of xylene cyanol. Run until the xylene cyanol dye reaches the bottom of the gel (it may go out). It usually takes ~1.5 to 2 h to run with medium-size gels.

Silver Staining of Nucleic Acids (Silver Stain Plus; Bio-Rad)

Follow the manufacturer's instructions.

12% Urea-PAGE Gel for RNA Analysis

30% (acrylamide/bis = 37.5:1) acrylamide/bis	20 ml
10× TBE	5 ml
Urea	21 g

Bring the volume to 50 ml with Milli-Q water.

10% ammonium persulfate	300 μl
TEMED	30 μl

RNA Sample Buffer

10 M urea

0.025% bromophenol blue

0.025% xylene cyanol in 1× TBE

Make 1% dye solution and add 1/40 amount of bromophenol blue and xylene cyanol to 10 M urea to a final concentration of 0.025%.

10× TBE

900 mM Tris	108 g
900 mM boric acid	55 g
20 mM Na_2EDTA	40 ml of 0.5 M stock

Adjust final volume to 1 liter.

Interpretation

Detecting tRNAs in IP samples is not difficult; however, the only interpretable information is that the antibodies in the serum recognize autoantigen that has tRNA as a component; it is not possible to identify the specificity of tRNAs by IP. Nevertheless, it is reasonable and practical to conclude a specificity of anti-tRNA synthetase antibodies based on IP of protein of the right molecular weight corresponding to each tRNA synthetase combined with IP of the tRNA. Detection of 7SL RNA for anti-SRP is not difficult, though having a positive control is ideal. Detection of U small nuclear RNAs is easy with a positive control as well. Representative silver staining gel to analyze RNA components of autoantigens is shown in Fig. 2.

Quality Assurance, Quality Control, and Test Validation

Most small RNAs associated with MSA antigens can be detected easily with good-quality IP and staining. A positive control of anti-Jo-1 and negative controls are useful as a standard for quality control.

ELISA USING RECOMBINANT MYOSITIS AUTOANTIGENS

The following protocol is based on ELISA using recombinant Jo-1 antigen, but the same protocol can be used for any other myositis autoantibody ELISA using recombinant autoantigens.

When ELISA is performed for the detection of autoantibody, there will be differences in the reactivity between samples. However, it should be noted that it does not mean the observed OD reflects the actual recognition of the target autoantigen. Quality/purity of recombinant proteins and ELISA varies, and validation for each ELISA compared with results from a previously established standard method such as IP is necessary.

1. Coat a 96-well microtiter plate (Nunc Immobilizer Amino; Nunc/Thermo Fisher Scientific, Roskilde, Denmark) with purified recombinant Jo-1 antigen by adding 0.5 μg/ml in PBS, 50 μl/well, at room temperature for 2 h or at 4°C overnight. The concentration of antigens may need to be optimized by titration analysis and adjusted for each assay. Any other type of high-binding microtiter plate may be used. The Immobilizer Amino plate covalently cross-links proteins to the plastic surface at amino acid positions of lysine and cysteine. This cross-linking process will not work in the presence of amines such as Tris or glycine. Other types of plate may not be affected by amine.

2. Tap the side of the plate gently and make sure that the whole bottom area of the wells is covered with coating solution. With 50 μl/well it will not spread, but 100 μl/well or more will spread without tapping. Using 50 μl/well will save on the cost of reagent.

3. After the coating/cross-linking step, remove antigen solution completely by tapping on dry paper towels.

4. Block with 0.5% bovine serum albumin (BSA) NET/IGEPAL (150 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl [pH 7.5], and 0.3% IGEPAL CA-630)–0.05% NaN_3, 150 μl/well, for 1 to 3 h at room temperature (or 4°C overnight).

5. Dump blocking buffer and tap on paper towels.

6. Add 100 μl/well of serum diluted in blocking buffer (0.5% BSA NET/IGEPAL–0.05% NaN_3, usually at 1:250 to 1:500, i.e., 2 μl of serum in 500 or 1,000 μl of blocking buffer). Incubate for 1 to 3 h at room temperature.

7. Wash plate three times with Tris-buffered saline (TBS)/Tween 20. There are many alternative types of washing buffer in common use.

8. Add 100 μl/well of alkaline phosphatase-conjugated goat F(ab)′2 anti-human IgG (γ-chain specific) second antibody at 1:1,000 in 0.5% BSA NET/IGEPAL–0.05% NaN_3. Incubate for 1 to 2 h at room temperature.

9. Wash three times with TBS/Tween 20.

10. Prepare the developing solution ~10 min before needed by adding 1 tablet (5 mg) of Sigma 104 substrate/5 ml

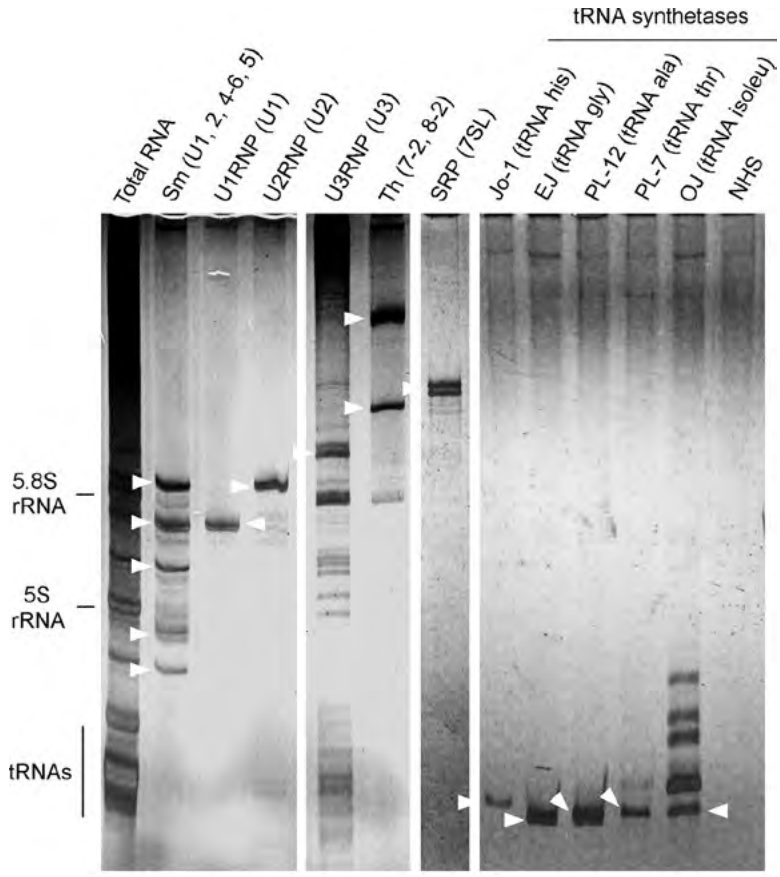

FIGURE 2 IP analysis of RNA components of autoantigens recognized by autoantibodies. K562 cell extract was immunoprecipitated by autoimmune sera. RNA components were extracted, fractionated on urea-PAGE, and silver stained. RNA components of autoantigen are indicated by arrowheads. Sm (U1, 2, 4-6, 5), U1, 2, 4-6, 5 small nuclear RNAs; U1RNP (U1), U1 small nuclear RNA; U2RNP (U2), U2 small nuclear RNA; U3RNP (U3), U3 small nucleolar RNA; Th (7-2, 8-2), 7-2 and 8-2 RNAs; SRP (7SL), 7SL RNAs; NHS, normal human serum. Y1-5 RNAs are also seen in the anti-OJ lane due to coexisting anti-Ro60 antibodies.

diethanolamine buffer. You need 10 ml/plate (100 μl/well × 96 wells = 9.6 ml). Put on rotator or shaker in a 50-ml tube to dissolve. Sigma 104 tablets are sensitive to temperature and humidity. Take the necessary amount quickly and do not leave the vial at room temperature for an extended period of time.

11. Develop plates by adding 100 μl/well of developing solution.

12. Read OD_{405} on a microtiter plate reader. Read OD more than once, including the one when the highest OD is ~1.5 to 2. If samples with low OD are important, overdevelop and read it again. Try to read at least twice for each plate.

VERY IMPORTANT: Do not push pipette all the way when developing solution is added because this will leave bubbles in the wells, which will directly and significantly affect the absorbance of wells. Depending on the operator's pipetting technique, there may still be bubbles. Check all wells for bubbles after pipetting and break them using a 25-gauge needle.

Blocking Buffer: NET/IGEPAL, 0.5% BSA, 0.05% NaN_3

0.15 M NaCl
2 mM EDTA
50 mM Tris-HCl (pH 7.5)
0.3% IGEPAL
0.5% BSA
0.05% NaN_3

Washing Buffer (TBS/Tween 20)

20 mM Tris-HCl (pH 7.5)
150 mM NaCl
0.1% Tween 20

Diethanolamine Buffer

Diethanolamine	97 ml
$MgCl_2 \cdot 6H_2O$	0.1 g
NaN_3	0.2 g
dH_2O	

Adjust pH to 9.6 with concentrated HCl, adjust volume to 1 liter with dH_2O, and filter using a 0.45-μm bottle-top filter.

Interpretation

It is ideal to set a cutoff in each laboratory based on a ROC (receiver operating characteristic) curve established by a large number of positive and negative controls.

Quality Assurance, Quality Control, and Test Validation

Quality and reliability of ELISA depend heavily on the quality of recombinant protein used. Positive controls selected based on conventional methods and negative controls are essential as standards for quality control. Weak but true positive versus false positive is often not easy to differentiate with ELISA.

FUTURE DIRECTIONS OF MSA TESTING

Currently anti-Jo-1 ELISA is the only widely available testing for most clinicians. Line immunoassay is available in certain countries but has not been used extensively. Although IP is a very powerful and reliable technique, it has been performed at only a limited number of laboratories and is not likely to become a routine assay in clinical practice. ELISAs and bead-based assays for several MSAs are currently under development and will become available in the future. Recently identified autoantibodies such as anti-TIF1 and anti-MDA5 have strong clinical significance, and development of commercial immunoassays is in progress. They will be widely available in the near future, and tests for these autoantibodies may become a part of standard tests in clinical practice for inflammatory myopathy.

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EIGHTH EDITION

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Chapter 25

Antihistone and Antispliceosome Antibodies

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List of Abbreviations

ANA Antinuclear antibody
DID Double immunodiffusion
DIL Drug-induced lupus
ELISA Enzyme-linked immunosorbent assay
ENA Extractable nuclear antigens
MCTD Mixed connective tissue disease
PHA Passive hemagglutination
snRNA U-rich small nuclear RNAs
snRNP Small nuclear ribonucleoprotein
UCTD Undifferntiated connective tissue disease

Autoantibodies directed against intracellular antigens are characteristic features of systemic lupus erythematosus (SLE) and other systemic autoimmune diseases. Studies have provided strong evidence that autoantibodies are produced by antigen-driven responses and they can be reporters from the immune system, revealing the identity of antigens involved in the pathognomonic mechanism. Some of these autoantibodies serve as disease-specific markers and are directed against intracellular macromolecular complexes or particles such as nucleosomes, small nuclear ribonucleoproteins (snRNPs), and Ro and La cytoplasmic ribonucleoproteins (RNPs). This chapter discusses the finer specificity of these autoantibodies.

ANTIHISTONE ANTIBODIES

The identification of lupus erythematosus (LE) cell phenomenon by Hargraves et al.[1] led to the recognition of autoimmune reactivity as a major feature of SLE. The LE cell phenomenon—which was based on the observation that cellular components released during cell death (particularly nuclei) can be phagocytosed by neutrophils in the milieu of certain plasma factors—is a classic immunoassay that was included in the previous criterion for classification of SLE.[2] Subsequent studies showed that antibodies to histones were a key requirement for the LE cell phenomenon and they hold a distinguished position in the recognition of the autoimmune nature of SLE, which lead to the discovery of many other antinuclear antibodies (ANA). It was concluded from these early studies that autoantibodies to deoxyribonucleoprotein recognize histone–DNA complexes, and this specificity was directly related to the LE cell factor.

Histones are Key Protein Components of Chromatin

Histones are bound to genomic DNA and organized into a macromolecular complex referred to as native chromatin. In addition to DNA and histones, which constitute 80% of its mass, chromatin contains nonhistone proteins, many of which are also autoantibody targets in rheumatic diseases—proteins such as the centromere and high-mobility group proteins.[3] Histones and DNA constitute the repeat subunit of chromatin called the nucleosome, which consists of two molecules of each of the "core" histones—H2A, H2B, H3, and H4—forming an octamer, along with a histone H1 molecule and of approximately 200 base pairs of DNA. An artificial complex of the H2A-H2B dimer and DNA can be formed in vitro at physiological conditions. This (H2A-H2B)-DNA complex is an important antigenic target in patients with SLE and drug-induced lupus (DIL).

Assays for Antihistone Antibodies

In SLE, most autoantibodies to the histone-DNA complexes can be detected as a homogeneous or diffuse indirect immunofluorescence staining pattern of the nuclei and the condensed mitotic chromatin (Figure 1(A)). A similar pattern is also seen with other autoantibodies, notably those directed

Systemic Lupus Erythematosus. http://dx.doi.org/10.1016/B978-0-12-801917-7.00025-5

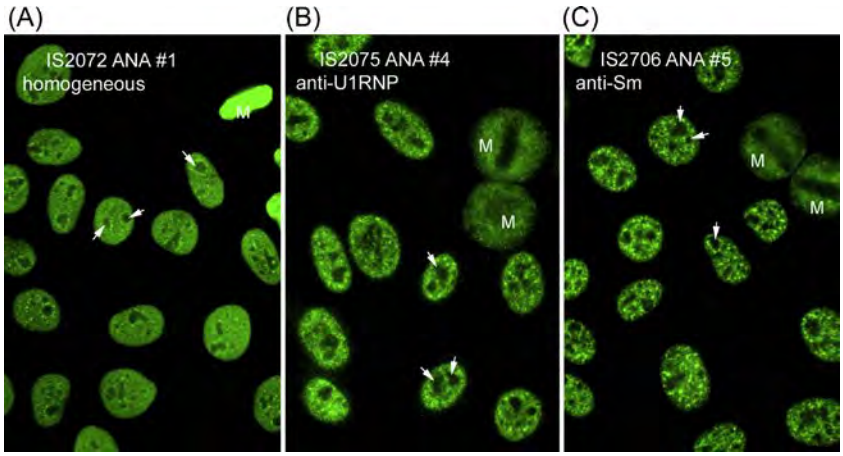

FIGURE 1 Indirect immunofluorescence of antihistone and antispliceosome antibodies on human HEp-2 cells. Staining patterns of standard sera from the Centers for Disease Control and Prevention (CDC) IS2072 for anti-DNA homogeneous pattern (A), IS2075 anti-U1RNP (B), and IS2076 anti-Sm (C). *HEp-2 substrate was obtained from INOVA Diagnostics, Inc., San Diego (CA, USA). M, mitotic cell; arrows, nucleoli. Original magnification 400×. Note that variations in staining patterns may depend on HEp-2 cell substrates (manufacturer and lot).*

against dsDNA. This pattern must be discriminated from the dense fine speckled pattern which also stains interphase nuclei and metaphase chromatin and is the hallmark of autoantibodies to DFS70/LEDGF antigens. Sera with antibodies to certain histone classes (e.g., H1, H3, H4) or hidden determinants (cryptotopes) on native or denatured histones may show a weak or even negative ANA; thus, more specific assays employing purified analytes are preferable.

Solid Phase Enzyme-Linked Immunosorbent Assays for Antihistone Antibodies

Enzyme-linked immunosorbent assays (ELISAs) are often used to detect specific antihistone and antichromatin antibodies, and this platform can be adapted to measure reactivity to individual histones, macromolecular histone complexes, and chromatin. Most immunoassays for the detection of antihistone antibodies in the past two decades have relied on histones purified from various cells or tissues, such as calf thymus. Subnucleosome structures have also been adapted to ELISA formats, which allow measurement of autoantibodies requiring these higher-ordered structures.

Problems and Discrepancies in Measuring Antihistone Antibodies

There are many possible explanations for discrepancies in the literature on the prevalence and fine specificity of antihistone antibodies. The quality of histones used as antigens can be highly variable, and histones from commercial sources were often degraded or contaminated with nonhistone proteins. In addition, the propensity for histones to bind nascent DNA in serum and other biological fluids can result in artifacts, such as false-positive reactions with anti-DNA antibodies. Such factitious binding to histone/DNA complexes that are formed in vitro is a phenomenon that is generally indistinguishable from bona fide antihistone antibody reaction.[4] DNA existing in serum in the form of mono- and oligonucleosomes may also have pathologic significance in that circulating nucleohistone binding to the negatively charged residues on heparin sulfate of the glomerular basement membrane may mediate the binding of DNA and anti-DNA antibodies to the glomerulus.

Antibodies to denatured purified histones detected by most immunoassays are common in systemic rheumatic diseases and appear to have limited value as specific diagnostic marker.[4] Other assays which use histone-DNA complexes, including LE cells, chromatin, soluble (H1-stripped) chromatin, (poly) nucleosomes, and (H2A-H2B)-DNA complexes would more likely be detecting reactivity to native autoepitopes. It is possible that in some immunoassays there is overlap of available epitopes in denatured histones and native (DNA-bound) histones; however, for the most part, autoantibodies directed to histones and to nucleosome-related antigens should be considered distinct and one cannot be substituted for the other. In the context of autoimmunity, the terms chromatin, nucleosome, and polynucleosome tend to be used interchangeably.[5]

Prevalence and Disease Association of Antihistone and Antinucleosome Antibodies

Reports of antihistone antibodies in various diseases are summarized in Table 1. Although antihistone antibodies have been observed in various rheumatic diseases, most studies have focused on SLE or DIL. Reported prevalence ranged from 17% to 95% in SLE and 67–100% in DIL.[4] Antihistone antibodies have also been consistently observed in rheumatoid arthritis and juvenile idiopathic arthritis. In some cases, a remarkably high prevalence was observed in other diseases, especially in primary biliary cirrhosis, autoimmune hepatitis, and ANA-positive neoplastic diseases.

In addition to the antibodies reactive with isolated histones, patients with up to ~75% lupus-like disorders

TABLE 1 Prevalence of Antihistone/Antinucleosome Antibodies in Human Diseases

Disease/Syndrome[a]	Prevalence[b]
Rheumatic Diseases	
SLE	24–95%
Drug-induced lupus	50–100%
Drug-induced ANA	22–95%
Rheumatoid arthritis	0–80%
Vasculitis	31–75%
Felty's syndrome	79%
Juvenile chronic arthritis	42–75%
Mixed connective tissue disease	45–90%
Sjögren's syndrome	8–67%
Systemic sclerosis	23–67%
Polymyositis/dermatomyositis	17%
Other Diseases	
Primary biliary cirrhosis	50–81%
Hepatic cirrhosis/autoimmune hepatitis	35–50%
Inflammatory bowel disease/ulcerative colitis	13–15%
Neoplastic diseases	14–79%

[a]*Modified from Fritzler and Rubin.*[4]
[b]*Prevalence of elevated IgG and/or IgM antibody to total histone or to at least one histone class.*

commonly have autoantibodies to chromatin (nucleosomes). In this context, it is important to appreciate that antinucleosome and anti-dsDNA responses appear to be particularly sensitive to corticosteroid therapy. Other studies from various geographic regions reported 38–86% sensitivity of antinucleosome antibodies in SLE. Most patients with lupus induced by procainamide, penicillamine, isoniazid, acebutolol, methyldopa, timolol, and sulfasalazine also have antinucleosome antibodies, predominately reactive with the (H2A-H2B)-DNA complex. Several groups reported antinucleosome antibodies in almost half the patients with autoimmune hepatitis. Antinucleosome antibodies have also been reported in 25–50% of patients with SSc (systemic sclerosis, scleroderma), but the specificity of these antibodies may be different from bona fide antichromatin antibodies as discussed below. A high prevalence of antinucleosome antibodies have generally not been reported in other rheumatic diseases and are remarkably lower in healthy cohorts, resulting in an overall sensitivity for SLE of 63% and when compared to other rheumatic diseases, a specificity for SLE of 95%.[5]

Antihistone in SLE

Studies on the association of antihistone antibodies with disease activity or severity, or with specific clinical features or organ involvement, have been inconsistent.[4] In recent years, studies that used nucleosome antigens have shown strong correlation with symptomatic SLE and a clinical specificity in such patients that was 95–99%. Antichromatin and anti-[(H2A-H2B)-DNA] antibodies were significantly correlated with glomerulonephritis and were more specific for this feature than anti-DNA. A higher prevalence and/or amount of antichromatin antibodies in SLE patients with kidney disease, progression to renal failure, or overall disease activity score have been reported, although association with disease activity was not seen in all studies (see Ref. 6 and references therein). Other studies have shown that nucleosomes can be found in the circulation due to aberrations during apoptosis and/or an ineffective clearance in SLE and during apoptosis, histones can be modified through acetylation and possibly other modifications, thereby possibly making them more immunogenic. While more definitive comparative studies are still required, current studies indicate that native nucleosomes are substantially better analytes for monitoring SLE than assays using purified histones, and many studies have concluded that antinucleosome antibodies are a better biomarker for SLE than anti-DNA, although this concept has been challenged.[7]

Antihistone in Drug-Induced Lupus

Histone-reactive antibodies have been reported in 50–100% of patients with DIL (Table 1), depending on the drug and the assay employed. The drugs most commonly implicated in DIL include procainamide, hydralazine, quinidine, and isoniazid; however, these drugs are currently rarely used, except isoniazid, and a variety of other drugs have been implicated as well. However, most patients who are treated with procainamide and other lupus-inducing drugs eventually develop antihistone antibodies, even though symptomatic disease occurs in only 10–20% of patients. Thus, most antihistone antibodies in this clinical setting are apparently relatively benign, consistent with the seemingly innocuous occurrence of histone-reactive antibodies in many rheumatic and nonrheumatic diseases (Table 1). However, examination of their class and fine specificity has revealed that antihistone antibodies in asymptomatic patients are predominantly IgM and display broad reactivity with all the individual histones. In contrast, patients with symptomatic DIL develop predominantly IgG antihistone antibodies that display pronounced reactivity with the H2A-H2B complex, especially when bound to DNA. In fact, anti-(H2A-H2B) has been observed to precede overt clinical symptoms and therefore may have predictive as well as diagnostic value. Anti-(H2A-H2B) has a sensitivity approaching 100% and a specificity of >90% for symptomatic procainamide-induced lupus compared to asymptomatic procainamide-treated

patients and an even higher specificity when an (H2A-H2B)-DNA complex is used as the screening antigen.

A convincing argument that chromatin drives the bulk of the histone-reactive antibody response in SLE and most DIL can be made from the data that compare the antigenicity of various forms of histones. Antibodies from patients with SLE and DIL as well as antibodies from murine lupus bound prominently to a structural epitope in the (H2A-H2B)-DNA complex. Antibodies in some patients with SLE also bound native DNA and $(H3\text{-}H4)_2$-DNA, but reactivity with individual histones was much lower. In murine lupus, antibodies to (H2A-H2B)-DNA were found early in disease, before antibodies to native DNA and $(H3\text{-}H4)_2$-DNA arose. Absorption with chromatin removed most of the antibody reactivity to subnucleosome structures, indicating that regions buried in chromatin were not antigenic in SLE, DIL, or murine lupus. Thus, antihistone antibodies in SLE can be most readily explained by autoimmunization with native chromatin accompanied by sequential loss of tolerance first to the (H2A-H2B)-DNA region and then to $(H3\text{-}H4)_2$-DNA and native DNA. Loss of immune tolerance to epitopes on DNA-free histones, which can be considered "denatured histones," and to "denatured DNA" (and to other nuclear antigens) may accompany the immune dysregulation associated with lupus-related disorders; however, because of the complexity of these epitopes and the heterogeneity of this immune response, only with nucleosome-reactive antibodies can a strong case be made for the putative in vivo existence of a chromatin-like immunogen.[4] More recent evidence links the antichromatin response to the release of DNA neutrophil extracellular traps (NETs).[8]

ANTISPLICEOSOME ANTIBODIES

The major autoantigens in spliceosome are snRNP and autoantibodies to snRNPs have been a focus of research and clinical immunological studies related to autoantibodies in rheumatic diseases for over three decades. Anti-Sm antibodies, identified over 40 years ago, were one of the first described to target nonhistone proteins in systemic autoimmune rheumatic diseases. Anti-Sm antibodies are highly specific (>90%) markers for SLE, although the prevalence in SLE is 5–30%. The Sm antigen was defined as proteins bound to U-rich small nuclear RNAs (snRNAs) and these autoantibodies have served as useful probes to help investigate the molecular and cellular functions of the spliceosome, which is responsible for pre-mRNA splicing of heterogeneous nuclear RNA to mature messenger RNA (mRNA). In this section, we discuss the major classes of autoantibodies to snRNP including anti-Sm (Smith), anti-U1RNP (also known as anti-nRNP), anti-U1/U2RNP, and briefly review minor autoantibodies to other classes of UsnRNPs, such as LSm (Like Sm) proteins.

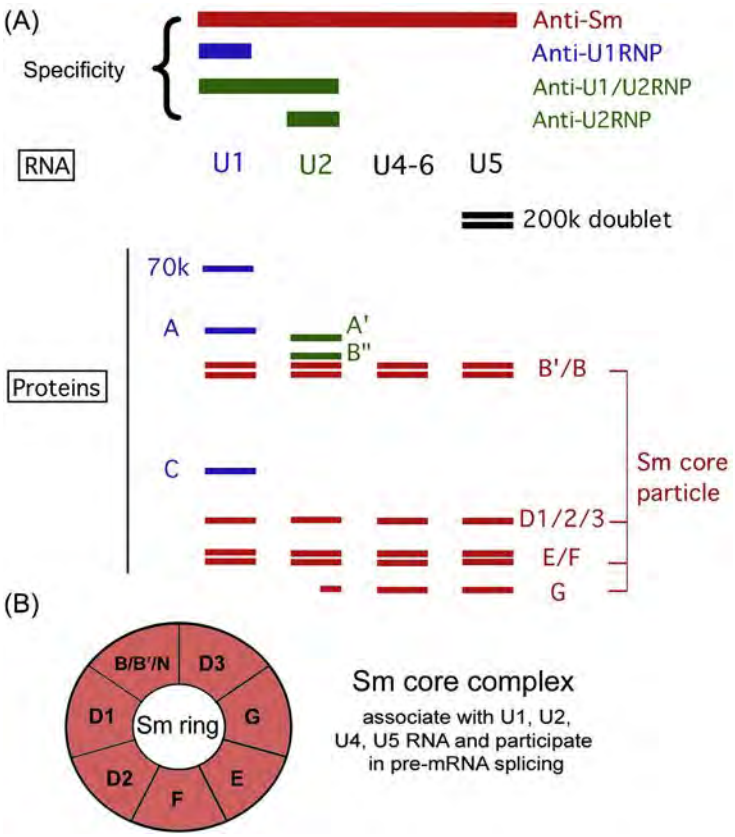

FIGURE 2 Structure and components of snRNPs. (A) RNA and protein components of snRNPs. Specificity of anti-Sm (red), -U1RNP (blue), -U1/U2RNP, and -U2RNP (green), and RNA and protein components of each snRNP are shown. (B) Components of Sm ring. Seven-member Sm ring has structural similarity to a doughnut. The center of the structure is involved in binding to single-stranded RNA.

Cellular Localization and Function of snRNP

Components of snRNPs. snRNPs are classified by association with specific snRNAs including the most abundant U1, U2, U4, U5, and U6 RNAs (Figure 2(A)). U6snRNP uses one of the LSm rings, which are structurally similar to the Sm ring, as its core complex (Figure 2(B)). Each snRNP is an RNA-protein macromolecule of corresponding UsnRNA complexed with several proteins. Common anti-snRNPs autoantibodies are classified into anti-U1RNP that recognize U1snRNPs and anti-Sm that recognize U1, U2, U4-6, and U5 snRNPs (Figure 2(A), see specificity). The Sm core proteins B or B' (27/28 kDa), D1/D2/D3 (14 kDa), E (12 kDa), F (11 kDa), and G (9 kDa), which are organized as seven-member ring structures (Figure 2(B), Sm ring, Sm core particle) are shared by U1, U2, U4/U6, and U5 snRNPs. Because these shared Sm core proteins are recognized by anti-Sm antibodies, U1, U2, U4/U6, and U5 snRNAs are immunoprecipitated by anti-Sm antibodies versus only U1RNA immunoprecipitated by anti-U1RNP antibodies (Figure 2(A)).

In addition to the Sm core particle, each snRNP is associated with several unique proteins. U1snRNPs (U1RNP) has U1snRNP specific proteins U1-70k (68/70kD), A (33kD), and C (22kD). U2snRNP has two unique proteins, U2-A′ and B″. U4/U6snRNP and U5snRNP have several unique proteins in addition to the Sm core particle that are not included in Figure 2(A), except the U5-200kD doublet.

Reactivity of Anti-snRNPs Autoantibodies

Following immunoprecipitation (IP) using sera with anti-snRNP autoantibodies, RNA and protein components can be analyzed (Figure 3). U1RNA is seen in anti-U1RNP immunoprecipitates (lane U1RNP), while U1, U2, U4, U5, and U6 RNAs are detectable with anti-Sm serum (lane Sm, Figure 3(A)). Protein components are usually analyzed by IP using ^{35}S-methionine labeled cell extract (Figure 3(B)).

To identify which proteins are directly recognized, western blot using affinity-purified snRNPs is the standard method. Anti-U1RNP sera frequently react with U1-70k, A, B′/B, and less frequently with C, in various combination

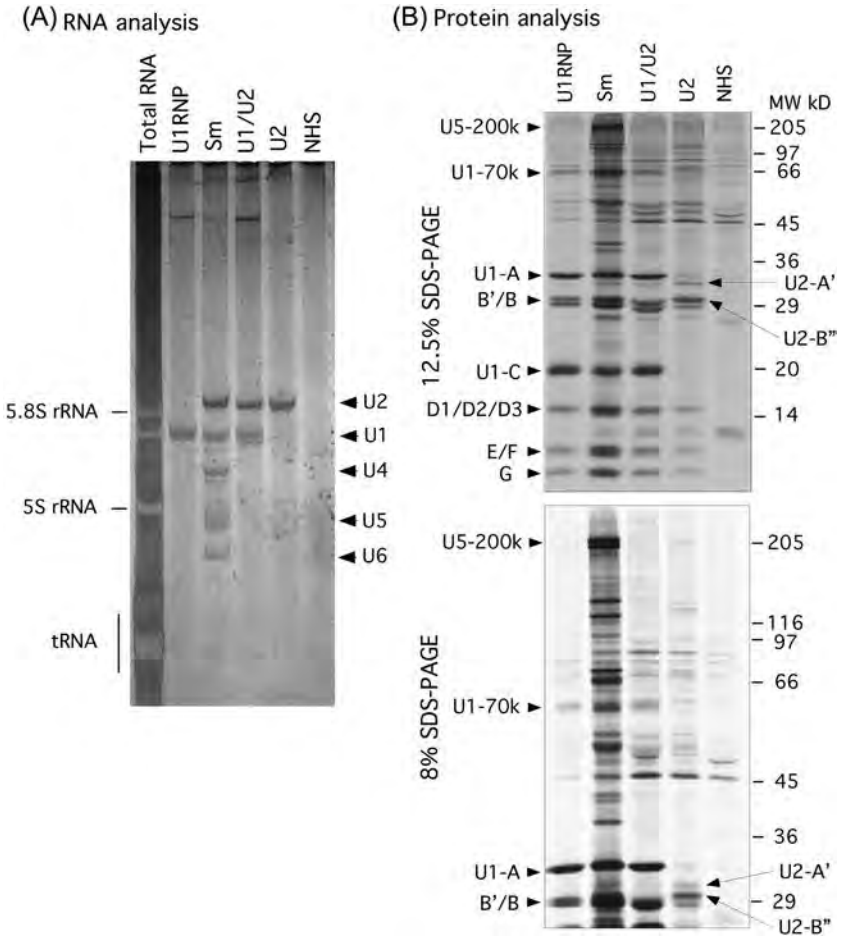

FIGURE 3 **Immunoprecipitation using anti-snRNPs antibodies.** A. RNA components immunoprecipitated from cell extract by human sera were extracted, run on urea-polyacrylamide gel, and identified by silver staining. B. snRNP associated proteins metabolically radiolabeled with ^{35}S-methionine were immunoprecipitated from cell extracts of K562 cells by human autoimmune sera and fractionated by 12.5% or 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography.

of reactivity. Anti-Sm antibodies react with Sm-B′/B and D1/D2/D3 proteins. Because virtually all human anti-Sm sera also have anti-U1RNP antibodies, reactivity with U1 unique proteins are also seen in anti-Sm positive sera. In contrast to short linear epitopes for T-cells, classic characteristic of autoimmune B-cell epitopes are discontinuous conformational epitopes as shown within each polypeptide of the snRNPs.[9] In addition, autoantibodies that recognize the conformational structure of the multiprotein complexes, possibly quaternary structure, have been described, including EFG complex[10] and U1-C-Sm core particle.[11] Autoantibodies to LSm4 and LSm complex also were reported.[12]

History of Detection of Autoantibodies to snRNPs and Potential Problems

Our understanding of the difference in reactivity of anti-U1RNP versus anti-Sm is incomplete, and changes in technology further complicate the issue. Assay technologies started with Ouchterlony double immunodiffusion (DID) followed by passive hemagglutination (PHA), IP detection of UsnRNA and protein components, western blot detection of reactivity to individual protein components, eventually evolving to line immunoassays (LIA), ELISA, addressable laser bead immunoassays (ALBIA), chemiluminescence immunoassay (CIA), and other multiplexed immunoassays. The source of antigens (analytes) used in various immunoassays also has been changed; antigens have spanned the spectrum from calf or rabbit thymus extract to human cell lines and recombinant proteins.

Historically, DID was one of the first assays used to detect anti-Sm reactivity. Anti-U1RNP was originally reported as anti-Mo that recognizes soluble nuclear ribonucleoprotein (nRNP) and makes a distinctive precipitin line from anti-Sm in DID. At about the same time, the same group of specificities (anti-snRNPs) were reported as anti-ENA that was based on a classification into RNase sensitive anti-ENA (correspond to anti-U1RNP) and RNase-resistant anti-ENA (correspond to anti-Sm). After this period, the source of antigens used for immunoassays had gradually shifted from calf or rabbit thymus tissues to human culture cell lines and recombinant human proteins. This was paralleled by a switch in ANA substrates from cryopreserved animal tissue to human culture cell lines such as HEp-2 (laryngeal cancer).

Detection of Antibodies to snRNPs in Clinical Practice

Antibodies to U1RNP and anti-Sm are autoantibody specificities that show a typical nuclear speckled pattern in ANA by immunofluorescence (Figure 1(B) and (C)). There are many other autoantibody specificities that show a similar ANA pattern but, certainly, anti-U1RNP and Sm are specificities that should be considered when the nuclear speckled pattern is observed. The titer of ANA is often very high, 1:1280 or even higher, particularly in patients with overlapping features of SLE, SSc, PM/DM—referred to as mixed connective tissue diseases (MCTD)—as well as in certain SLE or undifferentiated connective tissue disease (UCTD).

To confirm anti-U1RNP, -Sm specificities, a commonly used assay in the diagnostic laboratory is ELISA using recombinant or affinity purified antigens. Although ELISA will identify the majority of true positives, it may be troubled by a significant percentage of false positives, in particular anti-Sm.[13] Improved sensitivity of the anti-U1RNP ELISA using recombinant U1-70k, A, and C proteins was accomplished by adding U1RNA, via the formation of new epitopes resulted from interaction of U1RNA with the proteins antigens.[14] Other improvements in ELISA were the use of symmetric dimethylarginine modified Sm-D derived peptide to detect anti-Sm antibodies[15] based on earlier studies indicating the importance of this posttranslational modification in anti-Sm antibody reactivity.

DID was the original method to detect and define the anti-U1RNP and Sm specificities, but it was less commonly used after the 1990s. With the recent emergence of large, high-throughput laboratories, the emphasis has shifted to assays, such as ELISA and ALBIA, which can be automated, effecting cost savings and rapid turnaround times.[16] More recently, LIA, which is similar to dot blot immunoassay but contains multiple autoantigens individually printed on membrane strips, has been adopted by some laboratories. Other new types of assays include ALBIA, in which individual antigens are covalently bound to beads of different composition. Advantages of these assays include the detection of multiple autoantibodies in a single assay and the requirement for only small amounts (typically 10 μl) of serum sample.

Clinical Significance of Antibodies to snRNPs

Distribution and Coexistence of Anti-U1RNP and Anti-Sm Antibodies. Although anti-U1RNP and Sm often coexist, there are major differences in clinical significance of these two specificities. Anti-Sm antibodies are highly specific for the diagnosis of SLE, whereas anti-U1RNP can be found in patients with various diagnoses, particularly MCTD, as well as unclassified conditions, such as UCTD, and is not specific for SLE (Figure 4). The prevalence of anti-U1RNP in UCTD is 6–22% while anti-Sm is only 1–3%.[17]

Anti-U1RNP and anti-Sm antibodies have a unique interrelationship. Virtually all anti-Sm positive patients also have anti-U1RNP, with anti-Sm alone rarely found. However, only a fraction of patients with anti-U1RNP have anti-Sm antibodies. In SLE, usually 25–40% of anti-U1RNP positive patients have anti-Sm. This ratio (anti-Sm/anti-U1RNP) becomes much lower when all anti-U1RNP positive cases are analyzed because anti-U1RNP can be seen in diseases other than SLE and unclassified conditions (Figure 4).

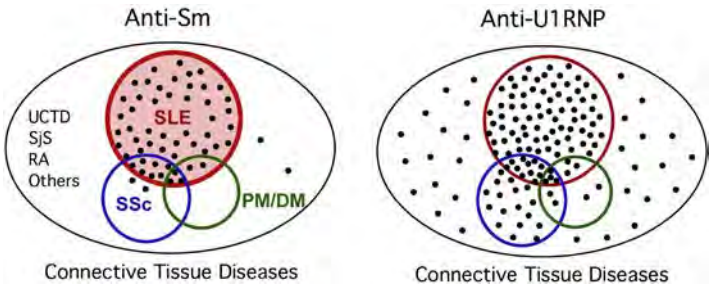

FIGURE 4 Distribution patterns of anti-Sm versus U1RNP in patients with connective tissue diseases. Typical distribution pattern of anti-Sm versus anti-U1RNP in SLE, SSc, PM/DM, and other connective tissue diseases is illustrated. Data are only to illustrate the trend and not based on actual numbers from two reports.[13]

Clinical Association of Anti-Sm and U1RNP Antibodies. Anti-Sm is highly specific for the diagnosis of SLE and is one of the most established and widely utilized disease marker antibodies. It is under the immunologic criteria, along with anti-dsDNA and anti-phospholipid antibodies, in the SLICC Classification system for SLE.[18] Many anti-Sm positive patients have typical SLE; however, anti-Sm is often seen in patients with SLE-overlap syndromes, which also have features of SSc and/or PM/DM (Figure 4) and some of them have more than one disease marker antibody. Because the production of specific autoantibodies usually precedes the development of typical clinical manifestation,[19] a small number of patients may not meet the classification criteria for SLE. In contrast, although anti-U1RNP antibodies are common in SLE patients, they can be found in SSc, PM/DM, Sjögren's syndrome and other conditions, and were associated with certain clinical manifestations such as Raynaud's phenomenon, swollen hand, sausage-like fingers, leukopenia, regardless of the diagnosis. Although, by definition, virtually all patients with MCTD are positive for anti-U1RNP antibodies, they are not a specific biomarker for MCTD. Why different clinical diagnosis and features are associated with anti-U1RNP versus anti-Sm, with both recognizing closely related UsnRNPs, is not known.

The clinical association and significance of anti-U1RNP and anti-Sm described above appears to be consistent regardless of ethnicity and certain other demographic variables; however, studies on detailed clinical association are more inconsistent. The reasons may be due to the differences in immunoassays used, genetic and environmental backgrounds of patients, focus and methods in clinical analysis, as well as treatment and follow-up.

The fact that most anti-Sm positive sera are also positive for anti-U1RNP makes anti-U1RNP antibodies a major confounding factor in clinical analysis. The only practical option to analyze the clinical associations of anti-Sm may be an analysis of anti-Sm+U1RNP versus anti-U1RNP cohorts. Similarly, clinical associations and immunological cross-reactivity of anti-Sm with anti-ribosomal P or with anti-dsDNA may also bias the data on clinical associations of anti-Sm. This might be part of the reason why clinical manifestations associated with anti-Sm were also associated with either anti-U1RNP or anti-dsDNA antibodies. Some reports suggested that the titers of anti-Sm antibodies correlate with the disease activity, milder renal and central nervous involvement, or late-onset renal disease, but these findings are controversial.[20]

A clinical concept of MCTD characterized by overlapping features of SLE, SSc, and PM/DM was proposed. MCTD has a high prevalence of Raynaud's phenomenon, edema of the fingers, arthritis/arthralgia, myositis, serositis, favorable response to steroid treatment, and a relative absence of renal disease. Although early studies emphasized a good prognosis of MCTD, long-term follow-up studies reported premature death in a subset of patients. Pulmonary hypertension and interstitial lung disease are particularly known as life-threatening comorbidities associated with anti-U1RNP antibodies.[21]

When anti-U1RNP antibodies are present alone in high titer, patients often present with typical features of MCTD; however, anti-U1RNP antibodies are also detected in other systemic rheumatic diseases. Because of the clinical relevance of the titers observed in systemic rheumatic diseases, consideration of the levels of antibodies may be important because patients with high levels of anti-U1RNP antibodies tend to have more typical features of classical MCTD.

Some SLE and MCTD sera bind most of the snRNP polypeptides, whereas others bind to little, if any, U1-70k or C polypeptides. The observed high frequency of anti-U1-70k antibodies in MCTD has been supported in several studies and reported to be as high as 76–95% in MCTD, while the range of reactivity in SLE is 8–50%, although the frequency is highly variable. It has been suggested that the presence of anti-U1-70k is primarily associated with classical features of MCTD, such as Raynaud's phenomenon, esophageal dysmotility, and myositis, and it is a negative indicator for the presence of renal disease. Although antibodies to the U1-70k, A, or C protein quantitatively vary during the disease course, there is little evidence that they correlate with disease activity or that they are involved in disease pathogenesis.

An association of the presence of anti-U1RNP in cerebrospinal fluid (CSF) and increased anti-U1RNP index [(CSF anti-U1RNP/serum anti-U1RNP)/(CSF IgG/serum IgG)] with neuropsychiatric symptoms in SLE and MCTD patients has been reported.[22] Anti-U1RNP antibodies in

CSF also were correlated with increased interferon-α and MCP-1 in CSF.[23] These observations suggest intrathecal production of anti-U1RNP antibodies and the possible role of anti-U1RNP antibodies in neuropsychiatric symptoms.

Other Anti-snRNPs Antibodies

Anti-U1/U2RNP antibodies were reported in patients with overlapping feature of SLE, SSc, and/or PM/DM. Antibodies to U4/U6RNP, U5RNP, and trimethylguanosine cap structure of UsnRNA that recognize U1-U5RNAs were also reported in some cases. Antibodies to UsnRNPs were originally thought to target only protein components; however, later studies showed that patients with autoantibodies to snRNPs often (35–38%) have antibodies directed to U1RNA.

Immunologic Characteristics of Anti-U1RNP and Sm Antibodies

Anti-U1RNP and Sm antibodies have been associated with hypergammaglobulinemia, with reported levels up to 20–30%, 8.6 mg/ml, of the total IgG, in striking contrast to relatively low concentrations of anti-dsDNA antibodies, 1–3 μg/ml. It has been emphasized in early studies that the titers of anti-U1RNP do not fluctuate significantly over time. Although this is true for many patients with MCTD, the levels of anti-U1RNP antibodies may not be as stable as originally believed, particularly in SLE patients. This issue was revisited recently because of the new concept of autoantibody production by short-lived plasmablasts in lymphoid tissue versus long-lived plasma cells in bone marrow[24] and of B-cell depleting therapy using rituximab.[25] B-cell depletion dramatically reduced the levels of anti-dsDNA antibodies but did not affect the levels of anti-U1RNP, Sm, Ro, and La, or total immunoglobulin levels.[25] These data are consistent with the interpretation that anti-U1RNP and Sm antibodies are mainly produced by long-lived plasma cells and may explain their relatively stable production.

Mechanism of Production

Despite various hypothesis and observations, the mechanisms of production of autoantibodies in SLE, and in particular the mechanism of selection of the target antigens, are incompletely understood. Different mechanism may play a role in breaking tolerance, spreading epitopes, and sustaining autoantibody production. Molecular mimicry may be a trigger, while intermolecular-intrastructural help mechanisms may be responsible for the epitope spreading. Apoptosis, microbody release, and Toll-like receptor (TLR) stimulation are also likely key factors in these processes. Thus, the actual mechanism is likely multifactorial and, to a certain extent, specific to individual patients based on genotype, environment, and epigenetic factors.

Production of anti-snRNPs antibodies in murine models of SLE appears to be dependent on IFN-γ, IL-12, and type I IFN (I-IFN). Levels of I-IFN inducible genes Mx1 and others were higher in patients with autoantibodies to U1RNP/Sm versus patients without antibodies to RNA-protein complex,[26,27] suggesting the association between I-IFN production and anti-U1RNP/Sm antibodies. However, it is not known whether high I-IFN in these patients is responsible for anti-snRNPs antibody production or if this is a result of I-IFN induction by anti-snRNPs immune complex. Studies indicate that anti-U1RNP immune complexes can be internalized via Fc receptor and released U1RNA stimulate TLR-7 to induce I-IFN.[28]

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ストレス科学　31(1)：29－36，2016

［特別企画：職業性ストレス研究のトピックス
―代表的な職業性ストレスモデルの動向と注目されている心理社会的要因―］

企業における組織的公正と労働者の健康：修飾要因に着目した近年の研究動向

井　上　彰　臣[1)]

抄録：組織的公正は(1)分配的公正（職場組織における意思決定［評価や処遇］の結果に関する公正性），(2)手続き的公正（意思決定に至るまでのプロセスに関する公正性），(3)相互作用的公正（上司の部下に対する接し方に関する公正性）に大別される。国内外の先行研究によって，組織的公正（主に手続き的公正，相互作用的公正）が損なわれた職場環境下では，労働者の身体疾患（虚血性心疾患，死亡），精神疾患（うつ病），疾病休業等の健康障害のリスクが高まることが報告されているが，近年では，組織的公正と労働者の健康との関連を修飾する要因についても，その関心が集まっている。本稿では，組織的公正の概念とその測定方法について説明した後，組織的公正と労働者の健康との関連を修飾する要因に着目した，近年の国内外の研究について紹介する。

Key words：分配的公正，手続き的公正，相互作用的公正，健康影響，修飾効果

Ⅰ．はじめに

我が国では，企業における年功序列・終身雇用制が崩壊し，成果主義の導入が進んでいる[1)]。それに伴い，企業と労働者との関係にも大きな変化が生じ，企業が人事考課や資源配分を行う際に，その公正性を担保することが重要な課題となってきている。ヨーロッパ諸国（主にイギリスやフィンランド）では，このような企業組織の公正性（組織的公正/組織公正性：organizational justice）にいち早く着目し，組織的公正が労働者の健康に及ぼす影響について，既に多くの知見が報告されている[2)~4)]。我が国においても，2010年頃から組織的公正と労働者の健康との関連に関する知見が報告され始め[5)~12)]，その一部は，本誌の特別企画[13)]や他誌の総説等[14)~16)]で紹介されている。一方で，近年では，組織的公正と労働者の健康との関連を修飾する（強めたり，弱めたりする）要因についても，その関心が集まっている。本稿では，組織的公正の概念とその測定方法について説明した後，組織的公正と労働者の健康との関連を修飾する要因に着目した近年の国内外の研究について紹介し，最後に実践への応用について考察する。

Ⅱ．組織的公正の概念とその測定方法

組織的公正は主に，(1)分配的公正（distributive justice）[17)]，(2)手続き的公正（procedural justice）[18)]，(3)相互作用的公正（interactional justice/relational justice）[19)]の3つに大別される。分配的公正は，「職場組織における意思決定（評価や処遇）の結果に関する公正性」を表す概念であり，産業保健の領域では，「努力－報酬不均衡モデル」[20)]として，労働者の健康との関連が調べられている。手続き的公正は，「意思決定に至るまでのプロセス

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に関する公正性」を表す概念であり，①意思決定に一貫性があること（一貫性のルール），②私利私欲や先入観により偏った意思決定にならないこと（偏向抑制のルール），③情報や意見が正確に集められていること（正確性のルール），④色々な視点から訂正や修正をする機会が与えられていること（修正可能性のルール），⑤さまざまな関係者の意見が反映されること（代表性のルール），⑥道徳的，倫理的に適切な意思決定であること（倫理性のルール）の6つの基準を満たすことが重要とされている[21]。相互作用的公正は，「上司の部下に対する接し方に関する公正性」を表す概念であり，①上司が部下を1人の人間として尊敬し，大切にしていること，②上司が部下に対し，適切に情報提供を行っていることの2要素が重要とされている。研究者によっては，これらの2要素のうち，前者を対人的公正（interpersonal justice），後者を情報的公正（informational justice）として区別する場合もある[22]。前述のように，産業保健の領域では，分配的公正は「努力－報酬不均衡モデル」として扱われていること，また，手続き的公正と相互作用的公正は，分配的公正を生み出す上位概念と考えられていることから，主に手続き的公正と相互作用的公正に着目して，労働者の健康との関連が調べられている[23]。

組織的公正を測定する評価尺度は，これまでに数多く開発されているが，産業保健の領域では，主にMoormanの尺度[24]が使用され，産業・組織心理学の領域では，主にColquittの尺度[25]が使用されている。前者は手続き的公正と相互作用的公正のみを測定することが可能だが，後者は分配的公正，手続き的公正，対人的公正，情報的公正のすべての下位概念を測定することが可能である。いずれも5件法のリッカート尺度であり，高得点であるほど，回答者が知覚している公正性が高いことを表す。これらの尺度はいずれも邦訳され，前者はInoueら[26]，後者はShibaokaら[27]によって，その信頼性と妥当性が報告されている。

Ⅲ．組織的公正と労働者の健康との関連を修飾する要因に着目した国内外の研究動向

組織的公正と労働者の健康（主に精神的健康や疾病休業）との関連を修飾する要因に着目した研究は，国内で4件[28)～31)]，海外で3件[32)～34)]報告されている。ここでは修飾要因を，(1)職場関連要因，(2)個人要因，(3)生活習慣の3つに分類して，その詳細を紹介する。

1　職場関連要因による修飾効果

組織的公正と労働者の健康との関連を修飾する職場関連要因として，雇用の不安定性（job insecurity），雇用形態，勤務時間の裁量権（work-time control），職場環境の変化，職場のソーシャル・キャピタル（職場の一体感），企業規模による修飾効果が検討されている。

Kaustoら[33]は，フィンランドの自治体の技術部門に勤務する職員1,443名（本文中には男女別の人数に関する明確な記載はないが，表中のデータから算出すると，男性1,010名，女性433名）を対象に，手続き的公正，相互作用的公正と情緒的消耗感（emotional exhaustion）およびストレス症状（stress symptoms）との関連における，雇用の不安定性の修飾効果を検討している。その結果，雇用が不安定であるほど，手続き的公正とストレス症状との関連が強くなることが明らかになっ

ている。一方で，相互作用的公正に対する雇用の不安定性の修飾効果は性別特異的であり，女性においてのみ，雇用が不安定であるほど，相互作用的公正と情緒的消耗感およびストレス症状との関連が強くなることが明らかになっている。

これに関連し，Inoue ら[28]は，日本の製造業に勤務するホワイトカラー労働者 1,017 名（男性 373 名，女性 644 名）を対象に，手続き的公正，相互作用的公正と心理的ストレス反応（psychological distress）との関連における，雇用形態の修飾効果を検討している。その結果，女性では，正規社員よりも非正規社員のほうが，手続き的公正，相互作用的公正と心理的ストレス反応との関連が強いことが明らかになっている（なお，本研究では，男性の非正規社員が僅少であったため，男性労働者における雇用形態別の検討はできていない）。社会心理学の領域では，「人は先行き不透明な状況に置かれると，公正性に対する知覚が特に敏感になる」ことが「不確実性マネジメント理論（uncertainty management theory）」[35]として知られているが，上記 2 つの研究は「不確実性マネジメント理論」を実際の労働者のデータを用いて実証したものであるといえよう。

Elovainio ら[32]は，フィンランドの自治体に勤務する職員 31,400 名（男性 7,083 名，女性 24,317 名）を対象に，手続き的公正，相互作用的公正と疾病休業との関連における，勤務時間の裁量権および職場環境の変化の修飾効果を検討している。その結果，勤務時間の裁量権が低い職場環境に置かれている人ほど，また，過去 1 年間にネガティブな（好ましくない）職場環境の変化を経験した人ほど，手続き的公正，相互作用的公正と疾病休業との関連が強くなることが明らかになっている。

Oshio ら[30]は，日本の企業（製造業，情報通信業，運輸業，医療等，多様な業種を含む 12 企業）に勤務する労働者 9,350 名（男性 7,268 名，女性 2,082 名）を対象に，手続き的公正，相互作用的公正と心理的ストレス反応との関連における，職場のソーシャル・キャピタルの修飾効果を検討している。その結果，ソーシャル・キャピタルが高い職場環境に置かれている人ほど，相互作用的公正と心理的ストレス反応との関連が弱くなることが明らかになっている。一方で，手続き的公正に対しては同様の修飾効果は認められず，組織的公正に対する職場のソーシャル・キャピタルの修飾効果は要素特異的である可能性が示唆されている。

Cheng & Chen[34]は，台湾の全国代表サンプルから抽出した労働者 17,042 名（男性 9,636 名，女性 7,406 名）を対象に，組織的公正（分配的公正，手続き的公正，対人的公正，情報的公正のすべての下位概念を測定しているが，全体的な組織的公正のみに着目しており，下位概念別の検討は行われていない）とバーンアウトおよび主観的（不）健康感（[poor] self-rated health）との関連における，企業規模の修飾効果を検討している。その結果，女性では，より大規模な企業で働いている人ほど，組織的公正とバーンアウトおよび主観的（不）健康感との関連が強いことが明らかになっている。

2　個人要因による修飾効果

組織的公正と労働者の健康との関連を修飾する個人要因として，年齢（年代）およびストレス対処行動による修飾効果が検討されている。

前節で紹介した Cheng & Chen の研究[34]

では，同様のサンプルを用いて組織的公正とバーンアウトおよび主観的(不)健康感との関連における，年齢（年代）の修飾効果についても検討している。その結果，若い年代ほど，組織的公正とバーンアウトおよび主観的(不)健康感との関連が強いことが明らかになっている。

Nakagawa ら[29]は，日本の製造業に勤務するホワイトカラー労働者1,235名（男性471名，女性764名）を対象に，手続き的公正，相互作用的公正と心理的ストレス反応との関連における，6つのストレス対処行動（「積極的問題解決」，「解決のための相談」，「発想の転換」，「気分転換」，「他者を巻き込んだ情動発散」，「逃避と抑制」）[36]の修飾効果を検討している。その結果，ストレス対処行動として「他者を巻き込んだ情動発散」を用いやすい傾向がある人ほど，手続き的公正，相互作用的公正と心理的ストレス反応との関連が強く，「発想の転換」を用いやすい傾向がある人ほど，これらの関連が弱い（ただし，相互作用的公正に対する「発想の転換」の修飾効果は女性においてのみ認められている）ことが明らかになっている。

3　生活習慣による修飾効果

組織的公正と労働者の健康との関連を修飾する生活習慣として，喫煙習慣による修飾効果が検討されている。

Inoue ら[31]は，日本の製造業に勤務する労働者2,838名（男性2,093名，女性745名）を対象に，手続き的公正，相互作用的公正と重度の心理的ストレス反応（serious psychological distress）との関連における，喫煙習慣の修飾効果を検討している。その結果，非喫煙者（喫煙未経験者と前喫煙者を含む）よりも喫煙者のほうが，手続き的公正と重度の心理的ストレス反応との関連が強いことが明らかになっている。一方で，相互作用的公正に対しては同様の修飾効果は認められず，組織的公正に対する喫煙習慣の修飾効果は要素特異的である可能性が示唆されている。

Ⅳ．実践への応用

ここまで，組織的公正と労働者の健康との関連を修飾する要因に着目した国内外の研究について紹介したが，これらの知見を実際の産業現場に応用する際には，「その修飾要因が（理論的かつ現実的に）修正可能であるかどうか」に着目する必要がある。

例えば，ストレス対処行動や喫煙習慣は，セルフケア研修や喫煙対策（個人に対する禁煙指導や職場の禁煙化）によって，それぞれ修正することが可能であるし，勤務時間の裁量権や職場のソーシャル・キャピタルは，職場環境改善によって修正することが可能である。これに対し，年齢（年代）は修正することが不可能である。また，雇用の不安定性や雇用形態は，雇用の安定化を図ることによって，理論上は修正することが可能であるが，現在の低迷する経済状況下では，このような対策を即時に講じることは容易ではない。同様に，企業規模についても，労働者数の削減を図ることによって，理論上は修正することが可能であるが，労働者側から見れば，「雇用機会の縮小」を意味することから，受け入れられる対策とは言い難い。

このような各修飾要因の修正可能性や，我が国の経済状況，利害関係者への影響を考慮すると，組織的公正（が損なわれていること）による労働者への健康影響を軽減するための対策として最も現実的かつ簡便なのは，ストレス対処行動をテーマとしたセルフケア

研修の実施（「他者を巻き込んだ情動発散」をできるだけ控えることや，「発想の転換」を心がけるようにすること等を研修内容に盛り込む）や産業保健スタッフによる禁煙指導等の個人アプローチであろう。また，職場のソーシャル・キャピタルや勤務時間の裁量権等の職場環境に着目し，職場環境改善を推進することも現実的な対策の1つと考えられるが，このような職場環境へのアプローチには，トップマネジメントの理解が必要不可欠である。そのため，これまでに職場環境改善に取り組んだ経験がない企業では，前述のような個人アプローチを中心とした取り組みを起点とし，トップマネジメントの理解を徐々に得ながら，「個人」から「職場環境」へ，取り組みの範囲を拡大していくほうが効率的であろう。また，前述のように，年齢（年代）や雇用形態は，直接的に修正することが不可能ないし困難な要因ではあるが，研修等の機会を通じて，人事労務担当者や管理監督者に対し，若年層や非正規労働者といった，組織的公正が損なわれた環境に対して特に脆弱な集団への理解を促していくことは可能であろう。

V. おわりに

以上，組織的公正と労働者の健康との関連を修飾する要因に着目した近年の国内外の研究について紹介し，これらの知見に基づく実践への応用について考察した。本稿で紹介した研究は，いずれも組織的公正（が損なわれていること）による労働者への健康影響を軽減するための効果的な対策について，極めて有用なヒントを与えてくれるものである。しかし，実際に対策を講じる際には，修飾要因だけでなく，組織的公正そのものを高める（ないし担保する）ための対策にも目を向ける必要がある。特に，セルフケア研修や禁煙指導等の個人アプローチは，あくまでも組織的公正による労働者への健康影響を“間接的”に軽減するものであり，組織的公正そのものを高めるわけではない点に注意が必要である。近年，管理監督者を対象としたフェアマネジメント研修（教育セッションやグループワーク等から構成される）が，対人的公正の向上に一定の有効性を示すことが確認されているが，手続き的公正や情報的公正を含む，組織的公正全体の向上に対する有効性までは確認できていない[37]。今後，組織的公正全体の向上を目的とした，より効果的なアプローチ方法について検討するとともに，本稿で紹介した研究結果から有効と考えられた各種対策が，組織的公正による労働者への健康影響を軽減する効果があるか，介入研究によって明らかにしていく必要があるだろう。

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〔文　献〕

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【Special Issue】

Organizational Justice and Health of Employees: Recent Trends in Research on Effect Modifiers

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Abstract

Organizational justice consists of three dimensions which include (1) distributive justice: referring to the fairness of the decision-making outcomes, (2) procedural justice: referring to the fairness of the decision-making processes underlying the allocation of outcomes, and (3) interactional (or relational) justice: referring to the polite and considerate treatment of individuals by supervisors. Several domestic and overseas studies have reported that the lack of organizational justice (mainly procedural justice and interactional justice) is associated with greater risk of health impairment such as physical disorders (e.g., cardiovascular morbidity and mortality), mental disorders (e.g., depression), and sickness absence. In recent years, however, there has also been increased interest in factors that modify the association between organizational justice and health of employees. This article introduces the concept and measuring method of organizational justice as well as recent domestic and overseas studies on the effect modifiers of the association between organizational justice and health of employees.

Key words: Distributive justice, Procedural justice, Interactional justice, Health effect, Modifying effect

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〔特別企画：職業性ストレス研究のトピックス
—代表的な職業性ストレスモデルの動向と注目されている心理社会的要因—〕

仕事の要求度－コントロールモデル / 要求度－コントロール－社会的支援モデル

井　上　彰　臣[1)]

抄録：「仕事の要求度－コントロール（JD-C）モデル」は，「仕事の要求度（仕事の量的負担）」と「仕事のコントロール（仕事の裁量権・技能の活用度）」の組み合わせによって仕事のストレス要因を規定した理論モデルである。これに「職場（上司・同僚）の社会的支援」を第3の側面として追加し，3次元に拡張したものが「要求度－コントロール－社会的支援（DCS）モデル」である。JD-C/DCSモデルは，古典的な職業性ストレスの理論モデルの1つとして産業保健領域の研究と実践に多く用いられている。本稿では，JD-C/DCSモデルの概要とその測定方法について説明した後，本モデルを用いた疫学研究と職場のストレス対策（主に職場環境改善）に関する介入研究の知見について紹介する。

Key words：仕事の要求度，仕事のコントロール，仕事のストレイン，社会的支援，理論モデル

Ⅰ．はじめに

我が国では，情報化やサービス分野の成長，規制緩和の進展によって，経済・産業構造が大きく転換するとともに，少子化や高齢化の進展，女性労働者の増加，若年層を中心とした勤労者意識の多様化など，企業や労働者を取り巻く環境が大きく変化しつつある。それに伴い，職場におけるストレス対策についても，新しい目標や枠組みを求める声が高まっており，労働者個人の仕事の特徴だけでなく，組織的公正やハラスメント，雇用の安定やワーク・ライフ・バランスなど，人事労務管理と密接にかかわる領域へのアプローチも求められ始めている[1)]。そのようななか，「仕事の要求度－コントロールモデル(job demand-control model：以下，JD-Cモデル)」[2)]と「要求度－コントロール－社会的支援モデル(demand-control-support model：以下，DCSモデル)」[3)]は，古典的な職業性ストレスの理論モデルの1つとして，今なお，多くの疫学研究や職場のストレス対策に用いられている。本稿では，JD-C/DCSモデルの概要とその測定方法について説明した後，本モデルを用いた疫学研究と職場のストレス対策（主に職場環境改善）に関する介入研究の知見について紹介する。

Ⅱ．JD-C/DCSモデルの概要

1979年，アメリカの産業・労働社会学者Karasekは，作業量による健康影響が職種によって異なる（管理職では小さく，製造ラインの作業者では大きい）ことに注目し，“仕事の要求度（仕事の量的負担）による健康影響を，仕事のコントロール（仕事の裁量権・技能の活用度）が和らげる”とするJD-Cモデルを提唱した[2)]。本モデルでは，仕事の要求度が高く，かつ仕事のコントロールが低い

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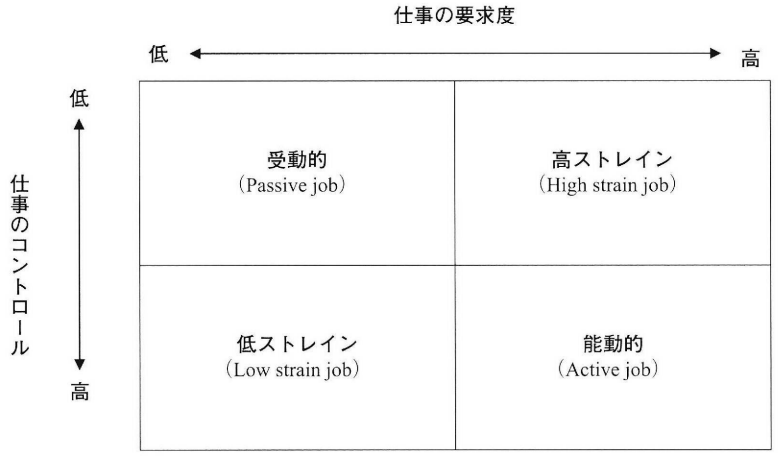

Fig.1 仕事の要求度－コントロールモデル
(Karasek (1979)[2] を邦訳・一部改編)

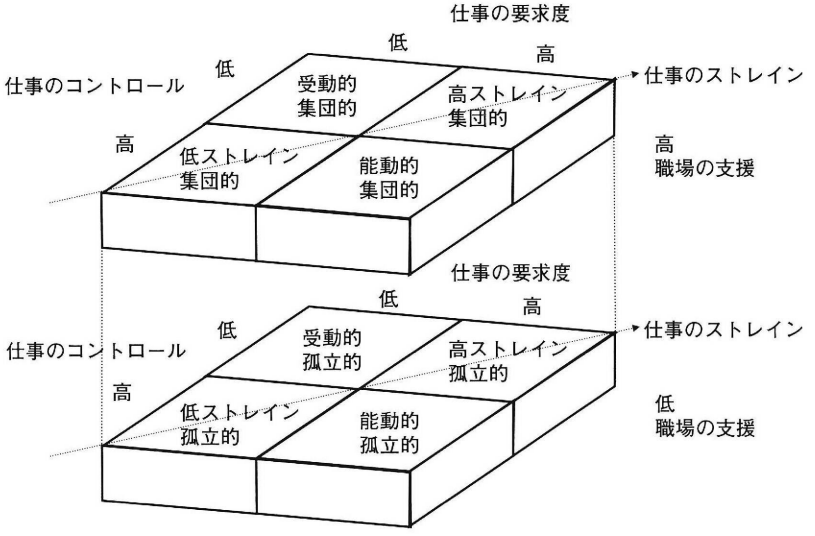

Fig.2 要求度－コントロール－社会的支援モデル
(Johnson & Hall (1988)[3] を邦訳)

状態を「高ストレイン (high strain job)」と定義し，高ストレイン群において，健康障害の発生リスクが最も高くなると考えている(Fig.1)。

一方で，JD-C モデルの研究と並行し，職場（上司・同僚）の社会的支援が仕事のストレス要因による健康影響を和らげたり，疾患の発症に直接影響を及ぼしたりすることが知られていたため，アメリカの社会・行動科学者 Johnson と Hall は，JD-C モデルに職場の社会的支援を加え，3 次元に拡張した DCS モデルを提唱した[3]。本モデルでは，仕事の要求度が高く，仕事のコントロールが低く，かつ職場の社会的支援が少ない三重苦の状態（このような，高ストレインで孤立的な状態を「アイソ・ストレイン (iso-strain)」と呼

ぶ場合もある）において，健康障害の発生リスクが最も高くなると考えている（Fig.2）。

これらの理論モデルは，仕事のストレス要因を 2 次元，ないしは 3 次元でとらえるというシンプルさから，労働者を対象とした多くの疫学研究に用いられている。また，現場では，本モデルに基づく職場環境改善も実施されており，極めて汎用性の高い理論モデルである。

Ⅲ．仕事の要求度，仕事のコントロール，職場の社会的支援を測定することができる代表的な自記式調査票

JD-C/DCS モデルに基づき，仕事の要求度，仕事のコントロール，職場の社会的支援を測定することができる代表的な自記式調査票として，（1）職業性ストレス簡易調査票[4]，（2）Job Content Questionnaire（JCQ）[5]，（3）National Institute for Occupational Safety and Health（NIOSH）職業性ストレス調査票（Generic Job Stress Questionnaire）[6] の 3 つが挙げられる。

職業性ストレス簡易調査票は，労働省（当時）「作業関連疾患の予防に関する研究」班によって作成されたもので，仕事のストレス要因，ストレス反応，修飾要因を全 57 項目で測定することができる自記式調査票である。少ない項目数で多面的な評価が可能であることから，我が国では多くの現場で使用されており，2015（平成 27）年 12 月 1 日に常時 50 人以上の労働者を使用する事業場で実施が義務付けられた「ストレスチェック制度」においても，その使用が推奨されている[7]。本調査票では，仕事の要求度を 3 項目，仕事の裁量権を 3 項目，技能の活用度を 1 項目，上司の支援を 3 項目，同僚の支援を 3 項目で，それぞれ測定することが可能である。また，本調査票の大きな特徴の 1 つとして，測定結果を個人や職場に返却するための定式化されたツール（書式）が作成されていることが挙げられる。個人向けには「あなたのストレスプロフィール」，職場向けには「仕事のストレス判定図」[8] を返却することが可能であるが，このうち，「仕事のストレス判定図」は，JD-C/DCS モデルに基づき，当該職場で健康障害が起きる可能性を全国平均と比較したものを「健康リスク」として算出するもので，後述する職場環境改善の支援ツールの 1 つとして活用されている。

JCQ は，仕事のストレス要因を測定する自記式調査票として，Karasek[5] によって開発されたものである。標準（推奨）版は全 49 項目で構成されているが，ここから抜粋して作成された最小版（全 22 項目）は JD-C/DCS モデルに準拠しており，仕事の要求度を 5 項目，仕事の裁量権を 3 項目，技能の活用度を 6 項目，上司の支援を 4 項目，同僚の支援を 4 項目で，それぞれ測定することが可能である。項目数が比較的多いことから，研究場面で使用されることが多い尺度である。JCQ の日本語版については，Kawakami ら[9] によって，その信頼性・妥当性が報告されている。また，測定結果を返却するための定式化されたツールとして，JCQ 用の「仕事のストレス判定図」が作成されており，職場向けに結果を返却することが可能である（個人向けの結果返却ツールは作成されていない）。

NIOSH 職業性ストレス調査票は，Hurrell と McLaney[6] が，今後標準となるべき信頼性・妥当性のある評価尺度を集めて作成した自記式調査票であり，仕事のストレス要因，ストレス反応，修飾要因などを全 253 項目で

Tab.1 仕事の要求度，仕事のコントロール，職場の社会的支援を測定することができる代表的な自記式調査票の項目数と特徴

自記式調査票（開発者または翻訳者，公表年）	仕事の要求度	仕事のコントロール‡		職場の社会的支援		特徴
		仕事の裁量権	技能の活用度	上司の支援	同僚の支援	
職業性ストレス簡易調査票（下光ら，2000）[4]	3	3	1	3	3	・国内で開発 ・現場で活用しやすい ・「ストレスチェック制度」で推奨 ・結果返却ツール：個人＋職場†
Job Content Questionnaire（Kawakami et al., 1995）[9]	5	3	6	4	4	・国際比較可能 ・研究向け ・結果返却ツール：職場のみ†
NIOSH 職業性ストレス調査票（Haratani et al., 1996）[12]	11	16	3	4	4	・国際比較可能 ・目的別に尺度を選択可能 ・研究向け ・結果返却ツール：なし

† 個人向け結果返却ツール：「あなたのストレスプロフィール」，職場向け結果返却ツール：「仕事のストレス判定図」[8]

‡ 職業性ストレス簡易調査票とNIOSH 職業性ストレス調査票では，「技能の活用度」を「仕事のコントロール」の下位概念としては位置付けず，「仕事の裁量権」のみを指して「仕事のコントロール」と表現している。

包括的に測定することが可能である（ただし，実際に使用する際は，必ずしもすべての項目を使用する必要はなく，目的に応じて必要な尺度を選択したり，新たな尺度を追加したりすることが可能となっている）。本調査票では，JD-C/DCS モデルに基づく評価尺度をCaplan ら[10] および Greenberger[11] が開発した尺度から抜粋（一部改編）し，仕事の要求度を 11 項目，仕事の裁量権を 16 項目，技能の活用度を 3 項目，上司の支援を 4 項目，同僚の支援を 4 項目で，それぞれ測定することが可能である。NIOSH 職業性ストレス調査票の日本語版については，Haratani ら[12] によって，その信頼性・妥当性が報告されている。ただし，測定結果を個人や職場に返却するための定式化されたツールが作成されていないことから，現場での活用が難しく，研究場面で使用されることが多い調査票である。

上記 3 つの自記式調査票の項目数と特徴を Tab.1 にまとめたので，ご参照いただきたい。

Ⅳ.「高ストレイン群」の選定方法

Ⅱ章で述べたとおり，JD-C モデルでは，仕事の要求度が高く，かつ仕事のコントロールが低い状態を「高ストレイン」と定義しているが，本章では，自記式調査票を用いて仕事の要求度と仕事のコントロールを測定した際の「高ストレイン群」の選定方法について紹介する。高ストレイン群を選定する代表的な方法として（1）象限を用いる方法（quadrant term）（2）比率を用いる方法（quotient term），（3）差分を用いる方法（linear term）の 3 つが挙げられる[13]。

Quadrant term では，仕事の要求度得点と仕事のコントロール得点の高低の組み合わせで，対象者を 4 つの群に分類し（Fig.1 参照），

「仕事の要求度（高）×仕事のコントロール（低）」の群に分類された者を「高ストレイン群」と選定する（仕事の要求度と仕事のコントロールの高低を決定する際のカットオフ値は，①調査対象集団の中央値を用いる方法と，②全国平均値を用いる方法の 2 パターンがある）。

Quotient term では，仕事の要求度得点を仕事のコントロール得点で除した「仕事の要求度/コントロール比」を算出し，仕事の要求度/コントロール比が大きい上位 1/4 ないし 1/3 の者を「高ストレイン群」と選定する。

Linear term では，仕事の要求度得点から仕事のコントロール得点を引いた値を算出し，その値が大きい上位 1/4 ないし 1/3 の者を「高ストレイン群」と選定する。

以上，「高ストレイン群」の選定方法について紹介したが，実際の疫学研究では quadrant term と quotient term が用いられることが多い。なお，quotient term と linear term については，必ずしも「高ストレイン群」を選定する必要はなく，仕事の要求度と仕事のコントロールのバランス（仕事のストレイン）を連続変数として扱うことも可能であり，より多様な統計解析に用いることができる。

Ⅴ．JD-C/DCS モデルを用いた疫学研究

JD-C/DCS モデルを用いた疫学研究として，冠動脈疾患および精神疾患の発症をアウトカムとしたメタ分析とシステマティックレビューの知見を紹介する。

Kivimäki ら[14] は，ヨーロッパで実施されている，未発表のものも含めた 13 の大規模労働者コホート（197,473 名）のデータベースを統合し，高ストレインが冠動脈疾患の発症に及ぼす影響を調べるメタ分析を実施している。その結果，高ストレインの職場環境に置かれている人は，そうでない人に比べ，冠動脈疾患を発症するリスクが 1.23 倍高くなることが明らかになっている。また，属性別の検討では，女性，50 歳以上，社会経済的地位の高い者において，そのリスクが高いことが明らかになっている。本メタ分析では，学術誌に公表された縦断研究の結果ではなく，労働者コホートのデータベースを分析対象としていることから，出版バイアスによる影響を最小限に抑えた，より正確なリスクを算出できている。なお，本研究では，仕事の要求度と仕事のコントロールが単独で冠動脈疾患の発症に及ぼすリスクや，DCS モデルに基づく（職場の社会的支援を含めた）リスクの検討は行われていない。

Stansfeld と Candy[15] は，仕事の要求度，仕事のコントロール，職場の社会的支援を含む，種々の仕事のストレス要因が精神疾患（構造化面接によって判定されたものだけでなく，自己評定による軽症なアウトカムも含む）の発症に及ぼす影響を調べた 11 の縦断研究を対象にメタ分析を実施している。その結果，高ストレインの職場環境に置かれている人は，そうでない人に比べ，精神疾患を発症するリスクが 1.82 倍高くなることが明らかになっている。また，仕事の要求度，仕事のコントロール，職場の社会的支援が単独で精神疾患の発症に及ぼすリスクは，それぞれ 1.39 倍，1.23 倍，1.32 倍であることが報告されている。更に，本メタ分析では，研究数が多かった仕事の要求度と職場の社会的支援について，男女別の解析も行っている。その結果，いずれも男性のほうが精神疾患の発症リスクが高く，性差が認められている（仕事の要求度：男性 1.55 倍，女性 1.34 倍，職場の

Tab.2　仕事の要求度，仕事のコントロール，職場の社会的支援による労働者への健康影響

アウトカム（著者名，公表年）	発症リスク（性差）†
冠動脈疾患（身体的健康） （Kivimäki et al., 2012）[14]	・高ストレイン：1.2倍（男性＜女性）
精神疾患・うつ病（精神的健康） （Stansfeld & Candy, 2006; Bonde, 2008）[15][16]	・高ストレイン：1.2～2.4倍（男性＞女性） ・仕事の要求度（高）：1.3～1.4倍（男性＞女性） ・仕事のコントロール（低）：1.2倍（男性＜女性） ・職場の社会的支援（低）：1.3～1.4倍（性差は研究によって異なる）

† 性差については，研究数・対象者数が少ないため，正確に推定できていない可能性がある。

社会的支援：男性1.38倍，女性1.20倍）。

Bonde[16]は，仕事の要求度，仕事のコントロール，職場の社会的支援を含む，種々の仕事のストレス要因がうつ病（医師の診断，抗うつ薬の処方，構造化面接によって判定されたものだけでなく，自己評定による抑うつ症状も含む）の発症に及ぼす影響を調べた16の縦断研究を対象にシステマティックレビューを実施している。その結果，高ストレインの職場環境に置かれている人は，そうでない人に比べ，うつ病を発症するリスクが1.2～2.4倍高くなることが明らかになっている。また，仕事の要求度，仕事のコントロール，職場の社会的支援が単独でうつ病の発症に及ぼすリスクは，それぞれ1.31倍，1.20倍，1.44倍であることが報告されており，StansfeldとCandyのメタ分析[15]と比較的一致した結果が得られている。なお，本レビューでは男女別の詳細な解析は行われていないが，高ストレインと仕事の要求度によるうつ病の発症リスクは男性のほうが高い傾向にあるのに対し，仕事のコントロールについては女性のほうが高い傾向にあることが確認できる。一方，職場の社会的支援については明確な性差は認められず，StansfeldとCandyのメタ分析[15]とは異なる傾向を示しているが，研究数・対象者数が少ないため，性差が正確に推定できていない可能性がある。

以上の疫学研究の知見をTab.2にまとめたので，ご参照いただきたい。

Ⅵ．JD-C/DCSモデルに基づく職場環境改善の介入研究

職場におけるストレス対策では，職業性ストレスへの対処能力の向上を目的とした「労働者個人へのアプローチ」と，快適な職場環境づくりを目的とした「組織的アプローチ」が重要であることが指摘されているが[17]，そのなかでも「組織的アプローチ」は，「労働者個人へのアプローチ」による対策よりも効果が大きく，より永続的な改善に結び付きやすいと考えられており，その科学的根拠が蓄積されている[18]。この「組織的アプローチ」の1つとして，職場環境改善が注目されており，近年では，職場環境改善によってメンタルヘルス不調が改善したという科学的根拠が蓄積され，その有効性が明らかになりつつある（Tab.3）[19]～[32]。特に，我が国では，JD-C/DCSモデルに基づく労働者参加型の職場環境改善を実施するための支援ツールとして，「メンタルヘルスアクションチェックリスト（Mental Health Action Check List：以下，MHACL）」[33]が開発されている。MHACLは職場環境改善の好事例を収集し，その内容を

Tab.3　職場環境改善に関する介入研究の例

研究デザイン	改善方法（著者名，公表年）
無作為化比較対照試験	・会合の頻度の増加（Jackson, 1983）[19] ・役割の明確化（Schaubroeck et al., 1993）[20] ・グループ活動で作業者の自立性の増加（Orth-Gomér et al., 1994）[21] ・就業後のインフォーマルな会合の実施（Horan, 2002）[22] ・メンタルヘルスアクションチェックリストによる参加型職場環境改善（Tsutsumi et al., 2009）[23]
比較対照試験	・作業手順と指揮系統の改善（Kawakami et al., 1997）[24] ・バス運転手のスケジュール・ルートの改善（Evans et al., 1999）[25] ・医療従事者における参加型職場環境改善（Bourbonnais et al., 2006）[26] ・メンタルヘルスアクションチェックリストによる参加型職場環境改善（Kobayashi et al., 2008）[27]
前後比較による効果評価	・作業レイアウト，コミュニケーション等の改善（Wall & Clegg, 1981; Wallin & Wright, 1986; Smith & Zehel, 1992）[28]～[30] ・バス運転手の労働時間，職場組織等の改善（Kompier et al., 2000）[31] ・専門家が職場ごとにストレス調査の結果を返却・助言（Anderzén & Arnetz, 2005）[32]

6 領域 30 項目に分類してチェックリスト形式にまとめたものである。職場で取り上げる改善策を項目ごとに選択する形式になっているため，職場のメンバー同士によるグループ討議などで利用することが可能である。また，付録として，MHACL で挙げられている 30 項目と前述の「仕事のストレス判定図」との対応を示した一覧表が準備されているため，「仕事のストレス判定図」の結果と付録の一覧表を見ながらグループ討議を行い，効果的に職場環境改善を進めることが可能である。ここでは，MHACL を使用して職場環境改善を実施した 2 つの介入研究[23][27]を紹介する。

Kobayashi ら[27]は，製造業に勤務する労働者（45 部署：1,071 名）を介入群（9 部署：321 名）と対照群（36 部署：750 名）に割り付け，介入群に対し，MHACL を使用したグループ討議と，それに基づく職場環境改善を実施している。その結果，参加率が 50％以上だった介入群（6 部署：178 名）は対照群に比べ，1 年後の抑うつ得点（職業性ストレス簡易調査票で測定）が有意に低下したが，参加率が 50％未満だった介入群（3 部署：143 名）では，1 年後の抑うつ得点の有意な低下は認められなかった。また，参加率が 50％以上だった介入群では，「仕事のストレス判定図」で算出される「健康リスク」の有意な低下も認められ，職場環境と精神的健康の双方の改善が認められた。本研究では，介入群と対照群の無作為割り付けは行われていないが，ここで得られた知見は「職場環境改善は，より多くのメンバーが参加することで，初めて有効な取り組みになる」という，極めて重要な実践的示唆を与えるものである。

Tsutsumi ら[23]は，製造業に勤務する労働者（11 部署：77 名）を介入群（6 部署：35 名）と対照群（5 部署：42 名）に無作為に割り付け，介入群に対し，MHACL を使用したグループ討議と，それに基づく職場環境改善を実施している。その結果，介入群は対照群に比べ，1 年後の仕事のパフォーマンス得点（World Health Organization Health and Work

Performance Questionnaire; WHO-HPQ で測定)[34]が有意に上昇した。また，精神的健康度(General Health Questionnaire; GHQ で測定)[35]については，介入群では1年後もベースライン時（介入前）と同水準を維持したのに対し，対照群では1年後に有意に悪化したことから，職場環境改善は，仕事のパフォーマンスを上昇させ，精神的健康の悪化を予防する効果がある可能性が示唆されている。本研究は，介入群と対照群を無作為に割り付けることで，職場環境改善の効果について，よりエビデンスレベルの高い知見を提供したものであるといえる。

Ⅶ. おわりに

以上，JD-C/DCS モデルの概要とその測定方法，本モデルを用いた疫学研究と職場のストレス対策に関する介入研究の知見について紹介した。本稿で紹介した研究は，メタ分析や（無作為化）比較対照試験など，いずれもエビデンスレベルの高いものである。一方で，DCS モデルで規定されている「アイソ・ストレイン」が労働者の健康に及ぼす影響については，未だ十分に検討されておらず，今後の更なる知見の蓄積が期待される。また，近年の縦断研究（平均追跡期間：15.5 年）では，「仕事のコントロール」のうち，「技能の活用度」は死亡リスクを低下させるのに対し，「仕事の裁量権」は死亡リスクをかえって上昇させるといった知見が報告されており[36]，「仕事のコントロール」の取り扱いについても，更なる検討が必要である。介入研究については，「疾病予防」と「健康増進」の双方へのアプローチを目標に，ワーク・エンゲイジメント[37]などのポジティブなアウトカム指標を含めた，より包括的な効果評価を行っていく必要があるだろう。JD-C/DCS モデルを用いた疫学研究と介入研究の更なる発展が期待される。

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【Special Issue】

Job Demand-Control Model / Demand-Control-Support Model

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Abstract

The job demand-control (JD-C) model is a theoretical model that defines job stressors as a combination of job demands (quantitative job overload) and job control (decision authority and skill discretion). The demand-control-support (DCS) model is a three-dimensional model that expanded and reformulated the JD-C model by adding work-related social support (supervisor support and coworker support) as a third important aspect of the workplace environment. The JD-C/DCS models have commonly been used in the occupational health research and practice as one of the classical theoretical models of job stress. This article introduces the outline and measuring methods of the JD-C/DCS models as well as the findings from epidemiological and intervention studies (mainly workplace environmental improvement) based on the JD-C/DCS models.

Key words: Job demands, Job control, Job strain, Social support, Theoretical model

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心理社会的ストレスへのコルチゾールの反応性と脅威刺激からの注意解放[1]

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Cortisol Reactivity to Psychosocial Stress and Attentional Disengagement from Threat Stimuli

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The present study examined whether or not the elevation of cortisol elicited in response to an acute psychosocial stress relates to a difficulty in disengaging attention from threat stimuli. For this purpose, we measured attentional bias for angry faces using a spatial cueing task in which the duration of the cue presentation was relatively long (1,000 ms). The participants engaged in the acute psychosocial stress task, and then the

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spatial cueing task. We divided the participants into cortisol responders and non-responders based on the elevation of salivary cortisol elicited in response to the acute psychosocial stress task. The results showed that cortisol responders had a difficulty in disengaging attention from angry faces, whereas non-responders rapidly disengaged attention from angry faces. These results suggest that the elevation of cortisol elicited in response to the acute psychosocial stress is associated with a difficulty in disengaging attention from threat stimuli.

Key words: cortisol, attentional bias, psychosocial stress, attentional disengagement

【要　約】本研究は，急性心理社会的ストレスに対するコルチゾールの反応性が脅威刺激からの注意解放の困難さと関連するか否かを検討した。この目的のため，手がかり刺激の提示時間を比較的長くした(1,000 ms)空間手がかり課題を用いて怒り顔への注意バイアスを計測した。実験参加者は，急性心理社会的ストレス課題を行った後空間手がかり課題を行った。本研究では，急性心理社会的ストレス課題により上昇した唾液中コルチゾール値に基づき，実験参加者をコルチゾール反応者と非反応者に分けた。その結果，反応者は怒り顔に対する注意の解放困難を示したのに対して，非反応者は怒り顔からの迅速な注意解放を示した。これらの結果は，急性心理社会的ストレスに対するコルチゾールの反応性の高さが脅威刺激からの注意解放の困難さと関連することを示唆する。

ヒトを含む霊長類は社会的動物であり，他者からの評価や拒絶といった社会的脅威に対して心理・生理的ストレス反応を生起する。このようなストレス反応の中でも，社会的脅威に対する主要なストレス反応は，視床下部-下垂体-副腎皮質系(hypothalamus-pituitary-adrenal cortex axis, 以下HPA系)の活性による副腎皮質からのグルココルチコイドの放出である(Dedovic et al., 2009; Dikerson & Kemeny, 2004)。ヒトにおける主要なグルココルチコイドはコルチゾールであり，社会的脅威を根源とする心理社会的ストレスは，日常的な状況であれ，実験室的な状況であれ，血中及び唾液中のコルチゾールを上昇させることが繰り返し報告されている(Dickerson & Kemeny, 2004)。

心理社会的ストレスに対するコルチゾールの反応性は，社会的脅威に対する感受性や他者評価への恐怖といった個人差に影響を受ける(Kemeny, 2009; Gruenewald et al., 2004)。このような個人差要因の背景にある認知・感情メカニズムとして，脅威刺激への注意バイアスがあげられる。注意バイアスとは，特定の刺激に対して選択的に注意が向けられる傾向を指し，情動ストループ課題やドットプローブ課題といった認知課題により測定される(MacLeod et al., 1986; Mathews & MacLeod, 1985)。これらの認知課題を聴衆の前でのスピーチや暗算といった心理社会的ストレス課題と組み合わせることで，コルチゾールのストレス反応性と注意バイアスの関連性が検討されている。たとえば，Roelofs et al. (2007)は，心理社会的ストレス負荷中に怒り顔を用いた情動ストループ課題を実施することで，コルチゾールの増加と怒り顔への注意の引きつけに関連があることを報告している。

脅威刺激への注意バイアスとコルチゾールの関連を検討する研究では，情動ストループ課題が多く用いられている(van Honk et al., 1998, 2000)。しかし，情動ストループ課題では，刺激に注意を向ける定位段階で生じるバイアスと刺激から注意をはずす解放段階で生じるバイアスの区別が困難である(Fox et al., 2002)。これに対して，空間手がかり課題では，注意の定位段階と解放段階のバイアスを分離して測定することが可能とされている。この課題では，手がかり刺激として脅威刺激か感情価のない中性刺激が左右いずれかの位置に提示された後，標的刺激が左右のどちらかに提示される。実験参加者の課題は，標的刺激の位置を検出して反応することである。この課題では，手がかり刺激と標的刺激の位置が一致するときの脅威刺激提示時の反応時間と中性刺激提示時の反応時間の差が脅威刺激への注意の定位段階におけるバイアスの強さを表すと考えられている。対して，手がかり刺激と標的刺激の位置が一致しないときの脅威刺激提示時の反応時間と中性刺激提示時の反応時間の差が脅威刺激から注意を解放する段階におけるバイアスの強さを表すと考えられている。

Ellenbogen et al. (2010)は，空間手がかり課題を用

い，コルチゾールのストレス反応性が注意のどの段階におけるバイアスと関連するのかを検討した。その結果，心理社会的ストレスによるコルチゾールの増加は注意の定位段階におけるバイアスの強さとは関連する一方，注意の解放段階におけるバイアスの強さとは関連しなかった。Roelofs et al. (2007) やEllenbogen et al. (2010) の研究が心理社会的ストレス負荷中の注意バイアスを検討しているのに対して，近年の研究は，ストレス負荷のない状態における注意バイアスがコルチゾールのストレス反応性とどのように関連するかに焦点をあてている。Pilgrim et al. (2010) は，心理社会的ストレス負荷の直前に空間手がかり課題を実施した。その結果，ストレス負荷によるコルチゾールの増加は，脅威関連単語への注意の定位段階におけるバイアスの強さとは関連したが，注意の解放段階におけるバイアスの強さとは関連しなかった。同様の結果は，心理社会的ストレス負荷とは別日に注意バイアスを測定した研究においても報告されている (Fox et al., 2010)。

ここまで概観してきたとおり，これまでの研究は，心理社会的ストレス負荷の有無にかかわらず，コルチゾールのストレス反応性の高さが脅威刺激への注意の定位段階におけるバイアスの強さと関連することを示してきた。一方で，コルチゾールのストレス反応性の高さと脅威刺激からの注意の解放段階におけるバイアスの強さには関連が見出されてこなかった。先行研究では，空間手がかり課題における手がかり刺激の提示時間は200 ms (Pilgrim et al., 2010) や300 ms (Fox et al., 2010) と比較的短い。HPA系の障害を特徴とする抑うつ者は，比較的後期の情報処理段階にバイアスが生じるため，脅威刺激の提示時間が500 ms以下のときには注意の解放に遅延は生じないが，脅威刺激の提示時間が1,000 ms以上のときには注意の解放に遅延が生じることが知られている (Koster et al., 2005)。このことから，先行研究においてコルチゾールのストレス反応性の高さと脅威刺激からの注意の解放段階におけるバイアスの強さに関連が見られなかったのは，手がかり刺激の提示時間が短かったことが原因かもしれない。

そこで，本研究では手がかり刺激の提示時間の比較的長い空間手がかり課題を用いることで，コルチゾールのストレス反応性の高さと注意の解放段階におけるバイアスの強さに関連があるか否かを検討する。このため，実験参加者は急性心理社会的ストレス課題を行った後，表情 (怒り顔，笑顔，真顔) を手がかり刺激とした空間手がかり課題を行った。抑うつ者における注意解放の困難さを報告した研究に従い，手がかり刺激の提示時間は1,000 msとした (Leyman et al., 2007)。コルチゾールのストレス反応性と注意バイアスの関連を検討するため，心理社会的ストレス課題に対するコルチゾールの増加に基づき実験参加者を反応者と非反応者に分けた。もしコルチゾールのストレス反応性の高さと注意の解放段階におけるバイアスの強さに関連があるのなら，怒り顔からの注意解放は反応者において非反応者よりも遅延すると考えられる。反対に，もしコルチゾールのストレス反応性の高さと注意の解放段階におけるバイアスの強さに関連がないのなら，怒り顔からの注意解放に反応者と非反応者で差はないと考えられる。

方　法

実験参加者　本研究は，Izawa et al. (2013) の研究において空間手がかり課題を遂行した実験参加者のデータを報告する。Izawa et al. (2013) では急性心理社会的ストレスが唾液中サイトカインに及ぼす影響に焦点を当てており，空間手がかり課題のデータは使用しておらず，注意バイアスへの言及もしていない。実験参加者は，健康な大学生および大学院生43名 (男性35名，女性8名) であり，平均年齢は21.5歳，範囲は18―28歳であった。喫煙者及びHPA系，免疫系に影響を及ぼす薬物やサプリメントを使用している参加者は除外した。性ホルモンがHPA系へ及ぼす影響を排除するため，女性参加者は卵胞前期，黄体期後期に実験に参加した。全ての参加者が実験内容の説明を受けた上で参加同意書に記入した。実験プロトコルは早稲田大学研究倫理委員会の承認を受けた。

空間手がかり課題　空間手がかり課題の提示にはディスプレイの大きさが15.4インチのノートパソコンを使用した。視覚刺激の提示，データの記録にはE-Prime (Psychology Software Tools社製) を用いた。実験参加者とディスプレイまでの距離は約60 cmとした。

手がかり刺激として，ATR顔表情画像データベー

スDB99 (http://www.atr-p.com/face-db.html) から選択した6名（男女各3名）の怒り顔，笑顔，真顔を用いた。画像は正面顔であり，顔の中心が入る位置でトリミングして顔部分の平均輝度が等しくなるよう明るさを調整した。手がかり刺激は，視角で縦6.7度×横5.7度となるよう提示した。固視点は白色であり大きさが1.2度の十字，標的刺激には1.2度の黒色の正方形を用いた。

実験参加者には正方形の標的刺激が左右どちらの位置に提示されたかを検出し，出来る限り早く標的刺激の位置に対応するキー（'F' と 'J'）を押して答えるように求めた。3種類の手がかり刺激（怒り顔，笑顔，真顔）について，24試行が手がかり刺激の位置と標的刺激の位置が同じvalid試行，24試行が手がかり刺激と標的刺激の位置が異なるinvalid試行，12試行が標的刺激の提示されないcatch試行であった。注意解放におけるバイアスを検討した先行研究に従い，本研究ではvalid試行とinvalid試行の頻度を等しくした (Leyman et al., 2007)。

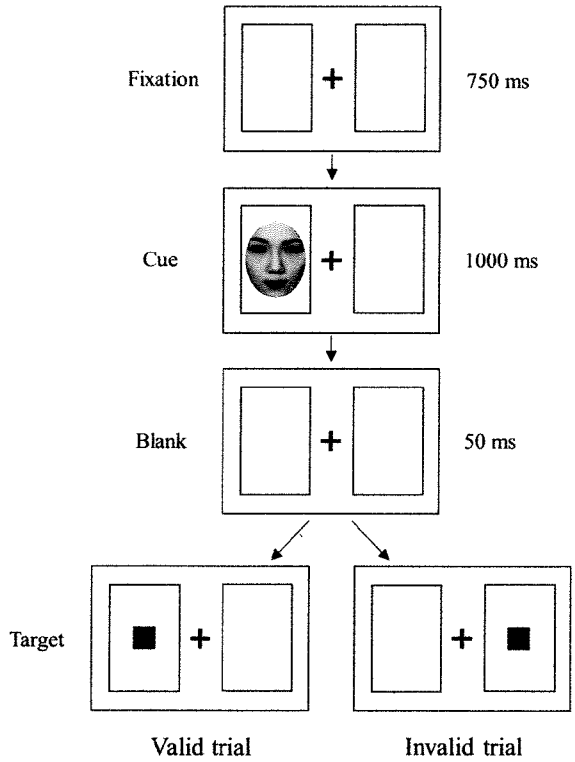

Figure 1. Schematic illustration of the stimuli and procedure in the spatial cueing task. The participants were asked to respond to the target as quickly as possible.

空間手がかり課題の1試行の流れをFigure 1に示す。課題では固視点から左右に視角で4.5度離れた位置を中心として白色の長方形を提示した。各試行のはじめに固視点が750 ms提示された後，左右どちらかの長方形の中央に手がかり刺激が1,000 ms提示された。手がかり刺激の消失から50 ms後に標的刺激が左右いずれかの長方形の中央に提示された。標的刺激は実験参加者が反応するまで提示された。試行間間隔は1,500 msであった。catch試行では標的刺激は提示されないため，手がかり刺激の消失から1,000 ms後に次の試行に移った。実験参加者には常に画面中央の固視点を注視することを教示した。手がかり刺激への注意に認知的制御が影響を及ぼさないよう，手がかり刺激については提示されること以外の教示は与えなかった。

質問紙　実験期間中の主観的ストレス感の尺度として，0%（まったくストレスを感じない）から100%（非常にストレスを感じる）としたVisual Analogue Scale (VAS) を用いた。加えて，実験前に米国国立精神保健研究所疫学的抑うつ尺度 (Center for Epidemiological Self-Depression Scale，以下CES-D) の日本語版への回答を求めた。

心拍数　ホルター心電計（フクダ電子社製）を用いて心電図を記録した。

唾液中コルチゾール　唾液中のコルチゾール濃度を計測するためPassive Drool法を用いて実験参加者の唾液を採取した。Passive Drool法は，自然分泌された唾液を2分間口の中で貯め，短いストローを使って収集容器に移す採取方法である。採取された唾液試料は−20℃で凍結保存した後，解析時に解凍して3,000 rpmで15分間の遠心分離を行った。唾液中コルチゾールは酵素結合免疫吸着測定法に基づく分析キット (IBL International社製) を用いて解析し，コルチゾール濃度を算出した。

手続き　コルチゾールの日内変動の影響を排除するため，実験は14時以降に開始した。実験参加者には実験開始の1時間前から飲食と運動をしないように教示した。本研究は，心理社会的ストレス負荷としてTrier Social Stress Test (TSST) を用いた。TSSTは実験参加者に2名の審査者の前で就職面接を模したスピーチと計算課題を求める実験プロトコルであり，コルチゾールを上昇させるために有用であることが確認

されている（Kirschbaum et al., 1993）。

実験参加者は，実験室に入室後，実験の説明を受けて参加同意書へ記入した。その後，ホルター心電計を装着し，一人で10分間安静にした（以下，安静期）。安静の直後，これから審査者の前でスピーチを行うことを教示した。スピーチの内容は，就職を希望する企業の面接者に対して自分の長所と短所を述べることと伝えた。その後，実験参加者は10分間のスピーチ準備（以下，準備期），5分間のスピーチ（以下，スピーチ期），5分間の計算課題（以下，計算期）を行った。TSST終了後，実験参加者は60分間安静にした（以下，回復期）。唾液採取及び主観的ストレス感の測定は，安静期，準備期，スピーチ期，計算期の直後及び回復期開始後10, 20, 30, 45, 60分（以下，それぞれ回復期10分，回復期20分，回復期30分，回復期45分，回復期60分）に実施した。

60分の回復期間後，空間手がかり課題を実施した。実験参加者は課題についての説明を受け，12試行の練習試行の後に本試行を行った。

データ解析　空間手がかり課題における反応時間データとして，誤反応及び反応時間が100 ms未満あるいは1,000 msより長いデータは分析から除外した。本研究では，注意定位と注意解放におけるバイアスを分離して検討するため，怒り顔と笑顔のそれぞれについて注意定位インデックスと注意解放インデックスを算出した。注意定位インデックスとして怒り顔，笑顔提示時のvalid試行の反応時間から真顔提示時のvalid試行の反応時間を引いた値を算出した。注意解放インデックスとして怒り顔，笑顔提示時のinvalid試行の反応時間から真顔提示時のinvalid試行の反応時間を引いた値を算出した。両インデックスとも正の値は真顔提示時よりも怒り顔あるいは笑顔提示時で反応が遅延したことを意味する。このことから，注意定位インデックスでは負の値が手がかり刺激への注意定位の促進を表すのに対して，注意解放インデックスでは正の値が手がかり刺激からの注意解放の遅延を表す。

心電図データは専用ソフトを用いてR波を検出した後に心拍数を算出した。その後，各期間における平均心拍数を求めた。

本研究では，二つの段階で統計的な検討を行った。はじめに，TSSTが実験参加者に急性ストレス反応を惹起していることを確認するため，主観的ストレス感，心拍数，唾液中コルチゾール濃度に対して測定時点（安静期・準備期・スピーチ期・計算期・回復期10分・回復期20分・回復期30分・回復期45分・回復期60分）を要因とする繰り返しのある分散分析を行った。次に，コルチゾールのストレス反応性と注意バイアスの関連を検討するため，TSSTによる唾液中コルチゾール濃度の増加量に基づき実験参加者を反応群と非反応群に分けた。先行研究は，抑うつと脅威刺激からの注意解放の困難に関連があることを報告している（Leyman et al., 2007）。このため，反応群と非反応群のCES-Dの得点を対応のないt検定を用いて比較することで両群の抑うつの程度に差があるかを検討した。反応群と非反応群においてストレス反応に差があるかを検討するため，唾液中コルチゾール，主観的ストレス感，心拍数に対して群（反応群・非反応群）×測定時点（安静期・準備期・スピーチ期・計算期・回復期10分・回復期20分・回復期30分・回復期45分・回復期60分）の2要因分散分析を行った。分散分析において有意な効果が認められた場合にはShafferの方法に基づく多重比較を行った。繰り返しのある分散分析において自由度調整が必要な場合はGreenhouse-Geisserの自由度調整法を用いた。

空間手がかり課題において注意バイアスが生じているかを明らかにするため，注意定位と注意解放の各インデックスについて0との差があるかを群別，表情別に1標本t検定を用いて検討した。加えて，注意バイアスが確認された条件において，注意の促進や遅延に群間で差があるかを明らかにするため，注意バイアスの確認された条件のインデックスについて対応のないt検定を用いて群間差を検討した。

結　果

急性ストレス反応の確認　Table 1に各測定時点における主観的ストレス感，心拍数，唾液中コルチゾール濃度を示す。主観的ストレス感に対して測定時点を要因とする繰り返しのある分散分析を行った結果，測定時点の主効果が有意であった（$F'(8, 336) = 60.61$, $\varepsilon = .48$, $p < .01$）。多重比較の結果，主観的ストレス感は準備期，スピーチ期，計算期において安静期及び回復期の全時点に比べて高かった。心拍数に対して測定時点を要因とする繰り返しのある分散分析を行った結

Table 1. Means (standard error of means) of perceived stress, HR, and cortisol in all participants.

	BL	PR	SP	MA	RE10	RE20	RE30	RE45	RE60
Perceived stress (VAS)	2.47	6.17	6.54	5.91	3.29	2.96	2.74	1.91	1.84
	(0.26)	(0.37)	(0.39)	(0.36)	(0.38)	(0.38)	(0.36)	(0.31)	(0.29)
HR (bpm)	66.51	71.44	68.37	69.37	66.91	66.44	65.51	65.65	63.93
	(1.88)	(2.25)	(2.14)	(1.82)	(1.82)	(1.73)	(1.76)	(1.76)	(1.79)
Cortisol (nmol/L)	7.47	7.46	9.76	12.21	13.34	11.77	10.12	8.76	7.85
	(0.64)	(0.58)	(0.87)	(1.25)	(1.42)	(1.1)	(0.88)	(0.74)	(0.56)

Note. BL: baseline period; PR: preparation period; SP: speech period; MA: mental arithmetic period; RE10: recovery period 10 min; RE20: recovery period 20 min; RE30: recovery period 30 min; RE45: recovery period 45 min; RE60: recovery period 60 min.

果，測定時点の主効果が有意であった（$F(8, 336) = 53.44, \varepsilon = .29, p < .01$）。多重比較の結果，心拍数は準備期，スピーチ期，計算期において安静期及び回復期の全時点に比べて高かった。唾液中コルチゾール濃度に対して測定時点を要因とする繰り返しのある分散分析を行った結果，測定時点の主効果が有意であった（$F(8, 336) = 19.16, \varepsilon = .29, p < .01$）。多重比較の結果，唾液中コルチゾール濃度はスピーチ期，計算期，回復期10分で安静期に比べて高かった。これらの結果は，本研究で用いたTSSTが急性ストレス反応を惹起したことを示す。

群分け　コルチゾールの増加と注意バイアスの関連性を検討するため，TSSTに対する唾液中コルチゾール濃度の変化に基づき実験参加者を反応群と非反応群に分けた。先行研究同様，唾液中コルチゾール濃度は，心理社会的ストレス負荷終了後10分の時点で最大値を示した（Dickerson & Kemeny, 2004）。これに基づき，コルチゾールの反応性は安静期における唾液中コルチゾール濃度から回復期10分における濃度への変化量と定義した。コルチゾールの反応性に基づき43名の参加者を中央値分割により反応群22名（男性19名，女性3名，平均年齢21.9歳，範囲18—28歳）と非反応群21名（男性16名，女性5名，平均年齢21.2歳，範囲19—27歳）に分けた。反応群と非反応群のCES-Dの得点はそれぞれ11.50（$SD = 7.37$）と13.10（$SD = 7.69$）であり群間差は観察されなかった（$t(41) = 0.70, p = .49$）。

Figure 2に反応群と非反応群の主観的ストレス感，心拍数，唾液中コルチゾール濃度を示す。主観的ストレス感について群（反応群・非反応群）×測定時点（安静期・準備期・スピーチ期・計算期・回復期10分・回復期20分・回復期30分・回復期45分・回復期60分）の2要因分散分析を行った結果，測定時点の主効果は有意であった（$F(8, 328) = 60.22, \varepsilon = .48, p < .01$）が，群の主効果及び交互作用は有意ではなかった。心拍数について群（反応群・非反応群）×測定時点（安静期・準備期・スピーチ期・計算期・回復期10分・回復期20分・回復期30分・回復期45分・回復期60分）の2要因分散分析を行った結果，測定時点の主効果は有意であった（$F(8, 328) = 14.14, \varepsilon = .64, p < .01$）が，群の主効果及び交互作用は有意ではなかった。唾液中コルチゾールについて群（反応群・非反応群）×測定時点（安静期・準備期・スピーチ期・計算期・回復期10分・回復期20分・回復期30分・回復期45分・回復期60分）の2要因分散分析を行ったところ，群×測定時点の交互作用が有意であった（$F(8, 328) = 18.94, \varepsilon = .33, p < .01$）。観察された一次の交互作用について群ごとに単純主効果の検定を行ったところ，反応群において単純主効果が有意であり（$F(8, 168) = 29.15, \varepsilon = .32, p < .01$），準備期と回復期45分を除く全ての測定時点の唾液中コルチゾール濃度が安静期よりも有意に高かった（$ps < .05$）。一方，非反応群においても単純主効果が有意であった（$F(8, 160) = 3.50, \varepsilon = .23, p < .05$）ものの，測定時点間に有意な差は観察されなかった。各測定時点におけるコルチゾール濃度は，安静期，準備期以外の測定時点で非反応群よりも反応群で高かった（$ps < .05$）。

注意定位・解放インデックス　Table 2に全参加者及び各群における条件ごとの平均反応時間を示す。Figure 3に群および表情別の注意定位インデックスと

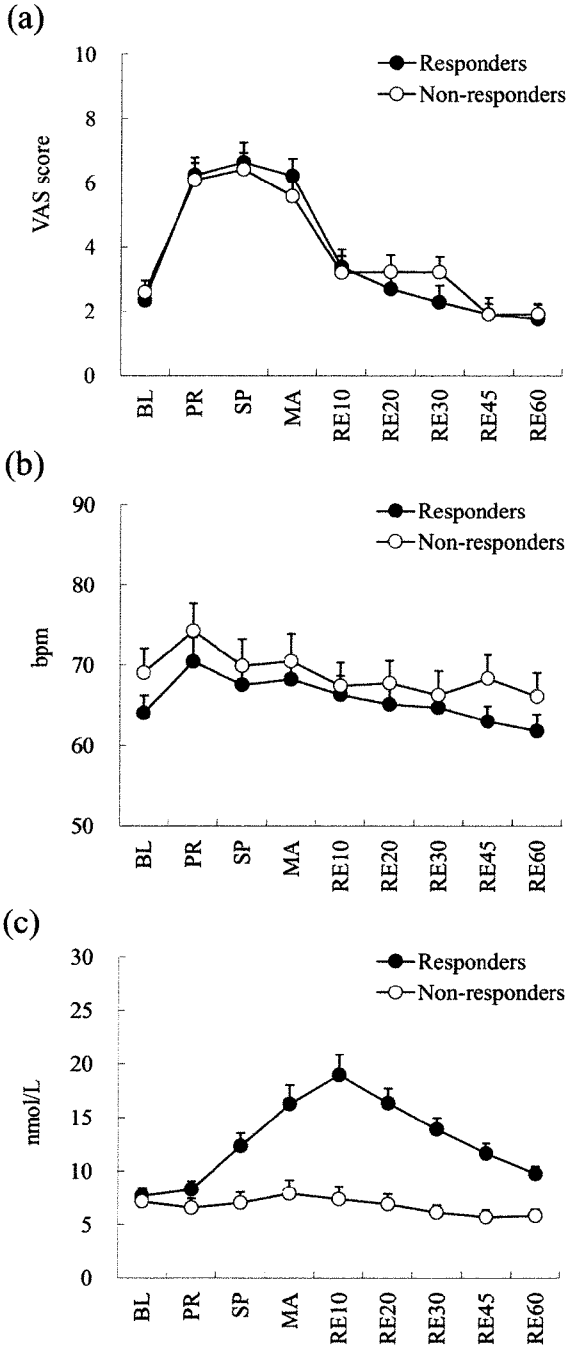

Figure 2. Means for (a) perceived stress, (b) HR, and (c) concentrations of salivary cortisol in cortisol responders and non-responders. Error bars represent standard error of the mean. BL: baseline period, PR: preparation period, SP: speech period, MA: mental arithmetic period, RE10: recovery period 10 min, RE20: recovery period 20 min, RE30: recovery period 30 minutes, RE45: recovery period 45 min, RE60: recovery period 60 min.

注意解放インデックスを示す。1標本t検定を注意定位，注意解放のインデックス別，表情別，群別に実施した。その結果，注意定位インデックスについては，いずれの条件においても有意な0との差は観察されなかった。一方，注意解放インデックスについては，反応群の怒り顔提示時で有意に0より大きく（$t(21)=2.50, p<.05$），非反応群の怒り顔提示時で有意に0より小さかった（$t(20)=2.51, p<.05$）。笑顔提示時の注意解放インデックスにはいずれの条件においても有意な0との差は観察されなかった。有意な促進と遅延が生じていた怒り顔提示時の注意解放インデックスについて，反応群と非反応群に差があるかを対応のないt検定を用いて検討した。その結果，怒り顔提示時の注意解放インデックスは非反応群に比べて反応群で大きかった（$t(41)=3.55, p<.01$）。

考　察

本研究は，コルチゾールのストレス反応性の高さと注意の解放段階におけるバイアスの強さに関連があるか否かを検討した。この目的のため，TSSTによる心理社会的ストレス負荷から60分後に手がかり刺激の提示時間の比較的長い空間手がかり課題を実施した。実験の結果，TSSTは主観的ストレス感，心拍数，唾液中コルチゾールを一過性に増加させた。TSSTに対する唾液中コルチゾールの反応性に基づき実験参加者を反応群と非反応群に分けたところ，両群の注意バイアスには異なる特徴が観察された。すなわち，反応群では怒り顔提示時の注意の解放インデックスが正の値を示したのに対して，非反応群では負の値を示した。

心理社会的ストレス課題によりコルチゾールの増加した反応者では，増加しなかった非反応者に比べて怒り顔に対する注意バイアスが強かった。この結果は，先行研究の結果と一致する（Ellenbogen et al., 2010; Fox et al., 2010; van Honk et al., 1998, 2000; Pilgrim et al., 2010）。重要な点は，コルチゾール反応者における怒り顔への注意バイアスが注意の解放段階でのみ生じており，注意の定位段階では生じていなかった点である。これは，注意の定位段階におけるバイアスの強さとコルチゾールの増加に関連を見出してきたこれまでの研究結果と一致しない。先行研究では，手がかり刺激の提示時間が500 ms以下（Fox et al., 2010;

Table 2. Mean RTs in ms (standard error of means) of the spatial cueing task.

	Angry		Neutral		Smiling	
	Valid	Invalid	Valid	Invalid	Valid	Invalid
All participants ($N=43$)	355.03 (7.16)	356.98 (6.54)	353.07 (7.54)	357.41 (6.75)	348.82 (6.78)	356.35 (7.04)
Responders ($N=22$)	353.70 (8.70)	364.26 (7.82)	357.58 (10.00)	355.08 (7.86)	351.77 (8.18)	358.69 (8.28)
Non-responders ($N=21$)	356.42 (11.71)	349.35 (10.54)	348.35 (11.51)	359.86 (11.27)	345.74 (11.10)	353.91 (11.71)

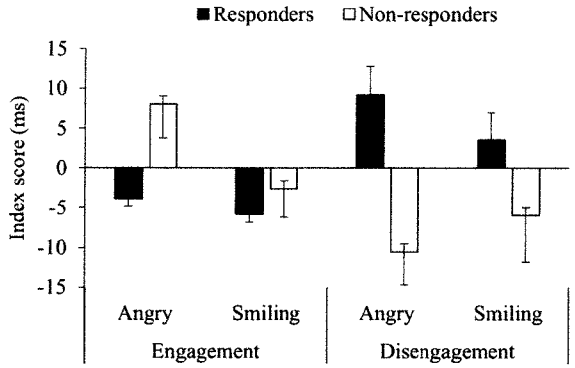

Figure 3. Mean engagement and disengagement scores for angry and smiling faces. Error bars represent standard error of the mean.

Pilgrim et al., 2010) であるのに対して，本研究では1,000 msという比較的長い提示時間を用いた。このことから，本研究の結果はコルチゾールの反応者において注意の解放段階におけるバイアスを検出するためには，手がかり刺激の提示に十分な時間が必要であることを示している。同時に，本研究の結果は，脅威刺激への注意バイアスが解放段階で生じるか定位段階で生じるかは，手がかり刺激の提示時間という実験課題のパラメータに依存することを意味する。比較的長い時間提示される手がかり刺激への注意バイアスは，自動的な初期の情報処理過程というよりは制御的な後期の情報処理過程におけるバイアスを反映しているとされる (Browning et al., 2010)。本研究の結果と先行研究の知見を合わせると，心理社会的ストレスに対するコルチゾールの増加は，脅威刺激への自動的な注意定位の促進と制御的処理過程における注意解放の困難さの両段階における注意バイアスと密接に結びついていると考えられる。

コルチゾール反応者が怒り顔からの注意の解放に困難を示したことに対して，コルチゾール非反応者では真顔よりも怒り顔からの注意の解放が早かった。この結果は，情動ストループ課題においてコルチゾール非反応者が脅威刺激から素早い注意回避を示すという報告と一致する (van Honk et al., 2000; Roelofs et al., 2007)。このことは，ストレス負荷によるコルチゾールの増加の少ない者は脅威刺激から積極的に注意を解放させる傾向があることを意味している。このような傾向は，先行研究において報告されている保護バイアス (protective bias) の一種と考えらえる。保護バイアスは，健康成人に観察される脅威刺激からの積極的な注意回避の傾向であり (Leyman et al., 2007)，ネガティブ情報への注意の促進を抑制することで抑うつや不安といったネガティブ気分の生起や持続を妨げる (McCabe & Gotlib, 1995; McCabe et al., 2000)。非反応者において保護バイアスが観察されたことは，社会的脅威刺激の積極的な無視が心理社会的ストレスに対するコルチゾールの増加を減衰する可能性を示唆している。

コルチゾール反応者，非反応者ともに笑顔に対しては注意バイアスを示さなかった。この結果は，快単語 (Pilgrim et al., 2010) や快画像 (Fox et al., 2010) に加えて，社会的な快刺激である笑顔への注意バイアスもコルチゾールのストレス反応性とは関連しないことを示す。同時に，この結果は，コルチゾールのストレス反応性が社会的刺激の中でも社会的脅威に関連する刺激の処理と特異的に結びついていることを示唆する。社会的脅威は，扁桃体の神経活動の上昇 (Jankord & Herman, 2008) や海馬の神経活動の減衰 (Pruessner et al., 2008) を介してHPA系からのコルチゾールの分泌を高める。このようなコルチゾールの分泌は，社会的

脅威からの回避を促進して生存を高めるための心理生物学的機構であると考えられている(Kemeny, 2009; Sapolsky, 2005)。一方，笑顔といった社会的受容に関連する刺激の処理はオキシトシンの分泌と関係する(Domes et al., 2013; Tollenaar et al., 2013)。このことから，社会的な脅威と受容では結びついている生物学的機構が異なると考えられる。このような観点から，コルチゾールのストレス反応性は，社会的脅威を意味する怒り顔への注意と密接な関連を示した一方，社会的受容を意味する笑顔への注意とは関連しなかったと解釈できる。

本研究では心理社会的ストレス負荷の60分後に空間手がかり課題を実施した。空間手がかり課題直前の主観的ストレス感や心拍数の結果から，TSSTによるネガティブ感情や覚醒度の上昇は空間手がかり課題実施時には回復していると考えられる。しかしながら，空間手がかり課題直前の唾液中コルチゾール濃度は，非反応群に比べて反応群で高かった。コルチゾールは脂溶性であり，血液脳関門を通過することで脳内のグルココルチコイド受容体，ミネラルコルチコイド受容体と結合する。これらの受容体は扁桃体に多く分布していることから(Patel et al., 2000)，循環中のコルチゾールの増加は，扁桃体の活動の調節を介して情動刺激の処理に影響を及ぼす(van Honk et al., 1998; van Stegeren et al., 2007)。よって，本研究で測定した注意バイアスには，増加した循環中のコルチゾールが影響している可能性がある。このため，ストレス負荷のない状態に注意バイアスを測定した研究の結果と同一視することはできない。今後は，ストレス負荷前と負荷後に繰り返し注意バイアスを測定する実験デザインを用いることで，本研究で観察されたコルチゾールのストレス反応性の高さと注意の解放段階におけるバイアスの強さの関連が，ストレス負荷のない状態における注意解放の遅延を反映しているのか，ストレス負荷により増加したコルチゾールが注意解放を遅延させたことを反映しているのか，詳細に検討する必要がある。

本研究にはいくつかの制限がある。はじめに，本研究では，注意解放のバイアスを測定するために空間手がかり課題を用いた。しかし，空間手がかり課題では，手がかり刺激の提示された位置に標的刺激が提示されることから注意の定位段階と解放段階におけるバイアスを完全に分けることができないという指摘がある(伊里・望月，2012)。このため，コルチゾールの増加と注意解放の困難さの関連については，ギャップ・オーバーラップ課題のような注意解放のバイアスを検出するのに特化した課題を用いて詳細に検討することが必要である。

次に，研究結果の一般化には注意が必要である。本研究では健康な大学生・大学院生を対象として実験を実施したが，コルチゾールのストレス反応性と注意バイアスの関連は実験参加者のプロフィールに大きく影響を受ける(Ellenbogen et al., 2010)。よって，今後の研究では，健康成人以外を対象とした実験を行うことで本研究の結果を一般化する試みが必要である。

最後に，本研究の結果は，注意バイアスとコルチゾールの相関関係を示すものであり，その因果関係について言及することはできない。脅威刺激への注意バイアスがコルチゾールの増加を調節する(Dandeneau et al., 2007)一方，コルチゾールの投与は注意バイアスを促進して脅威刺激に対する扁桃体の神経活動を高める(Henckens et al., 2012)。このことは，脅威刺激の処理がコルチゾールを増加させるとともに，増加したコルチゾールが脅威刺激の処理を促進するといった，脅威への行動的反応を促進するためのフィードフォワード機構の存在を示唆する。興味深いことに，コルチゾールと脅威刺激の処理の関連は表情といった社会的脅威刺激にはとどまらず，単語や画像といった幅広い刺激の種類でも観察される(Fox et al., 2010; Pilgrim et al., 2010)。加えて，上述のとおり，オキシトシンは社会的受容を意味する快刺激の処理を促進する(Domes et al., 2013)。これらは，体内の内分泌の状態が感情刺激の処理へと大きな影響を及ぼしていることを示している。このような観点から，今後は，ヒトの内分泌の状態がどのように感情刺激の評価，主観的な感じ方へと影響を及ぼすのかを検討することが必要である。

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テクニカルノート

爪に含まれるコルチゾールの定量手法の検討
——粉砕粒度と抽出時間の検討——[1]

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Quantitative Measurements of Fingernail Cortisol: Effects of Ground-fingernail Grain Size and Extraction Time

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Although cortisol levels in fingernails may reflect the hormone's long-term cumulative production, a standard measuring procedure has not yet been established. In this study, we investigated the effects of ground-fingernail grain size and cortisol extraction time on the measurement of fingernail cortisol. Fourteen healthy males provided fingernail samples, which were ground for 1, 4, or 16 minutes (coarse-, medium-, or fine-grading conditions, respectively). Subsequently, cortisol was extracted in pure methanol for 1, 6, 24, or 48 hours (four extraction time conditions). The clear supernatant was filtered and dried, and extracted cortisol was measured using an enzyme-linked immunosorbent assay. ANOVA results indicated that finer grading and longer extraction time was associated with higher cortisol levels. Furthermore, an interaction between grading and extraction-time conditions was observed: cortisol levels between medium- and fine-grading conditions were comparable in 48-hour extraction. This study clearly demonstrated that the grain-size of ground fingernails and extraction time could affect the amount of extracted cortisol obtained from fingernails and should therefore be considered during fingernail cortisol assay.

Key words: fingernail, cortisol, stress, enzyme-linked immunosorbent assay

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[1] 本研究は，JSPS科研費15K13169（爪試料を利用した慢性的・蓄積的なコルチゾール分泌の評価法の確立）の助成を受けた。

【要　約】 爪に含まれるコルチゾールは過去の比較的長期的なホルモンの動態を反映すると考えられているが，その標準的な定量方法についてはまだ確立されたものがない。本研究では爪試料の粉砕粒度や抽出時間が爪試料から抽出されるコルチゾール量に与える影響を検討した。健常な男性14名から手の爪を採取した。爪試料を1, 4, 16分間粉砕し，粉砕粒度の条件（粗い，中程度，細かい）を設定した。また，粉砕した検体はメタノールにより抽出処理を行うが，本研究では抽出時間を1, 6, 24, 48時間の4条件に設定した。上澄み液にろ過処理を施し，蒸発乾固させた後に，最終的にコルチゾールの抽出量を酵素免疫測定により評価した。粉砕粒度と抽出時間を要因とした分散分析を行った結果，粉砕粒度が細かいほど，また抽出時間が長いほど，コルチゾールの抽出量が多いことが示された。また，粉砕粒度と抽出時間の交互作用が有意であり，爪試料が中程度以上に粉砕されていて，かつ抽出時間が48時間の条件では，粉砕粒度の影響は小さいことも示された。本研究では粉砕粒度や抽出時間が爪からのコルチゾールの抽出量に影響を与えることを明確に示した。爪からコルチゾールを測定する際はこれらの要因にも留意する必要性が示された。

心理社会的ストレスは様々な生理学的な変化を引き起こす。視床下部-下垂体-副腎皮質系の賦活はその変化の一つであり，最終的に，副腎皮質からコルチゾールが血中に分泌される。現在まで多くの研究が行われており，急性ストレスは血中・唾液中のコルチゾールを増加させることが知られている（Dickerson & Kemeny, 2004）。従来，コルチゾールは血液や唾液から測定されることが多い。血液や唾液から測定されるコルチゾールは，数分から数十分の比較的短期間の急性のホルモンの動態を示すと考えられている。また，最近では毛髪からコルチゾールを測定する研究も増えてきている。毛髪は形成される際に毛細血管からコルチゾールが拡散すると考えられている。毛髪は1か月に1センチ伸びるため，例えば，根元から3センチ部分は最近3か月のコルチゾールの分泌を反映すると考えられている。毛髪は慢性的なコルチゾールの状態を把握する指標として注目されている（Russell et al., 2012）。

本研究では，爪に含まれるコルチゾールに注目する。毛髪と同様に，爪も形成される際に毛細血管からコルチゾールが拡散すると考えられている（de Berker et al., 2007; Warnock et al., 2010）。手の爪は10日で約1ミリ伸びるため（Gupta et al., 2005），1ミリの爪は約10日のホルモン状態を反映すると言われている。また手の爪は根元から先端に伸びるに数か月を要することから，先端の爪は数か月前の状態を反映すると言われている。最近の研究では，手の爪のコルチゾールは4, 5か月前の唾液中コルチゾールの値と中程度の相関を示したことが報告されている（Izawa et al., 2015）。爪は毛髪と比較しても，検体が少量で済む，自身でも採取できるなどの利点もあるため，慢性的なコルチゾールの状態を把握する指標として注目されている。

しかしながら，爪に含まれるコルチゾールの定量方法に関しては，まだ確立されたものがない。先行研究では毛髪のコルチゾールの測定方法を援用しており，爪のコルチゾールの定量方法としての妥当性については，まだ十分に検討されているとはいえない。毛髪や爪からコルチゾールを測定する際には，はじめに検体の外側に付着した物質（汗や脂など）を洗浄する。続いて，検体を粉状に粉砕し，メタノールなどの溶媒液の中で一定時間（多くの場合は24時間），コルチゾールを抽出する。コルチゾールなどのステロイドホルモンは水溶性であるため，この工程によって毛髪・爪の中に含まれるコルチゾールが溶媒液中に移動する。その後，溶媒液中に含まれるコルチゾールを質量分析や酵素免疫学的測定法によって評価する。多くの場合は，一度，溶媒液を乾固し，測定に適した溶液によって懸濁し，測定を行う。この一連の過程で問題となるのは，爪検体からコルチゾールが十分に抽出できているかという点である。例えば，検体の粉砕後の細かさ（粉砕粒度）が抽出に影響を与える可能性が考えられる。また，設定する抽出時間によっても回収できるコルチゾール量が異なる可能性も考えられる。現時点ではこのような基本的な技術要素さえ検討がなされておらず，今後，研究データを積み重ねていくためには共通したプロトコルの確立は必須である。また，爪の検体は，その個人の切り方など，もともとの個人差も大きい事が予想され，粉砕粒度や設定する抽出時間の影響を検討することは重要であると考えられる。本研究では，粉砕粒度と抽出時間を段階的に実験条件として

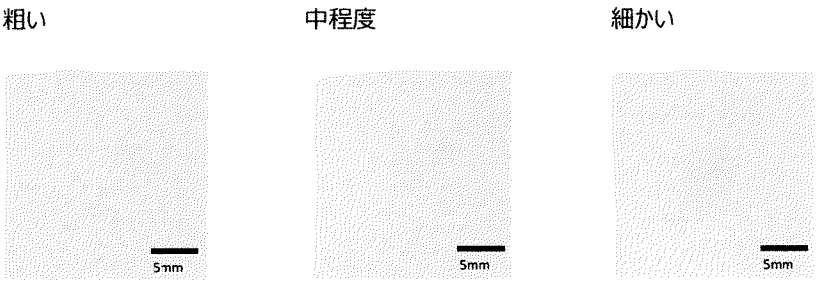

図1. 粉砕された爪の例：粗い条件の粒度（爪片の長さの平均値±標準偏差）は3.07±0.76 mm，中程度の条件では1.61±0.78 mm，細かい条件では0.05±0.02 mmであった．粒度の測定にあたっては，顕微鏡で撮影したデジタル画像から粒径の最大値を1つずつ計測した．

設定し，粉砕粒度や抽出時間が爪検体から抽出されるコルチゾール量に与える影響を検討することを目的とした。

方　法

実験参加者

健常な男性の大学生・大学院生14名を対象とした（20—25歳）。14名中3名が喫煙習慣を有し，14名のBody Mass Indexは20.2±2.5 kg/m^{2}であった。なお，本実験は，長岡技術科学大学の倫理審査委員会において実施を認められたものであった（承認番号H26-13号）。

爪の採取

両手の10本の指から爪を採取するように求めた。期間は4週間とし，その間に伸びた爪を実験参加者自身で爪切りによって切り，ジップロックに回収するように指示した。

爪コルチゾールの測定手続きならびに条件設定

爪コルチゾールの測定は，Izawa et al.（2015）や野村ら（2012）などの研究も参考にし，下記の工程によって実施した。粉砕と抽出の工程において実験条件を設定した。

洗浄　爪検体を2.0 mlのマイクロチューブの中にいれ，1.0 mlのメタノールを注ぎ，5秒間攪拌した。メタノールを排出した後，爪検体を24時間以上，自然乾燥させた。

粉砕　乾燥させた爪検体とメタルコーンをマイクロチューブ（2.0 ml凍結保存チューブ（品番72.608），ザルスタット株式会社，日本）の中にいれ，多検体細胞粉砕機（マルチビーズショッカー®，安井器械株式会社，日本）によって，回転数を2,000 rpmに設定して粉砕した。粉砕粒度を操作するために，粉砕時間を1分，4分，16分に設定した。利用した爪検体の量は一人当たり約135 mgであり，これを3本のマイクロチューブに45 mgずつ分けて，粉砕した。同じ実験参加者でも爪の部位によって濃度が異なる可能性を考慮して，1分粉砕した後に3本のチューブの爪粉を薬包紙上で混ぜた。1本分の爪粉を1分の条件にふり，残りの2本分の爪粉を再度，45 mgずつチューブに戻し，粉砕した。4分の時点で再度，2本分の爪粉を薬包紙上で混ぜた。1本分の爪粉を4分の条件にふり，最後の1本分の爪粉を16分の時点になるまで粉砕した。この手続きによって，一人の実験参加者の爪検体について，粉砕粒度が粗い条件（1分），中程度の条件（4分），細かい条件（16分）を設定した（図1）。

抽出　マイクロチューブ（2.0 ml凍結保存チューブ（品番72.608），ザルスタット株式会社，日本）の中に約40 mgの爪粉をいれ，そこに1.6 mlのメタノールを加えて，コルチゾールの抽出を行った。抽出は室温下（18—25℃）で行い，抽出の間，マイクロチューブをローテーターの上で攪拌した。抽出時間の条件を1時間，6時間，24時間，48時間に設定した。該当するタイミングで270 µlのメタノールの上澄みを，別の1.5 mlのマイクロチューブ（マキシマムリカバリーチューブ（品番BT-150 L），BIO-BIK，日本）に移した。なお，上澄みを移動させる前にマイクロチューブを6,000 rpmで10分間遠心した。またシリンジ（ディスポシリンジロック基，テルモ株式会社，日本）とフィルター（マイクレスフィルター，メルクミリポア，アメリカ）を利用して，上澄み液をさらに精製し

た。フィルターはポリフッ化ビニリデン製，孔径0.22 μmのものであった。メタノールに爪粉が含まれた場合，爪粉が後述の酵素免疫測定に影響を及ぼす可能性が懸念されるが，本研究ではこの手続きにより，その可能性を排除した。

蒸発乾固・酵素免疫測定　得られた上澄みは室温下(18—25℃)で蒸発乾固した。コルチゾールの測定には酵素免疫学的測定法の原理に基づく分析キット(Cortisol EIA kit salivary, 1-3002, Salimetrics LLC, USA)を用いた。キットに付随している希釈液30 μlを蒸発乾固したマイクロチューブに加えて，懸濁を行い，希釈液に含まれるコルチゾール量をキットの指定する手順によって測定した。キットの測定内変動係数は7%以下，測定間変動係数は11%以下であった。測定値は爪1グラム中に含まれるコルチゾール量で表した(pmol/g)。

統計

コルチゾール濃度を従属変数とし，粉砕粒度(粗い，中程度，細かい)，抽出時間(1時間，6時間，24時間，48時間)を被験者内要因とした2要因の分散分析を行った。球面性の仮定が支持されなかったため，Greenhouse-Geisser法による自由度の補正を行った。多重比較では対応のあるt検定を行い，ボンフェローニ法による有意水準の補正を行った。粉砕粒度条件では$p<.0167$ (.05/3)，抽出時間条件では$p<.0083$ (.05/6)を有意水準とした。また，各条件間のデータの対応をみるために，ピアソンの相関係数を全12条件(粉砕3条件×抽出時間4条件)の間で算出した。

結　果

粉砕粒度と抽出時間を要因とした2要因分散分析を行った結果，粉砕粒度($F(1.1, 14.9) = 31.6$, $p<.001$, $\eta^2 = .71$)と抽出時間($F(1.1, 14.0) = 82.3$, $p<.001$, $\eta^2 = .86$)の主効果，ならびに粉砕粒度と抽出時間の交互作用($F(2.7, 34.9) = 4.1$, $p<.05$, $\eta^2 = .24$)が有意であった。全般的に粉砕粒度が細かいほど，また抽出時間が長いほど，コルチゾールの測定値が高いことが示された。交互作用について単純主効果の検定を行った結果，48時間の抽出の条件では，粒度が中程度の条件と細かい条件の間ではコルチゾールの測定値に有意な差が認められなかった。それ以外の間では有意差が認められた(図2)。

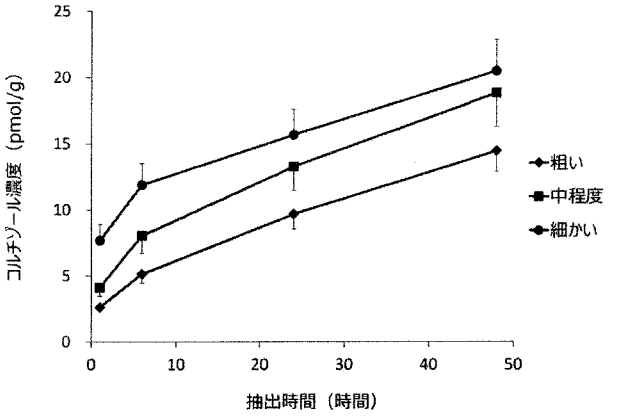

図2.　各粉砕粒度条件および抽出時間条件における爪コルチゾール濃度(平均値と標準誤差)

各条件間のデータの対応をみるために，全12条件の相関を算出した結果，.75から.98の強い相関が認められた。

考　察

本研究では爪に含まれるコルチゾールの定量方法において，粉砕粒度と抽出時間の影響を検討した。現在までに爪に含まれるコルチゾールの定量方法に関しては，まだ確立されたものがない。先行研究では毛髪のコルチゾールの定量方法を援用しており，爪のコルチゾールの定量方法としての妥当性については，まだ十分に検討されているとはいえない。例えば，Warnock et al. (2007)の研究では，2.5分と5分の粉砕時間を設定しているが，その設定時間毎のデータは報告されておらず，Izawa et al. (2015)の研究では，40分の粉砕時間を設定しているのみである。粉砕に用いる機器は研究間で異なるので，粉砕粒度に関する共通した見解は得られていない。また，抽出時間については，いずれの研究も24時間に設定しており，それ以外の抽出時間の条件は検討していない。本研究では，全体的に粉砕粒度が細かい程，また抽出時間が長い程，抽出されるコルチゾール量が多いことが示された。私たちの知る限り，爪のコルチゾールの測定において，粉砕粒度や抽出時間の影響を検討した研究は本報告がはじめてのものであり，定量方法に関する基礎的な情報を示したという点において本研究は意義があるものと考えられる。

また，本研究では，爪検体が中程度以上に粉砕され

ていて，かつ抽出時間が長い条件では，粉砕粒度の影響は比較的小さいことも示された。この結果は爪試料からコルチゾールを測定するような研究に対して方法論的な示唆を与える。基本的に人から得られる爪検体は個人差が大きい。例えば，爪の切り方はさまざまであり，得られる爪検体の細かさは個人によって異なり，これは粉砕粒度に影響を与える。本研究では中程度以上の細かさに粉砕し，かつ長い抽出時間の条件では，粉砕粒度の影響はみられなかった。したがって，一定以上の細かさに爪を粉砕して，抽出時間を長く設定することが爪検体の細かさの個人差の影響を少なくする一つの方法となるであろう。

本研究の結果の解釈における留意点として何点かあげることができる。一点目は，本研究では，粉砕粒度が細かいほど，あるいは抽出時間が長いほど，コルチゾールの抽出量が増加したが，このコルチゾールの増加が停滞するような現象（プラトー）は観察されなかった。したがって，粉砕時間や抽出時間をさらに長く設定すれば，より多くのコルチゾールを抽出できる可能性が考えられる。今後の研究では，設定する条件を増やし，場合によっては非線形関数のフィッティングなどの数学的手法も利用して，この点について検討が必要である。二点目に，本研究では各条件間において比較的高い相関を認めたが，これは粉砕粒度や抽出時間の条件が異なっても，条件間でコルチゾール値が一致することは示していない。どの条件が最も適切な測定条件であるかについては，前述のプラトーの条件を明らかにしつつ，相関分析や一致度の評価（Bland & Altman, 1986）などを通して検討が必要である。三点目に，本研究では爪の提供者の年代や性別が限定されていることである。したがって，提供者の年代や性別によって結果が異なる可能性も考えられる。

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自律神経バランスとセルフヘルスケア

（自律神経バランス測定のための副読本）

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2016 年 3 月

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１．はじめに

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ます。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（ホメオスタシス）を維持しています。ここでは、この自律神経バランスの簡便な測定方法と、それを活用したセルフヘルスケアについてお話いたします。測定方法は、最近開発されたウエアラブル生体センサ Silmee™ Bar Type（東芝製）を用いて心電図の R 波と R 波の間隔（R-R 間隔）を測定し、その結果をレーダ―チャートとして六角形の図に表します。さらに、更年期障害患者における自律神経バランスの変動と自律神経バランスによるセルフヘルスケアの意義について述べます。

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、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）的に働く神経です。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ります。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ます。

戦うか逃げるか、体の準備をするのが交感神経

アメリカの有名な薬理学の教科書 [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、（人又は動物が敵に遭

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す」と書かれています。すなわち、相手と戦う（又は逃げる）ためには、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ります。気管支を広げ、血糖値を上げることにより、酸素とエネルギー源のブドウ糖を筋肉へ送り、相手に負けしないようにします。また瞳孔を広げて、相手をよく見て戦うわけです。そう考えると、私達の体は非常に上手く出来ていることがわかります。例として、かつて小学校の運動会の時、かけっこ（徒競争）で自分の順番が回って来てスタートラインに立った時の事を思い出して下さい。あの時が、交感神経が最も興奮している状態です。

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、胃腸運動の亢進、消化腺分泌の亢進、栄養吸収の促進、排尿促進等が見られ、体全体として休息したりエネルギーを貯蔵させる方向に働きます[1)]。わかり易く説明しますと、例えば夕食後、自分の部屋で照明を少し落とし、好きな音楽のＣＤを聴いてリラックスしている時を想像して下さい。この時が、副交感神経が活動している状態です。

ライバルでありかつ親友の交感神経と副交感神経

活動時に働く交感神経と休息時に働く副交感神経は、基本的には一つの臓器（例えば心臓や気管支等）を２重支配し、お互い相反しながら協調的に作用し、体全体においてバランスを取りながら働きます（図 1）[2)]。従って、両神経はお互いにライバルであり、かつ大の親友でもあるわけです。両神経の機能が上

手く働くことによって、私たちの体は気温の変化や精神的ストレスなど、外界からの刺激に対応して体の状態を一定に保てるわけです。これを生体のホメオスタシス、または生体の恒常性と言い、この機能を維持するのが自律神経の役割です。このように交感神経と副交感神経は、その場その場において臨機応変に作用し、体がスムーズに働くように機能を調節しております。逆に言いますと、この自律神経が上手く働くことにより、私達は日常生活において何の違和感もなく生活出来ていることになります。

図１　自律神経の働き：交感神経と副交感神経の主な機能 [2)]

3　ストレスなど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きます。適度なストレスは生活のリズムを作るうえで大切ですが、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し、最悪の場合は死に至ることがあります。少し古い記事で毎日新聞[3]に掲載されたものですが、それは「その数年前に小泉首相による郵政民営化改革があり、自民党からかなりの人数が追い出され、その後大部分の人が自民党に復党しましたが、平沼氏の1人だけが郵政民営化にサインを拒否し復党しませんでした。結局これがストレスにつながったかどうかはわかりませんが、その後病に倒れてしまいました」というものでした。さらに「戦後、在任中に倒れた首相は4人いますが、いずれも総辞職をしています。故大平元首相は、1980年に激しい政争の末、総選挙中に不調を訴え心筋梗塞で入院し、70歳ぐらいで亡くなられました。病理解剖をした結果、冠動脈はまだ50代から60代の若さで、恐らく想像を超える精神的ストレスがあったのだろうと推測され、政治家のストレスが並大抵ではないと考えられます」という内容でありました。

もうひとつは野球選手のイチローの話です。これも毎日新聞（2009年4月）の記事ですが、2009年のWBC(ワールドベースボールクラッシック)で日本は優勝しました。韓国との決勝戦ではイチローの決勝打で勝利し、私達はイチローが大活躍したと思っていました。しかしWBCが終わった直後に、彼は胃潰瘍により米国メジャーリーグでの故障者リストに入り、私達を驚かせました。確かにイチローは2次予選で、彼にしては珍しく11打数無安打の打撃不振に陥っていました。彼にとっては自分のプライドが許さなかったのでしょうか、何とか塁に出ようとバントを試みますが、バントも失敗してしまいました。WBCの時の私達が想像する以上の苦しみやストレスが故障者リスト入りの引き金になったのではないかと、その記事には書かれていました。あの天才打者と言われるイチローでさえも、たかだか2、3週間の戦いで本職である打撃の不振でストレスを受けると胃潰瘍になってしまうわけであります。私達凡人は、

何をかいわんやということです。

ストレスがなぜ体に悪いかと言いますと、一つは強いストレスを感じると私たち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）という物質を大量に放出します。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、さらに免疫系も抑制され、最悪の場合は死に至るのです。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）に影響します（図 2）[4]。大脳辺縁系の真下には視床下部があるので、ストレスが大脳皮質からストレートにここに影響するわけです。その視床下部から自律神経は出発しており、交感神経や副腎を支配している腹部交感神経も出ています。従って、強いストレスを長期間受けますと、これらの神経が刺激されて大量のカテコールアミンを放出し、このような病気を引き起こしてしまうのです。

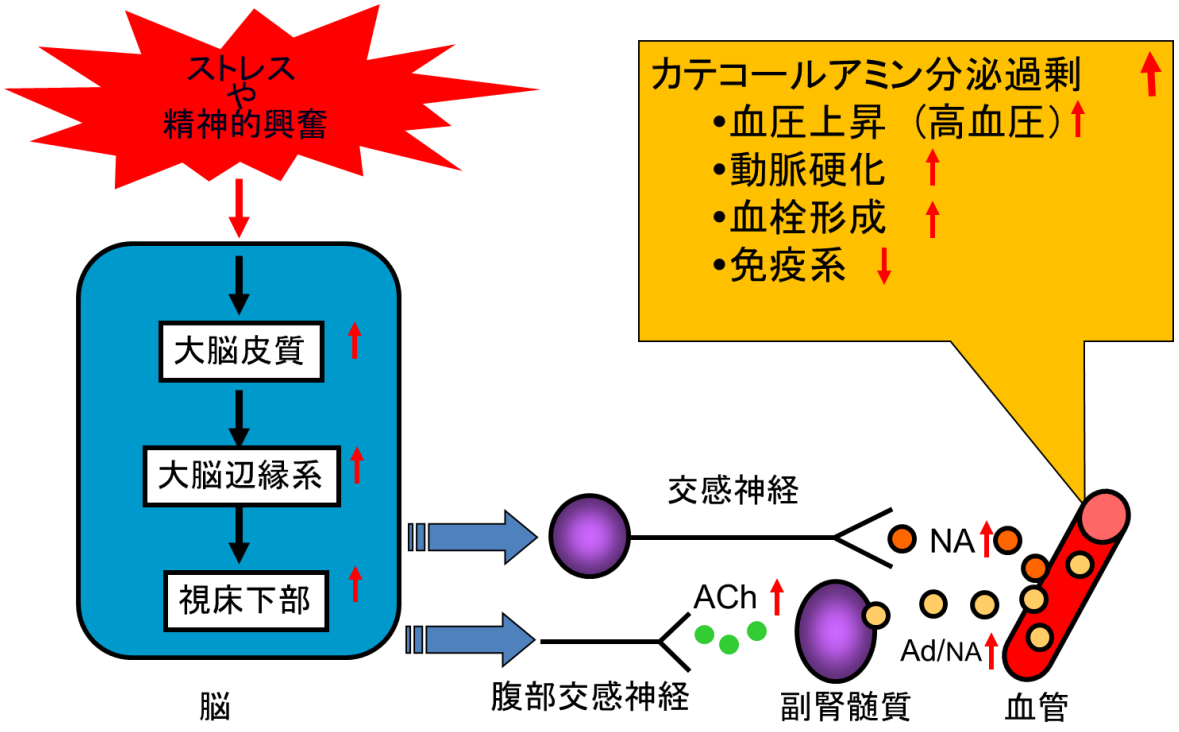

図２　ストレスによる生体カテコールアミン反応 [4]

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ます[5)]。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（安静抑臥位から立位への血圧の変動測定）、立位心電図（安静抑臥位から立位への心電図の変化）、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-R 間隔を測定）、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、多数あります。

今回紹介する測定法は、上記の電気的検査法の内の心拍変動検査の 1 つで、後藤幸生氏（福井大学名誉教授、産業医科大学非常勤講師）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[6-8)]です。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-R 間隔を測定します。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ますが、R-R 間隔をミリセカンドで表すと一定の変動があり、これを心拍変動のゆらぎと言います。健康な人であれば、誰でもこのようなゆらぎがあります。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します。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していますが、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をするという新しい方法です。R-R 間隔の経時変化を観察しますと、安静時から起立時に一時的に短くなり、これを交感神経被刺激度（瞬時反応度）と言います(図 3)[6-8)]。これはすぐに元のレベル近くまで戻りますが、起立した状態では安静時レベルまでは戻りません。これを交感神経興奮度（活性

化の持続度合い）と言います(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）で、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）です（図 4）[6-8]。

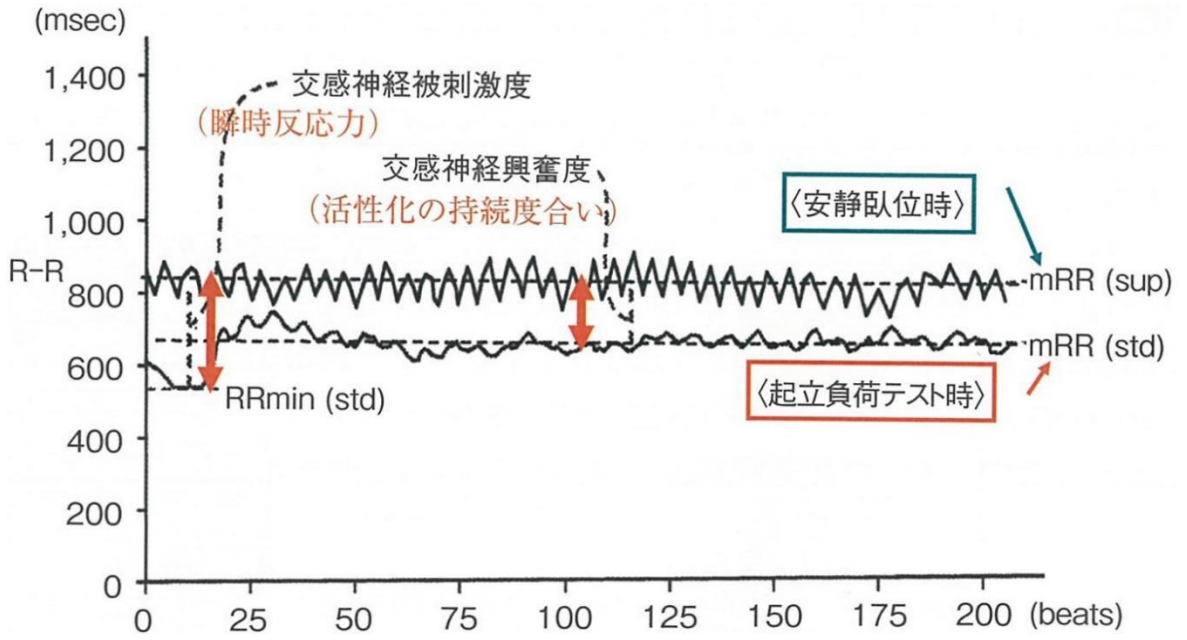

図３　安静仰臥位および立位での心電図 R-R 間隔の変化[8)]

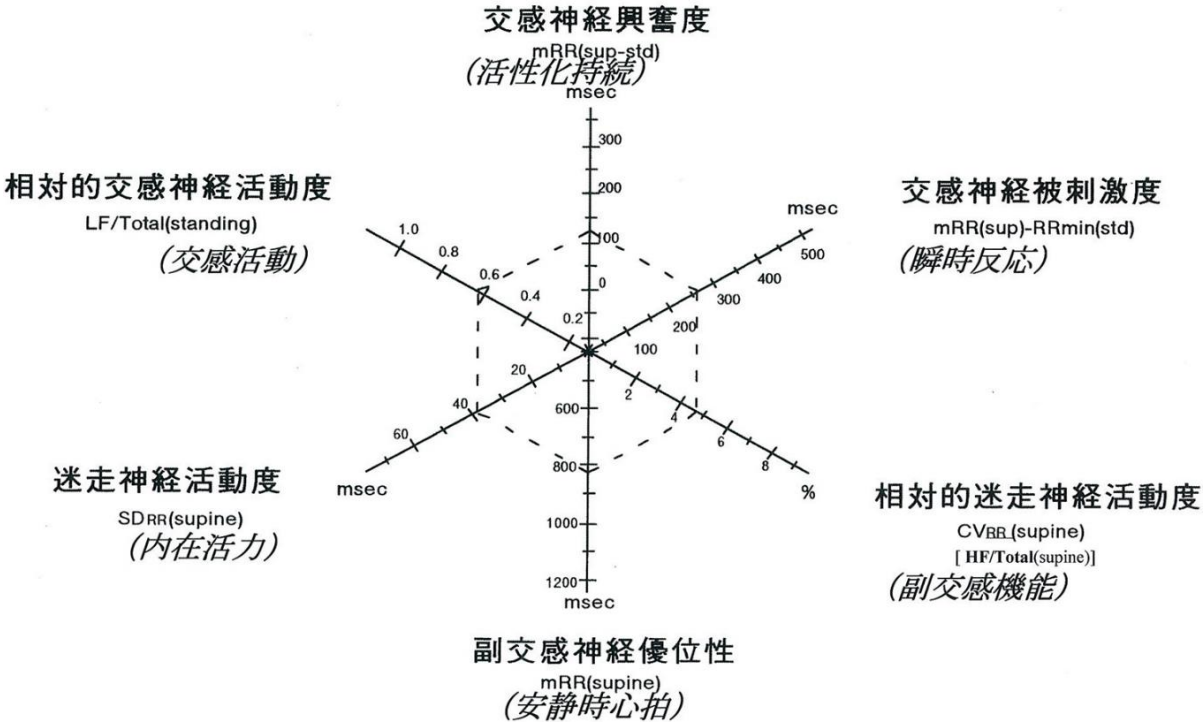

図４　自律神経バランス測定におけるレーダーチャート[8)]

5　測定操作の手順

測定操作の概略を説明します。まず注意すべき点として、この検査を実施出来ない人は、心臓ペースメーカーを使用している人、または中程度以上の不整脈や立ちくらみがある人です。

心電図計測器として、ウエアラブル生体サインセンサ Silmee™ Bar Type（東芝製、以下 Silmee）(図５A) を用います。

１. Silmee を左脇腹に貼る場合(図５B 左)　→　1-1 へ

（左脇腹で測定出来ない場合は、胸部で測定します。）

胸部に貼る場合(図５B 右)　　→　1-2 へ

1-1．Silmee を左脇腹に貼ります（図 5B 左)。

① 貼付部を薄いアルコール綿で拭きます。

（アルコールに皮膚がまける人は拭かないで大丈夫です）

② Silmee の電源を ON にします。

③ Silmee にゲルパッドを貼り付け、ゲルパッドを約 45 度傾けて貼ります。（ブラジャー直下の肋骨の上に、TOSHIBA の字を手前(上側)にして水平から 45 度傾ける）

1－2． 体質により、脇腹では測定できない場合があります。その場合は、鎖骨下中央部で測定します （図 5B 右)。（鎖骨の 5～10cm 下あたりに TOSHIBA の字を下にして貼り付ける）

図 5 Silmee™ Bar Type（A）とその装着イメージ(B)

２．ベッド上に仰向けになり、安静にしてください(図6A)。

① 目を閉じてください。腕は横に置いてください。

② 4秒に1回のリズミカルな呼吸※をしてください。

（１秒で吸って、２，３，４秒で吐く。深呼吸でなく普通に）

※メトロノームが1秒毎に鳴りますが、その内4秒に1回高い音があります。その高い音の時に軽く息を吸って、２，３，４秒でゆっくり息を吐きます。出来るだけ自然な呼吸をしてください。ベッドで横になってからしばらく練習をしてみて下さい。

③ 安静時のデータ採取をスタートします。

④ 測定は約1分です。

⑤ 1分経つと「立ち上がって下さい」の音声があります。

３．ベッドから速やかに起き上がり、壁に向かって立って下さい(図6B)。

① 目は開けてください。

② 速やかに立ち上がります。

③ 呼吸は安静時と同じく4秒に1回の呼吸です。

④ 立位時のデータ採取をスタートします。測定は約90秒間です。

⑤ 両腕は肩の力を抜いて、体に沿って下に垂らして下さい。

以上で測定は終了します。

４．自律神経バランスを測定後、タブレットに簡易更年期指数(SMI)テスト[9]をマークシート形式で回答します。そうすると、自律神経バランス測定の結果がレーダーチャートとして、またSMIをもとにした更年期障害のレベルが自動的にみられる仕組みになっております。

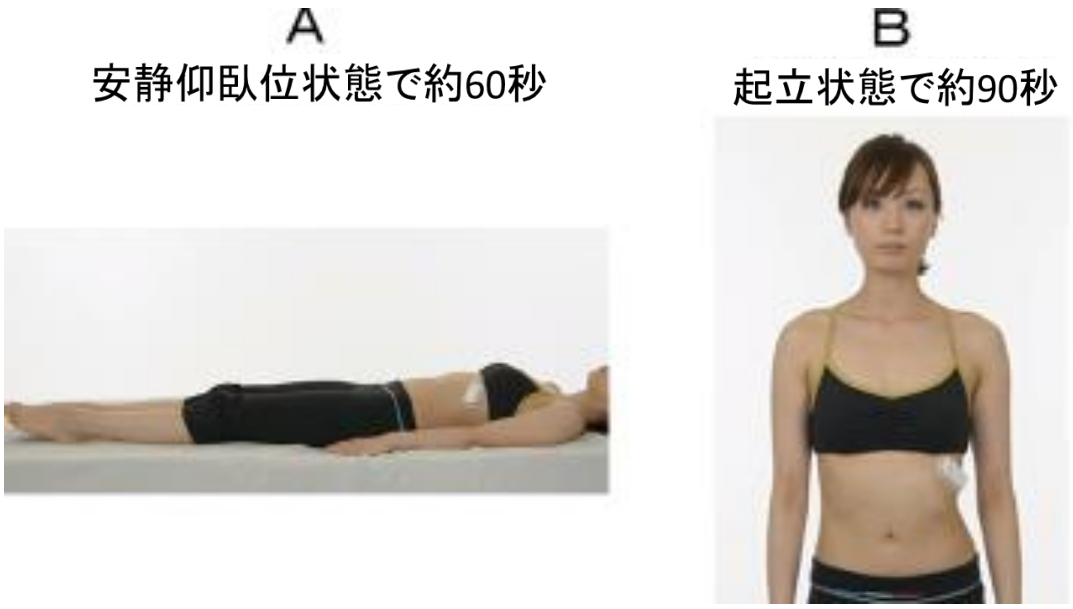

図６　安静仰臥位（A）と立位（B）での測定イメージ

6　測定結果の読み方

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と続く起立負荷テストを併せ、真の自律神経活動性を一つの六角形レーダーチャートで図示するものです。この方法は、単に交感・副交感の二つ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六つに分け、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ものです[6-8]。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）からなる標準正六角形のレーダ―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タブレット画面にて確認でき、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す。

その６つの指標（図 4）[6-8]とは、①起立負荷時の周波数解析データ低周波 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 その交感神経興奮度；mRR(sup-std)でその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)、すなわち安静時の心拍、⑤ 安静仰臥位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；SD_{RR}(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）[8] が表現され、これら６つの指標のバランスが標準図形と比較することで、その歪み具合が評価できます(表 1)。

交感神経機能		機能(指標)	基準値より低い時 (<80%)	正常(基準値±20%)	基準値より高い時 (>120%)
①	相対的交感神経機能	交感神経機能の強弱を示す。身体機能(筋肉収縮、血圧上昇など)だけでなくメンタル面(衝動、興奮など)への影響も含む。	交感神経の働きが弱い状態です。適度な運動と規則正しい生活で心身を活性化させましょう。	正常です	交感神経が過剰に働いている可能性があります。衝動的な行動を生まないよう、心身を意識して落ち着かせることを心がけましょう。
②	交感神経の瞬時反応性	負荷刺激に対する身体的反応の瞬発力を示す。起立という運動刺激に対し本来反応すべき血圧上昇などの交感神経機能が即時に働くかどうかを意味する。	運動刺激負荷に対して、その反応性が低下しています。適度な運動習慣をもつことを心がけましょう。副交感神経機能が低下しています。	正常です	運動刺激に対し交感神経が適切に反応しています。 一方、あまり高い場合は、刺激に対して交感神経が過敏な状態です。
③	交感神経の活性化持続	負荷刺激を受けた時に本来活性化する交感神経の持続度合いを示す。血圧の上昇など、運動に必要な身体機能を持続させる能力心拍変動のゆらぎを表し、自律神経活動の強弱をしめす。	運動負荷刺激時に交感神経の働きが持続しにくい状態です。適度な運動習慣をもつことを心がけましょう。	正常です	運動時に交感神経の働きを持続させることができています。一方、あまり高い場合は、刺激に対して交感神経の過敏な状態が長く続いています。
副交感神経		機能(指標)	基準値より低い時 (<80%)	正常(基準値±20%)	基準値より高い時 (>120%)
④	安静時平均心拍	心臓リズムの間隔時間を示す。	心拍が平均よりも速い状態です。	正常です	心拍が平均よりもゆっくりとなっています。
⑤	相対的副交感神経機能	副交感神経機能の強弱を示す。副交感神経は消化管等内臓機能を活性化させ、エネルギー補給に深く関与し、身体の調整・修復に働く。	休息を多く取り規則正しい生活を心がけましょう。	正常です	副交感神経機能が高い状態でリラックスしています。
⑥	内在活力	内在活力ともいい、被験者が秘める活力を意味する重要な指標である。	心拍変動のゆらぎが少なく活力が低下しています。休息を多く取り規則正しい生活を心がけましょう。	正常です	心拍変動のゆらぎは力強く、活力がみなぎり、充実した生活を送られていると思われます。

交感神経/副交感神経活動比 (S/P ratio)	機能(指標)	基準より低い (<0.9)	正常(1.0±0.1)	基準より高い (>1.1)	非常に高い (>1.5)
	交感神経と副交感神経のバランスを示す	副交感神経が優位です	自律神経バランスが良い	交感神経が優位です	交感神経が過度に高い

表１　自律神経バランスの項目別評価

図 7 は、レーダーチャートの様々なパターン[8)]を示したものです。中央の図は、100 人の健康な人の平均値で平均安定型です。その左図は、下部が大きいパターンで副交感神経優位型、その下の上部が大きいのは交感神経優位型となります。これらのパターンは人によって様々ですが、同じ人でも測定する時によって異なりますので、常に変化していると考えた方が良いと思います。何故ならば、自律神経バランスは日常生活において、その場その場で外的環境に即応しながら変化しているからです。

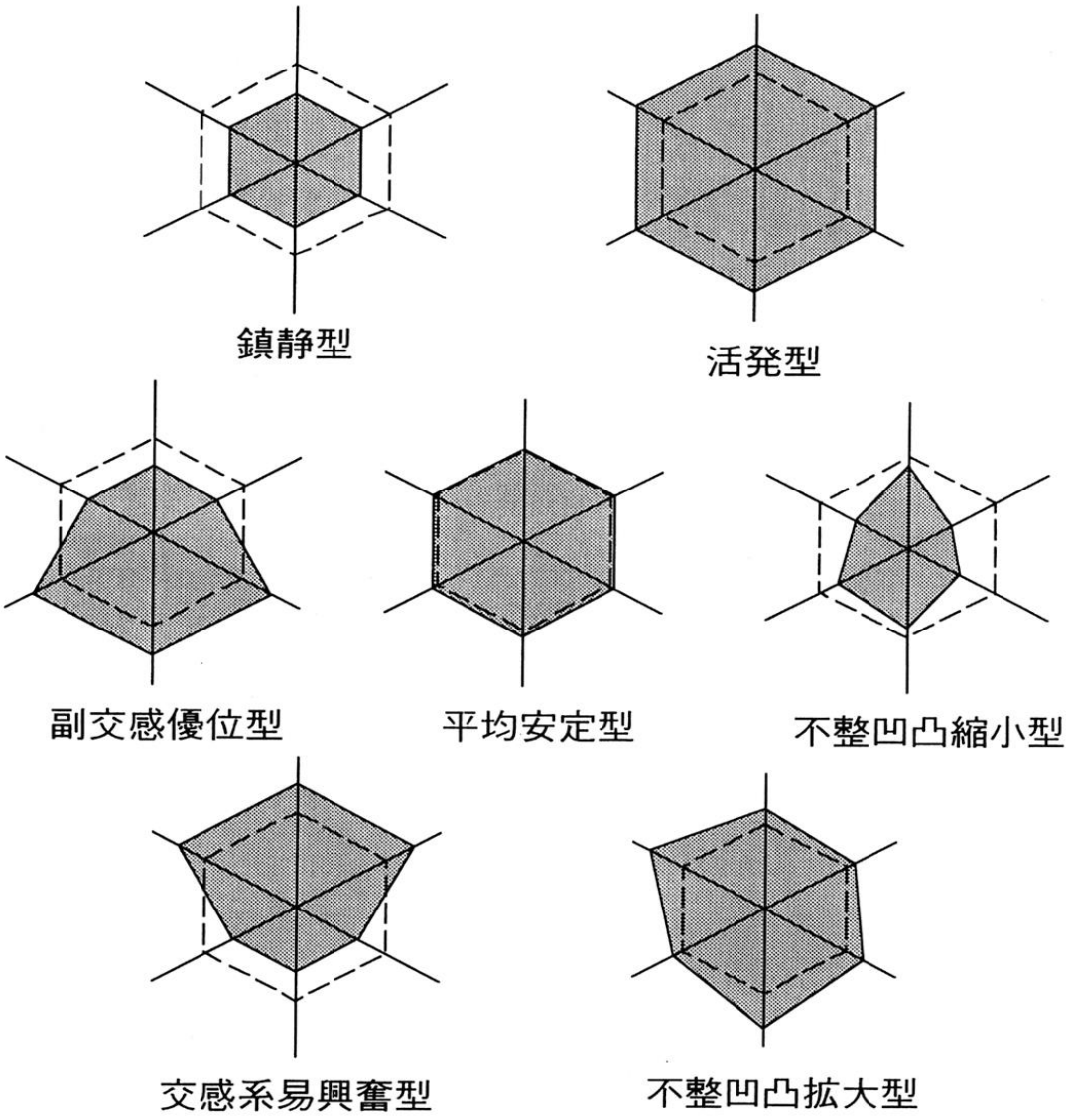

図 7　自律神経バランスの色々なパターン[8)]

7　更年期障害患者における自律神経バランス測定評価

更年期障害患者ではホットフラッシュ（ほてり）[10]、発汗、動悸、頭痛、手足のしびれなどの自律神経失調症状[9,11]や不眠、うつなどの更年期特有の症状が見られます。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す。以前の報告では、更年期障害患者において心臓での自律神経活動に２つの変化が報告されています。それは、(1) 心拍変動の低下と(2) 交感神経活動の増加です。前者は、閉経後の更年期障害患者において R-R 間隔の平均値と標準偏差が低下し、後者は交感神経緊張度が優位となり、交感神経バランスが増加するという報告[12,13]です。

筆者らの最近の研究[14,15]において、①コントロール群として更年期症状が認められない 40～60 歳の健常者女性と、②更年期障害患者群として産業医科大学病院の産婦人科外来を受診した 40～60 歳で、更年期障害と診断された女性の結果を比較した興味ある知見が得られました。なお、この患者群は少なくとも３ヶ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ます。更年期症状のある患者 45 名の中で、5 名は簡易更年期指数（SMI）が 51 以下であったために除外しました。残りの患者 40 名の SMI は 72.2 ± 2.2 でした。血中エストラジオール 17β-E_2 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（17β-E_2, 83 pg/ml; FSH, 13.5 pg/ml）[16]と比較して減増していました。また、更年期障害患者の中で、22 名、12 名、12 名、そして 6 名は各々エストリオール、ベンゾジアゼピン系睡眠薬、漢方薬、そして抗うつ薬を服用していました。

更年期障害患者においては、瞬時交感神経被刺激度(mRR(sup)-

RRmin(std))と交感神経興奮度の持続(mRR(sup-std))、さらに R-R 間隔平均値の標準偏差(SD$_{RR}$(sup)) が統計学的に有意に低下していました(各々P＜0.05, P＜0.01, P＜0.001）（図 8）[14,15]。このことは自律神経活動の反応性が鈍っていることを意味するので重要です。しかしながら、自律神経バランス比である交感神経活性/副交感神経活性の比(S/P ratio=1.11 ± 0.05) は、健常者(S/P ratio=1.13 ± 0.04)と比べて差はありませんでした。次に、SD$_{RR}$(sup)と更年期障害重症度の簡易更年期指数(SMI)[9]の相関性について検討しました。その結果、両者には統計学的に有意差を持って、やや相関性がある（相関係数 r = -0.363, P=0.0167）との結果を得ました(図 9)[14,15]。

Subjestc	Age	Autonomic nervous balance						S/P ratio
		Sympathetic nervous activity			Parasympathetic nervous activity			
		LF/Total	mRR(sup-std)	mRR-RRmin	HF/Total	mRR(sup)	SDRR(sup)	
Healthy subjects (n=40)	50.1±0.9	0.687 ±0.146	137±9	231±11	0.502 ±0.025	861±16	31.6±2.1	1.13 ±0.04
Climacteric (n=40)	51.5±0.7	0.679 ±0.161	96.7±7**	196±12*	0.506 ±0.032	822±20	20.5±1.4 ***	1.11 ±0.05

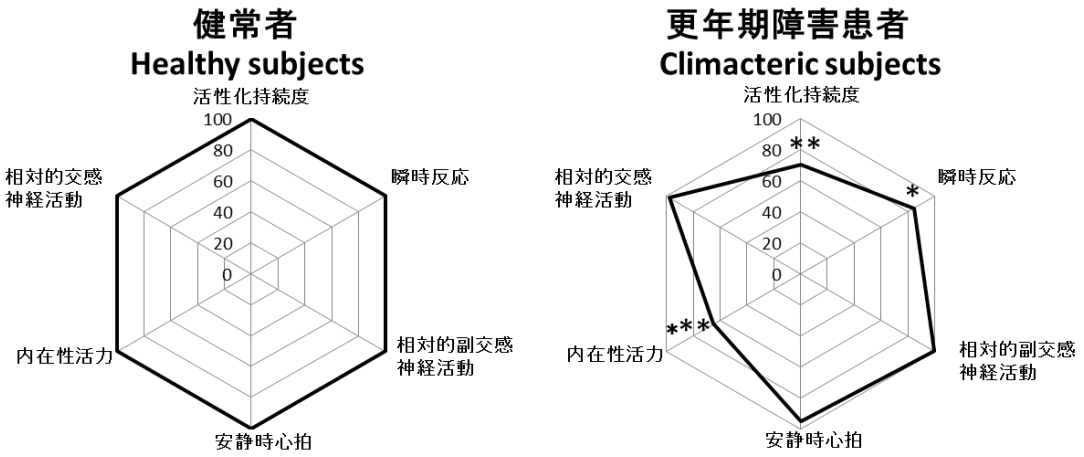

図８　更年期障害患者における自律神経バランス [14-15]を改変

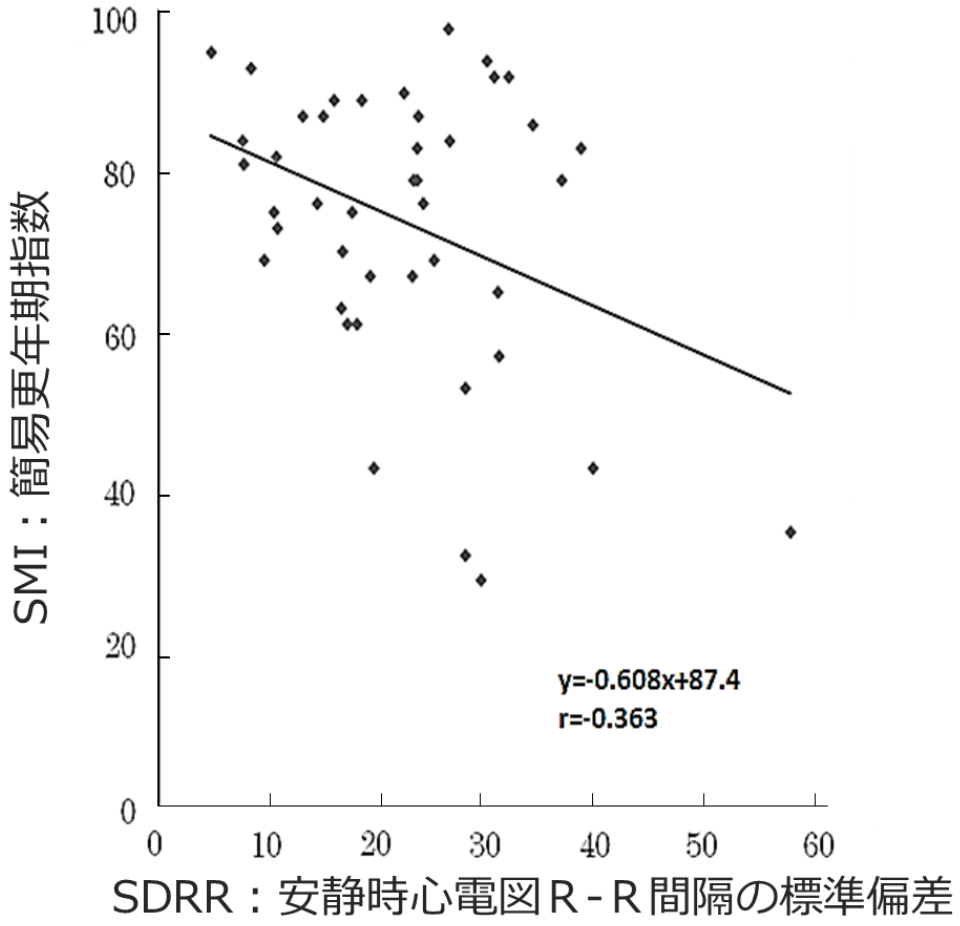

図９　簡易更年期指数（SMI）と安静時 R-R 間隔平均値標準偏差の逆相関性 [14, 15] を改変

8　セルフヘルスケアにおける自律神経バランス測定の意義

私達が日常生活をスムーズに過ごすには、自律神経系の働きは非常に大切です。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を上手く対応出来るように調節します。しかし、強いストレスなどが過度に長期間続いた時に交感神経系の過剰興奮が続き、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します。以前より

多くの研究[16-19]により、長期のストレスにさらされると消化性潰瘍や心臓血管病、免疫系の抑制や神経精神症状、さらには癌の発症などが生じる事が強く示唆されております。病気にならないためにも、ストレスをため込まないで適当に気晴らしをして、上手にストレス社会を過ごしたいものです。しかし、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ます。

従来の自律神経バランスの測定は、最低でも 1 人あるいは 2 人の測定者が側にいて測定する必要がありました。今回、紹介しました方法は、測定操作の大部分を自動化し、被験者自身で 1 人でも測定可能となる、いわゆるセルフヘルスチェックシステムとしました。今後、更年期障害のみならず自律神経失調症をきたすいろいろな病気や症状に対して、自律神経バランスを適切にコントロールし過重の負荷に対するブレーキや生活環境の改善に生かすようになればと期待しております。

9　まとめ

今回、東芝 Silmee による自律神経バランス測定について紹介しました。今後、日常生活において自律神経バランスをセルフチェックする有力なツールとして、活用できる可能性を示しました。おりしも従業員が 50 名以上の企業では、2015 年 12 月から健康診断でストレスチェックが法律により義務化され、精神健康管理の重要性が今後、益々増加するとみられております。セルフヘルスケアと言う観点から今回の自律神経バランス測定システムは、自分のストレス状態をチェックする手段の一つとして、「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おります。

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日本音響学会、電子情報通信学会

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Research Paper

Long working hours, job satisfaction, and depressive symptoms: a community-based cross-sectional study among Japanese employees in small- and medium-scale businesses

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ABSTRACT

Although long working hours have been suspected to be a risk factor for depressive symptoms (DS), it is not well understood the conditions under which long working hours are associated with it. This study investigated the moderating effect of job satisfaction on the relationship between working hours and DS. A total of 2,375 full-time non-shift day workers (73% men), aged 18–79 (mean 45) years, in 296 small- and medium-scale businesses were surveyed using a self-administered questionnaire evaluating working hours, job satisfaction, DS and covariates. The Center for Epidemiologic Studies Depression scale (CES-D) was used to assess DS. Risk of DS (CES-D ≥ 16) by working hours, job satisfaction, and both combined was estimated by multivariable logistic regression analysis. Compared to participants working 6–8 hrs/day, those working 12+ hrs/day had significantly higher odds of DS (adjusted odds ratio [aOR] 1.49), while participants with low satisfaction, as opposed to high satisfaction, had increased odds of DS (aOR 1.81). Furthermore, compared to those working 6–8 hrs/day with high satisfaction (reference group), participants working 6-8 hrs/day, > 8 to 10 hrs/day, and > 10 hrs/day combined with low satisfaction had dose-response increase of DS (aOR 1.48, 2.21 and 2.31, respectively, $p < 0.05$), whereas those working > 8 to 10 hrs/day and > 10 hrs/day combined with high satisfaction had not (aOR 0.93 and 1.39, respectively, $p > 0.10$). The results suggest that long working hours are associated with increased risk of DS only under reduced job satisfaction condition, which highlights the importance of improving job satisfaction, particularly among those working excessive hours.

INTRODUCTION

Depression/depressive symptoms (DDS) is one of the most common and most costly mental health disorders, contributing to work impairment and reduced productivity affecting a large number of working people [1]. According to a report by the World Health Organization in 2012, it was estimated that 350 million people suffered from depression worldwide [2]. In Japan, nearly 2 million people were thought to have suffered from depression in 2005 [3]. A study that addressed the economic impact of depression in Japan estimated that the yearly cost was approximately $11 billion and of this cost 62.8% was work-related depression [4].

Long working hours and overtime have been suspected to be a critical risk factor for DDS, although the findings are not always consistent [5]. To my knowledge, there are a total of 33 studies that have examined the relationship between long working hours/overtime and DDS, and of these reports, 21 studies found significant positive associations [6–26], while 12 studies reported insignificant or even an inverse relationship between the two [27–38]. Inconsistent findings across studies may be due partly to differences in sample sizes, characteristic of

study populations, definition of working hours/depression, analytic approaches, and covariates adjusted for, as well as study designs (leading to possible cause-effect reversals), as pointed out by some researchers [15, 18, 19, 35]. However, there are also other potentially important work-related factors that may have contributed to mixed results. It is becoming increasingly clear that the perception of psychosocial work conditions affect the relationship between work hours and psychological health [39–41]. For example, if employees are satisfied with or enjoying their job they may work long without suffering from DDS, whereas those who are dissatisfied with their job may not or could not work long because of depressed mood. Indeed, a meta-analysis on the relationship between job satisfaction and health confirmed that increased job satisfaction is protective against DDS [42]. Furthermore, a study that investigated the relationship between overtime and psychological health found that employees working overtime combined with low rewards had an elevated risk of poor recovery, burnout, negative home-to-work interference, and negative work-to-home interference compared to those with no overtime/high rewards (reference group), whereas those with overtime/high rewards did not show such an increase compared to the reference group [39].

Other work conditions that deserve attention are the influence of work schedule and status of employment. Regarding work schedule, a growing body of evidence suggests that night/rotating/irregular shift work is more harmful to mental health than daytime work condition [15, 37, 43–45], suggesting that the research on the relationship between work hours and DDS should rule out or at least statistically control for the influence of work schedules to produce more accurate estimates [5]. With respect to employment status, part-time employment has been reported to be more detrimental to health than full-time employment, especially among those involuntarily working part-time [32, 46, 47]. In accordance with these reports, one study confirmed the importance of distinguishing between overtime and long working hours among full- and part-time employees in relation to work-related outcomes, i.e., motivation and fatigue [48].

Based on these assumptions, the present study was designed to investigate the possible moderating role of job satisfaction in the relationship between working hours and depressive symptoms in full-time employees working under non-shift daytime condition. The aim of this study was to answer three research questions: 1) Is there an association between long working hours and depressive symptoms? 2) Is there an association between job satisfaction and depressive symptoms? 3) Do the combination of working hours and job satisfaction together relate to depressive symptoms? If so, which factor is mainly related to depressive symptoms? I address these questions using a sample of 2,375 full-time employees from 329 small and medium-scale businesses (SMBs) comprised of various industry sectors and occupations.

RESULTS

Descriptive statistics for participants are shown in Table 1. Roughly 73% and 27% of participants were men and women, respectively. Overall, 45% of participants were aged 50 years and older, 68% married, 80% had high school education or higher, 48% current smoker, 68% alcohol drinker, 91% coffee/tea drinker, 38% slept less than 6 hrs/day, 21% had BMI 25 or higher, 76% had no physical/psychological symptoms, and 14% used medications. Regarding occupational factors, 43% worked in production/manufacturing, 44% metalworking, and 38% working in a business with employees less than 18 people.

Overall prevalence and prevalence of depressive symptoms (CES-D $\geq$ 16) by working hours and job satisfaction are shown in Table 2. The prevalence of depressive symptoms among this population was 30.3% (95% CI 28.4–32.1). Working 12 hrs/day or more (compared to 6–8 hrs/day) and reduced job satisfaction were associated with increased depressive symptoms in a dose-response manner, but the strength of association with depressive symptoms seemed to be more pronounced for job satisfaction than for working hours. Prevalence of depressive symptoms among those who reported 'very satisfied' with their job had 16.8% while those reporting 'somewhat satisfied,' 'not too satisfied,' and 'not at all satisfied' had 27.6%, 36.0%, and 51.4%, respectively.

Direct associations of working hours and job satisfaction with depressive symptoms as estimated by multivariable logistic regression analyses are shown in Table 3. Participants working 12+ hrs/day had significantly higher odds of depressive symptoms than those working 6 to 8 hrs/day (reference group) even after controlling for confounders. Furthermore, the trichotomized analysis found that participants working > 10 hrs/day had significantly increased odds of depressive symptoms than the reference category.

Regarding job satisfaction, participants reporting 'not at all satisfied,' 'not too satisfied,' and 'somewhat satisfied' had significantly increased odds of depressive symptoms compared to those reporting 'very satisfied' with their job (reference group). The dichotomized analysis found that participants reporting low job satisfaction had 75% to 88% increase of depressive symptoms than those with high satisfaction.

The combined association of working hours and job satisfaction with depressive symptoms are shown in Table 4 and Figure 1. As compared with a reference group that had a 6 to 8 hrs/day working hours with high job satisfaction, the odds of depressive symptoms were significantly higher among participants working 6 to 8 hrs/day, > 8 to 10 hrs/day or > 10 hrs/day with low job satisfaction. Although participants working > 10 hrs/day with high job satisfaction had increased depressive symptoms compared with the reference group in models 1

Table 1: Sample descriptive statistics (N = 2,375)

Characteristics	*N*	(%)
Total participants	*2,375*	*(100)*
Sociodemographic and socioeconomic factors:		
Sex		
Men	*1,739*	*(73.2)*
Women	*636*	*(26.8)*
Age group, years		
18–29	*383*	*(16.1)*
30–39	*522*	*(22.0)*
40–49	*399*	*(16.8)*
50–59	*723*	*(30.4)*
60+	*348*	*(14.7)*
Marital status		
Married	*1,613*	*(67.9)*
Single	*605*	*(25.5)*
Separated/divorced/widowed	*157*	*(6.6)*
Educational level		
Junior high school	*486*	*(20.5)*
High school	*1,166*	*(49.1)*
Vocational/junior college	*336*	*(14.1)*
College/graduate school	*387*	*(16.3)*
Health indicators:		
Smoking status		
Lifetime nonsmoker	*987*	*(41.6)*
Former smoker	*240*	*(10.1)*
Current smoker (> 0 to ≤ 10 cigarettes/day)	*192*	*(8.1)*
Current smoker (> 10 to ≤ 20 cigarettes/day)	*639*	*(26.9)*
Current smoker (> 20 cigarettes/day)	*317*	*(13.3)*
Drinking habit		
Non-drinker	*757*	*(31.9)*
Occasional (> 0 to ≤ 3 times/week)	*578*	*(24.3)*
Frequent (≥ 4 times/week)	*1,040*	*(43.8)*
Caffeine intake (cups of coffee or tea/day)		
Almost none	*212*	*(8.9)*
1 to 2	*1,119*	*(47.1)*
3+	*1,044*	*(44.0)*
Sleep hours per day		
< 6	*912*	*(38.4)*
≥ 6	*1,463*	*(61.6)*
Body Mass Index		
< 20	*419*	*(17.6)*
≥ 20 to < 22.5	*772*	*(32.5)*
≥ 22.5 to < 25.0	*684*	*(28.8)*
≥ 25.0	*500*	*(21.1)*
Number of physical/psychological symptoms[a]		
None	*1,801*	*(75.8)*
1	*491*	*(20.7)*

2 or more	*83*	*(3.5)*
Use of medication[b]		
No	*2,035*	*(85.7)*
Yes	*340*	*(14.3)*
Occupational factors:		
Job type		
Managerial/clerical	*642*	*(27.0)*
Sales/service	*170*	*(7.2)*
Technical	*97*	*(4.1)*
Production/Manufacturing	*1,026*	*(43.2)*
Other	*440*	*(18.5)*
Industry sector		
Ceramic/clay/stone	*46*	*(1.9)*
Textile	*40*	*(1.7)*
Papermaking	*128*	*(5.4)*
Printing	*41*	*(1.7)*
Chemical	*308*	*(13.0)*
Leather	*15*	*(0.6)*
Metalworking	*1,033*	*(43.5)*
Food	*127*	*(5.3)*
Machinery	*376*	*(15.8)*
Other	*261*	*(11.0)*
Size of company by number of employees (in quintiles)		
1–8 workers	*412*	*(17.3)*
9–18 workers	*506*	*(21.3)*
19–31 workers	*495*	*(20.8)*
32–61 workers	*515*	*(21.7)*
62+ workers	*447*	*(18.8)*
Job control (in tertiles)		
High	*798*	*(33.6)*
Medium	*795*	*(33.5)*
Low	*782*	*(32.9)*
Quantitative workload (in tertiles)		
Low	*895*	*(37.7)*
Medium	*770*	*(32.4)*
High	*710*	*(29.9)*

[a]Physical/psychological symptoms include hypertension, hyperlipidemia, diabetes mellitus, menopausal syndrome, cardiovascular disease, cancer, stomach/duodenal ulcer, arrhythmia, gout, hyperuricemia, renal disease, liver disease, stroke, gynecologic diseases, hyperthyrodism, peptic ulcer, severe allergy, hernia, back pain, rheumatoid arthritis, and panic disorder. [b]Medications include aspirin, acetaminophen, β-blockers, cold/flu medicine, anti-hypertensives, naproxen, corticosteroids, and ibuprofen.

and 2 ($p < 0.05$), the significance disappeared after further adjustment for additional covariates (models 3 and 4).

DISCUSSION

The purpose of this study was to investigate the independent and combined association of working hours and job satisfaction with depressive symptoms in a large number of SMB employees in a suburb of Tokyo. The current study produced three main findings. First, long working hours, particularly those working 12+ hrs/day (compared to those working 6 to 8 hrs/day), were weakly but significantly associated with increased risk of depressive symptoms. Second, reduced job satisfaction was strongly related to depressive symptoms in a dose-response manner. Third and most importantly, the combination of

Table 2: Prevalence of depressive symptoms by working hours and job satisfaction (*N* = 2,375)

Variables	*N*	(%)	CES-D Score ≥ 16, % (95% CI)
Overall prevalence	*2,375*	*(100.0)*	30.3 (28.4 to 32.1)
Working hours per day:[a]			
6 to 8	*1,144*	*(48.2)*	28.4 (25.8 to 31.0)
9	*506*	*(21.3)*	28.9 (24.9 to 32.8)
10	*416*	*(17.5)*	31.5 (27.0 to 36.0)
11	*121*	*(5.1)*	37.2 (28.6 to 45.8)
12+	*188*	*(7.9)*	38.3 (31.3 to 45.2)
Working hours per day:[b]			
6 to 8	*1,144*	*(48.2)*	28.4 (25.8 to 31.0)
> 8 to 10	*922*	*(38.8)*	30.0 (27.1 to 33.0)
> 10	*309*	*(13.0)*	37.9 (32.5 to 43.3)
Job satisfaction:[c]			
Very satisfied	*280*	*(11.8)*	16.8 (12.4 to 21.2)
Somewhat satisfied	*1,306*	*(55.0)*	27.6 (25.2 to 30.1)
Not too satisfied	*614*	*(25.9)*	36.0 (32.2 to 39.8)
Not at all satisfied	*175*	*(7.4)*	51.4 (44.0 to 58.8)
Job satisfaction:[c]			
Very satisfied/Somewhat satisfied	*1,586*	*(66.8)*	25.7 (23.6 to 27.9)
Not too satisfied/Not at all satisfied	*789*	*(33.2)*	39.4 (36.0 to 42.8)

[a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.001$ (Chi-squared test).

working hours with job satisfaction had a synergistic association with depressive symptoms, but this association was observed only when different working hours were combined with low job satisfaction (compared to those working 6–8 hours/day with high satisfaction). Although the results of this study should be interpreted with caution in light of self-reporting and cross-sectional design, these data imply that job redesign/crafting aimed at enhancing job satisfaction may prevent, or at least reduce, occurrence of workplace DDS associated with long working hours.

Data from past studies have suggested that long working hours are associated with increased risk of

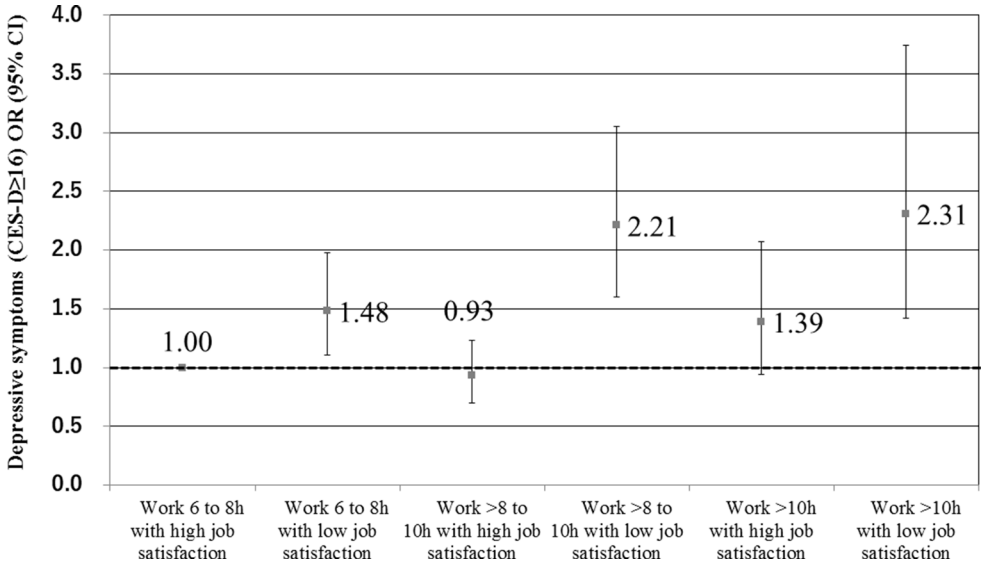

Figure 1: Combined association of working hours and job satisfaction on depressive symptoms.

Table 3: Association of working hours and job satisfaction with depressive symptoms (N = 2,375)

	Model 1[a]	Model 2[b]	Model 3[c]	Model 4[d]
Variables	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**
Working hours per day:				
6 to 8	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
9	1.02 (0.81 to 1.29)	1.04 (0.82 to 1.32)	1.03 (0.81 to 1.31)	1.09 (0.84 to 1.42)
10	1.16 (0.91 to 1.48)	1.25 (0.96 to 1.62)	1.20 (0.92 to 1.56)	1.16 (0.87 to 1.55)
11	1.49 (1.01 to 2.21)[e]	1.56 (1.04 to 2.35)[e]	1.45 (0.96 to 2.20)	1.51 (0.96 to 2.37)
12+	1.56 (1.14 to 2.16)[e]	1.64 (1.17 to 2.30)[e]	1.44 (1.02 to 2.04)[e]	1.49 (1.00 to 2.22)[e]
Working hours per day:				
6 to 8	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
> 8 to 10	1.08 (0.89 to 1.31)	1.12 (0.92 to 1.38)	1.10 (0.89 to 1.35)	1.12 (0.89 to 1.40)
> 10	1.54 (1.18 to 2.00)[f]	1.60 (1.20 to 2.13)[f]	1.43 (1.07 to 1.92)[e]	1.49 (1.07 to 2.08)[e]
Job satisfaction:				
Very satisfied	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Somewhat satisfied	1.89 (1.35 to 2.65)[f]	1.79 (1.28 to 2.52)[f]	1.82 (1.29 to 2.56)[f]	2.04 (1.42 to 2.92)[f]
Not too satisfied	2.79 (1.96 to 3.97)[f]	2.52 (1.76 to 3.61)[f]	2.55 (1.78 to 3.67)[f]	2.92 (1.99 to 4.30)[f]
Not at all satisfied	5.25 (3.41 to 8.08)[f]	4.52 (2.92 to 7.01)[f]	4.50 (2.90 to 7.00)[f]	5.51 (3.41 to 8.89)[f]
Job satisfaction:				
Very satisfied/Somewhat satisfied (high)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Not too satisfied/Not at all satisfied (low)	1.88 (1.57 to 2.25)[f]	1.75 (1.46 to 2.11)[f]	1.75 (1.45 to 2.11)[f]	1.81 (1.47 to 2.22)[f]

[a]Unadjusted.

[b]Adjusted for sex, age group, marital status, and educational level.[c] Adjusted for sex, age group, marital status, educational level, smoking, drinking, caffeine intake, sleep duration, and BMI.[d] Adjusted for sex, age group, marital status, educational level, smoking, drinking, caffeine intake, sleep duration, BMI, number of physical/psychological symptoms, use of medication (yes/no), job type, industry sector, company size, job control (high, medium, low), and male/female ratio.[e] $p < 0.05$, [f] $p < 0.001$.

DDS [6–26]. At the same time, there are also studies that reported no significant relationship [27–36] or even an inverse relationship between the two [37, 38]. As stated earlier in the Introduction, several plausible explanations have been proposed for its inconsistent findings. In addition to these explanations, this study yielded an alternative explanation as to why work hour-DDS relationship is not simple or straightforward. The results of this study suggested that long working hours do not necessarily have negative psychological health consequences if employees are working under favorable/positive working condition, i.e., high job satisfaction. Conversely, the risk of DDS associated with long working hours are more pronounced if employees are working under poor/negative condition, i.e., low job satisfaction. The finding is supported by several studies that considered working condition in relation to long work hours and health [39, 40]. One study reported that long work hours are not necessarily related to adverse psychological health if job rewards are high, even under high pressure to work overtime among postal service employees [39]. Another study observed a positive association between weekly working hours and poor physical health among train drivers who worked overtime with high pressure and low social support, whereas those under high pressure with high social support yielded an inverse relationship [40]. These findings, together with the current results, support the notion that psychosocial work condition could serve as a moderator in the relationship between working hours and health.

Table 4: Combined association of working hours and job satisfaction with depressive symptoms (*N* = 2,375)

			Model 1[a]	Model 2[b]	Model 3[c]	Model 4[d]
Variables	***N***	***(%)***	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**
Working hours and job satisfaction:						
Working 6 to 8 h/day with high job satisfaction	*768*	*(32.3)*	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Working 6 to 8 h/day with low job satisfaction	*376*	*(15.8)*	1.58 (1.21 to 2.07)[g]	1.48 (1.13 to 1.94)[f]	1.47 (1.12 to 1.93)[f]	1.48 (1.11 to 1.98)[f]
Working > 8 to 10 h/day with high job satisfaction	*618*	*(26.0)*	0.92 (0.72 to 1.18)	0.97 (0.75 to 1.25)	0.93 (0.72 to 1.21)	0.93 (0.70 to 1.23)
Working > 8 to 10 h/day with low job satisfaction	*304*	*(12.8)*	2.21 (1.67 to 2.92)[g]	2.16 (1.61 to 2.89)[g]	2.13 (1.59 to 2.86)[g]	2.21 (1.60 to 3.05)[g]
Working more than 10 h/day with high job satisfaction	*200*	*(8.4)*	1.49 (1.07 to 2.09)e	1.56 (1.09 to 2.22)e	1.41 (0.98 to 2.20)	1.39 (0.94 to 2.07)
Working more than 10 h/day with low job satisfaction	*109*	*(4.6)*	2.51 (1.66 to 3.78)[g]	2.45 (1.59 to 3.76)[g]	2.13 (1.38 to 3.30)[g]	2.31 (1.42 to 3.74)[g]

[a]Unadjusted.
[b]Adjusted for sex, age group, marital status, and educational level.
[c]Adjusted for sex, age group, marital status, educational level, smoking, drinking, caffeine intake, sleep duration, and BMI.
[d]Adjusted for sex, age group, marital status, educational level, smoking, drinking, caffeine intake, sleep duration, BMI, number of physical/psychological symptoms, use of medication (yes/no), job type, industry sector, company size, job control (high, medium, low), and male/female ratio.
[e]$p < 0.05$, [f]$p < 0.01$, [g]$p < 0.001$.

Interestingly, those who worked moderately long hours (> 8 to 10 hrs/day) with high job satisfaction had the lowest risk of depressive symptoms compared to those working 6–8 hrs/day with high job satisfaction (aOR=0.92 to 0.97). Although the results only yielded a small difference, it seems reasonable to think that this population is consisted of healthiest group of employees who are working voluntarily with high motivation. Two studies from Netherlands suggested that moderate overtime is not always harmful to health outcomes [49, 50]. For instance, a study using a representative sample of a Dutch full-time workforce found that voluntary overtime workers were non-fatigued and satisfied with their job even without rewards while involuntary overtime workers exhibited high fatigue level and less satisfaction [49].

More than a half of the participants who were 'not at all satisfied' with their job had depressive symptoms. This finding is in line with several empirical researches [51, 52] as well as a result of meta-analysis based on 485 studies of job satisfaction and health which reported that workers with low levels of satisfaction were more likely to experience DDS (ρ =.428) [42], indicating that job satisfaction is an important predictor of psychological health. Therefore, those with extremely low levels of job satisfaction may need an immediate care to prevent workplace depression.

In this study, 30.3% of participants had CES-D scores of 16 or higher. The prevalence is similar to several studies using same criteria among the working populations (ranging from 24.5% to 33.9%) [53–55]. In contrast, an estimate based on diagnostic criteria are often much lower. For example, a median 12-month prevalence of major depressive disorder based on 42 different studies yielded 5.3% with an interquartile range of 3.6% to 6.5% [56]. Similarly, lifetime and 12-month prevalence of major depressive disorder was reported to be 6.1% and 2.2%, respectively, based on the World Mental Health Japan Survey [57]. The prevalence gap between the former and latter studies could be attributable to different criteria for defining DDS, i.e., questionnaire vs. diagnostic criteria. In consideration of these facts, studies using both methodology/definition simultaneously may help understand the relationship between long working hour and DDS more precisely.

Strengths and limitations

A principal strength of this study is that it not only explored the independent association of working hours and job satisfaction with depression but also examined the combined associations of working hours and job satisfaction on depressive symptoms in a fairly large number of full-time employees of SMBs representing various industry sectors and occupations. Furthermore, participants under shift work and non-full-time condition, who reported major depressive disorders and anxiety disorders, as well as those working < 6 h/day and > 20 h/day were excluded to minimize selection bias leading to under- or overestimation. The limitations of this study are as follows. First, since this is a cross-sectional data, the association could be in either direction, i.e., long work hours and diminished job satisfaction may increase the risk of depressive symptoms or that undiagnosed depression or depressive personality traits may be the cause for reduced job satisfaction and short

working hours. Second, work hours, job satisfaction, and depressive symptoms were assessed by self-report rather than through the use of objective measures or diagnostic criteria. Third, response bias may have occurred if non-respondents differed from respondents; in particular, those who worked extremely long hours may have had less time available to respond to the questionnaire. Fourth, although the study included a fairly large number of confounders, information on unmeasured work-related factors such as work-family conflict and organizational justice and non-work-related variables such as personality traits and genetic components, as well as unknown common factors for both depressive symptoms and job satisfaction were not included in the analyses.

MATERIALS AND METHODS

Study participants and procedure

The study design was cross-sectional and data were collected using a self-administered questionnaire between August and December 2002. The study sample consisted of full-time employees of SMBs in a size ranging from 1 to 158 workers in the city of Yashio, Saitama, and in the Ohta ward of Tokyo. Yashio has the highest percentage of manufacturing plants in Saitama prefecture. The ward of Ohta, which is a so-called "industrial area," is unique for its number of SMBs. About 20% of SMBs in both areas were selected weighted by distribution of industry sector types, resulting in 329 SMBs from Yashio and 61 from the Ohta ward. An occupational health nurse/physician contacted each representative of the company to request participation in the questionnaire survey. Among these businesses, 248 in Yashio and 52 in Ohta agreed to participate. Questionnaires were distributed during visits to each business and were given to 2,591 employees in Yashio and 1,102 employees in Ohta (n = 3,693). Finally, responses were obtained from 2,884 employees (2,022 men and 862 women) from 296 businesses (response rate 78.1%). Those who had missing responses to sex, age, working hours, and job satisfaction were eliminated from the analyses (n = 126). Similarly, those who had 6 or more missing responses on the Center for Epidemiologic Studies Depression Scale (CES-D) (see 'Measurements' section for detail) and those who had been diagnosed with major depressive disorder or anxiety disorders were excluded from the analysis (n= 64). In addition, those who reported working < 6 hrs/day or > 20 hrs/day, working under non-day shifts or < 18 years old were excluded (n = 131). Since there were less than 5% missing responses for all the covariates in this study, Missing Value Analysis was performed using IBM SPSS Statistics 21.0 software (SPSS, Inc., Chicago, IL, USA) [58]. The 'expectation–maximization method' of imputing missing values was utilized. As a result, following variables, i.e., marital status, educational level, smoking status, drinking habit, caffeine intake, sleep hours, BMI, number of physical/psychological symptoms, use of medication, job type, job control, and quantitative workload were imputed. Thus, data on a total of 2,375 participants (1,739 men and 636 women) working under non-shift daytime condition were used in the final analyses. The study was approved by the Medical Ethical Committee of the University of Tokyo. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all participants for being included in the studies presented.

Variables

Working hours

Working hours were determined by an open-ended question: How many hours do you usually work in a typical working day? Number of hours were grouped into three categories (i) 6 to 8 hrs/day, (ii) > 8 to 10 hrs/day, and (iii) > 10 hrs/day.

Job satisfaction

Job satisfaction was assessed by a single-item assessment tool included in the Japanese version of the generic job stress questionnaire (GJSQ) developed by the U.S. National Institute for Occupational Safety and Health (NIOSH) which is a well-established means of measurement [59–61]. Item/response for the scale is as follows: All in all, how satisfied would you say you are with your job? (1) not at all satisfied, (2) not too satisfied, (3) somewhat satisfied, (4) very satisfied. The item has been frequently used in past studies to measure job satisfaction at the workplaces [51]. Job satisfaction was dichotomized into low (not at all satisfied/not too satisfied) and high (somewhat satisfied/very satisfied) levels.

Depressive symptoms

Depressive symptoms was measured using a Japanese version of the Center for Epidemiologic Studies Depression scale (CES-D) [62]. The 20-item depressive symptom scale measures the level of depressive symptoms experienced in the past week. The CES-D scale cut-off score is 16, which differentiates between those exhibiting high levels of depressive symptoms (score ≥ 16) and those with lower levels of such symptoms (score < 16) [63]. The internal consistency of the CES-D scale for the study sample was 0.84.

Covariates

Covariates considered included sociodemographic and socioeconomic factors, health behaviors, biological factors, medication usage, and occupational factors as

listed in Table 1. Daily sleep hours during the previous 1-year period were assessed by a following questionnaire: On average, how much sleep at night do you usually get? Response options were: < 5 hrs/5 to < 6 hrs/6 to < 7 hrs/7 to < 8 hrs/8 to < 9 hrs/9+ hrs. A previous study confirmed a strong convergent and discriminant validity as well as a high level of test-retest stability over 1 year for this question [64]. Information on height and weight were obtained to assess body mass index (BMI), calculated as weight (kg) divided by height (m) squared, and divided into four groups. Job control and quantitative workload were evaluated by the Japanese version of the NIOSH GJSQ. Job control measures how much the worker feels that tasks, workplace setting, and decisions at work are controllable and is assessed based on 16-items, while quantitative workload estimates how much work must be done on daily basis and is based on 4-items. Internal consistency (Cronbach's alpha) for these scales was 0.96 and 0.88, respectively.

Participants were asked if they were treated for any of the following disorders or symptoms: hypertension, hyperlipidemia, diabetes mellitus, major depressive disorder, menopausal syndrome, or other. If the participants reported 'other disorders,' they were asked to specify the condition. Participants reported various disorders as listed on the bottom of Table 1. The numbers of disorders among the participants were counted and were included as a covariate.

Statistical analyses

Prevalence of depression by working hours and job satisfaction was analyzed by Chi-squared test. The risk of depression by working hours and job satisfaction was estimated by multivariable logistic regression with odds ratios (ORs) and 95% confidence intervals (CIs) as measures of association. Combined associations of working hours and job satisfaction were examined by a similar analytic method. They were divided into six groups as follows: three groups of working hours (< 6 hrs/day, 6 to < 8 hrs/day, or 8+ hrs/day) × two groups of job satisfaction (low versus high). The interactive associations of working hours and job satisfaction on depression were also examined. Adjustments for covariates were made in a stepwise fashion. A crude OR was computed in Model 1. The second model included sociodemographic and socioeconomic factors as covariates (Model 2). The third model included health behaviors and biological factors in addition to model 2 covariates (Model 3). And finally, occupational factors were included in addition to model 3 covariates (Model 4). Quantitative workload was left out of multivariable logistic regression analyses because of a strong intercorrrelation with working hours and some recent studies indicated that work demands should be treated as an intermediate variable but not as a confounder [17, 22]. The significance level for all statistical analyses was $P < 0.05$ (two-tailed test). Data were analyzed using IBM SPSS version 21.0 software (SPSS, Inc., Chicago, IL, USA).

CONCLUSIONS

This study found independent associations of working hours and job satisfaction with depressive symptoms. However, when the combined associations of working hours and job satisfaction were tested, job satisfaction turned out to be the main factor related to depressive symptoms. Furthermore, a combination of long working hours with reduced job satisfaction exerted a reciprocal association on depressive symptoms, but a combination of long working hours with high satisfaction did not show such an effect. Prospective research is warranted to determine the causal mechanisms underlying the present findings.

Abbreviations

DDS: depression/depressive symptoms; CES-D: Center for Epidemiologic Studies Depression scale; SMB: small and medium-scale business; NIOSH: National Institute for Occupational Safety and Health; GJSQ: generic job stress questionnaire; OR: odds ratio; aOR: adjusted odds ratio; CIs: confidence intervals; BMI: body mass index

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CONFLICTS OF INTEREST

There is no conflicts of interest.

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