participants by means of baseline questionnaires, biochemical assays, hospital birth records, and four months post-partum health records. We finally used a total of 15266 participants, details of selection is shown in the flow chart (**Fig. 1**).

Certain repeated self-reported information obtained from birth records and postpartum questionnaires were used to compare with the baseline questionnaires in order to improve quality and missing information in the whole data. Otherwise, these data were not used in the analysis of this report.

Biochemical assays

Non-fasting whole blood samples were collected from participants, pre-treated and sera obtained. Sera were stored promptly at 4°C until they were transported on ice to a commercial laboratory (SRL Corporation Inc, Tokyo, Japan), for folate assay. The ADVIA Centaur Folate Assay Protocol is one of the automated Competitive Protein Binding (CPB) Immunoassay Technology. Folate is quantified by direct chemiluminescent acridinium ester technology[13]. This technique has an acceptable imprecision of less than 10.00%, with an advanced Quality Control (QC) package. It has an analytical sensitivity of 0.91nmol/L. It can detect from small volumes of as low as 10uL of biological specimen, thus, making it a method of choice in large epidemiological studies[14]. Specimen preparations, shipping, and assays, were done in batches, depending on new recruitments. All laboratory analysts were blinded to participants` information. Because there is no standard classification of folate status from automated immunoassay techniques, we adopted the World Health Organization's (WHO) classification guidelines[15]. Nicotine is the toxic chemical in tobacco products and its predominant metabolite is cotinine. Cotinine can be detected in biological specimens as biomarker of exposure to tobacco. In this study, we used plasma cotinine concentrations to quantitatively classify active and passive smoking status. The details of measurements of plasma cotinine are described in our previous report[16].

Definition of variables

The dependent variable was folate status. Folate status was classified as: folate deficiency, defined as (<6.80nmol/L) of serum folate; suboptimal status (6.80 -13.59nmol/L) and optimal folate status (≥13.60nmol/L)[15]. Folate deficiency was reported in 0.52% of the study population. To improve study power, and because non fasting serum was used for folate assay, we merged this group with the suboptimal category. Active and passive exposure to tobacco smoking statuses were classified based on plasma cotinine cut-off points established in a previous report[16]. A non-smoker was defined as having plasma cotinine concentration of less than 1.19nmol/L. A person exposed to environmental tobacco smoke (ETS) as having 1.19 – 65.21nmol/L; and active smoker as having greater than 65.21nmol/L of plasma cotinine concentration. Prenatal folic acid (FA) supplement use was defined as "a report on the use of FA supplements before or

after conception". Other nutritional supplements use was defined as "any report on intake of nutritional supplements other than FA, before or after conception". Ingestion of alcoholic beverages was categorized based on frequency of intake: "monthly, weekly or daily". Self-reported active tobacco smoking was categorized based on the number of cigarette sticks smoked per day. Light smokers (<10 cigarette sticks per day); moderate smokers (10-19 cigarette sticks per day); and heavy smokers (≥20 cigarette sticks per day). ETS exposure at home was defined as "living with one or more active smokers". ETS at work place referred to "working with one or more active smokers at work place". In this study, lifestyle habits considered were alcoholic beverage consumption, nutritional supplements use, and tobacco use. Potential predictors of folate status were identified based on previous reports. In this study, year of enrolment, maternal age, parity, BMI, educational level, household income, occupation, use of nutritional supplements, active and passive cigarette smoking, alcohol intake, season of the year, and geographical location, were identified as putative predictors.

Statistical analyses

Statistical tests of associations included Pearson's chi squared tests, and Fischer's exact tests for categorical variables. Skewed serum folate and plasma cotinine concentrations were log-transformed during the preliminary descriptive analyses, thereafter back-transformed. Differences in mean folate levels were explored using ANOVA with post-hoc analyses to correct for multiple comparisons. However, the main regression analyses were performed using qualitative folate status. We imputed the missing values present in the data via Multivariate Imputation by Chained Equations (MICE), as implemented in the R package *mice*, obtaining m = 10 imputed data sets. MICE is a Markov Chain Mote Carlo method that uses the correlation structure of the data and imputes missing data values for each incomplete variable m times by regression of incomplete variables on the other available variables iteratively. We used Bayesian logistic regression and fitted the model to the m＝10 imputed data set, with dichotomized folate status as the outcome variable, and the followings as potential predictor variables: age, BMI, parity, educational level, income, occupation, region, year of enrollment, season of the year at enrollment, folic acid supplements use, other nutritional supplements use, alcohol intake, active cigarette smoking, and exposure to environmental tobacco smoke (ETS) both at home and work place. We used results of plasma cotinine concentration to quantitatively classify active smoking and passive exposure to tobacco products, and regressed against folate status, with adjustment for all other potential predictors. We reported pooled estimates for the main effects of the predictor variables in the model. P-values for testing for the presence of a linear trend are also reported for predictor variables with more than two categories. Reported effects, confidence intervals and p-values are pooled over the m = 10 imputed data sets. Additionally, we

reported the value of the McFadden's pseudo-R^2 pooled over these data sets. All statistical analyses were performed using JMP 11 Pro Statistical Software Package (SAS, Cary, NC, USA), except for the binary logistic regression model which required multiple imputation of missing data and was performed using R version 3.2.2 (Vienna, Austria). An alpha level of significance was set at <0.05.

（倫理面への配慮）

The Institutional Ethical Board for Human Gene and Genome studies at Hokkaido University Graduate School of Medicine approved the study protocol.

C. 研究結果

Overall, geometric mean (standard deviation) of serum folate concentration was 17.77 (3.58)nmol/L. Among women with optimal folate status, the geometric mean (standard deviation) was 20.67 (3.26) nmol/l, and 10.83 (2.65) nmol/l among participants with suboptimal folate status. One sided lower limit tolerance interval at 95% of the population was 8.47nmol/L. Prevalence of folate deficiency was 0.52%. Suboptimal folate status constituted 25.65%, while optimal folate status was reported in 73.83% of the population (**Table 1**). Initial descriptive analyses using folate as a continuous variable revealed mean serum folate concentrations increased with increasing maternal age (p<0.001), educational status (p<0.001), annual income (p<0.001), FA supplements use (p<0.001), and other nutritional supplements use (p<0.001). Mean serum folate concentrations decreased with increasing number of cigarette sticks smoked per day (p<0.001), ETS exposure at home (p<0.001), and increasing plasma cotinine concentrations (p<0.001). Exposure to ETS at both home and at work was associated with low folate status, p <0.001. About 60.00% of those with folate deficiency were exposed to both ETS at home and at work place. Other associations were geographical region, year of enrolment into the study, and season of the year (data not shown). Serum folate inversely correlated with plasma cotinine concentration (r = -0.2000, p <0.001, data not shown). Significant differences were observed in mean plasma cotinine concentrations among nonusers of FA supplements and users, with geometric mean (SD) of 46.41 (23.23)nmol/L, and 25.27 (15.32)nmol/L, p <0.001, respectively. Also, geometric mean (SD) between nonusers and users of other nutritional supplements was 42.49 (21.91)nmol/L, and 34. 99 (20.17)nmol/L, p = 0.028, respectively **(Fig. 2).** Users of FA supplements were likely to be those with chronic inter-current medical conditions, those who had fertility treatments, and those who were also users of other nutritional supplements. 7.00% of folic acid users started intake more than 3months before conception. Another 8.00% started 1 month before conception, while majority (more than 60.00%) started use following confirmation of pregnancy. The average frequency of use per week was 3 times. Multivitamins reported were found to contain various doses of folic acid in the range of 100μg to 200μg per tablet (data not shown).

In the regression model, the value of the McFadden's pseudo-R^2 pooled over the m ＝10 imputed data sets was–8.69%. The

demographic determinants of low folate status identified were lower maternal age (AOR: 1.48, 95% CI: 1.32, 1.66, p < 0.001); lower educational level (AOR: 1.27, 95% CI: 1.17, 1.39, p < 0.001); lower annual income (AOR: 1.11, 95% CI: 1.01, 1.22, p = 0.024); residing in the south and eastern regions (AOR: 1.25, 95% CI: 1.14, 1.38, p <0.001), and (AOR: 1.15, 95% CI: 1.05, 1.25, p = 0.003), respectively. Being enrolled into the study between 2005 and 2007 was associated with an increase in the risk of low folate status (AOR: 1.23, 95% CI:-1.12, 1.35, p <0.001); while recruitment between 2008 and 2010 reduced the likelihood of having low folate status (AOR: 0.81, 95% CI: 0.73, 0.90, p < 0.001), respectively. Being enrolled during summer, autumn, and winter were associated with higher likelihood of low folate status (AOR: 1.12, 95% CI: 1.02, 1.24, p = 0.023); (AOR: 1.13, 95% CI: 1.02, 1.25, p = 0.015), and (AOR: 1.13, 95% CI: 1.01, 1.27, p = 0.037), respectively. Lower BMI (AOR: 0.84, 95% CI: 0.74, 0.94, p = 0.006; and unemployment were associated with risk reduction (AOR: 0.87, 95% CI: 0.80, 0.94, p = 0.001), **(Table 2).**

Lifestyle factors that reduced the odds of low folate status were the use of FA supplements (AOR: 0.19, 95% CI: 0.17, 0.22, p <0.001); other nutritional supplements (AOR: 0.55, 95% CI: 0.48, 0.64, p <0.001); and weekly alcohol consumption (AOR: 0.75, 95% CI: 0.62, 0.90, p = 0.003), respectively. Lifestyle factors that increased the odds of low folate status were active cigarette smoking and ETS exposure. Smoking <10 cigarette sticks per day was associated with increased odds (AOR: 1.42, 95% CI: 1.23, 1.64, p <0.001); while smoking between 10 to 19 cigarette sticks per day was associated with an increased risk (AOR: 2.28, 95% CI: 1.92, 2.71, p < 0.001). However, smoking ≥ 20 cigarette sticks per day was not statistically significant, but p_{trend} <0.001. Exposure to ETS at home increased the odds of low folate status (AOR: 1.23, 95% CI: 1.13, 1.34, p < 0.001), and exposure to ETS at workplace also increased the odds of low folate status (AOR: 1.16, 95% CI: 1.02, 1.31, p = 0.02565), **(Tables 2)**.

Using plasma cotinine concentrations to classify active and passive exposure to tobacco products, **Tables 3** shows that participants with plasma cotinine levels between 1.19 – 65.21nmol/L were 1.20 times more likely to have low folate status (AOR: 1.20, 95% CI: 1.10, 1.31, p < 0.001); while those with levels > 65.21nmol/L had a twofold increase in risk (AOR: 1.91, 95% CI: 1.70, 2.14, p < 0.001); p_{trend} <0.001.

D. 考察

To our knowledge, this report presents robust information on demographic and lifestyle predictors of folate status in a relatively large cohort of pregnant Japanese women. Majority (73.83%) of participants had optimal first trimester folate status. Only 0.52% had serum folate concentrations below 6.80nmol/L, a level clinically considered a negative folate balance, while 25.65% of the population had marginal folate status. Lower tolerance limit of 8.47nmol/L implies a negative folate balance for this population. Our findings contrast those from Tokyo where more than 50.00% of the study population of pregnant women

had low folate status.

Demographic predictors of folate status

Low folate status was associated with younger maternal age, higher BMI, educational level and annual income. Cigarette smoking rate is on the increase among young Japanese women, and a quest to achieve a lower BMI via dieting is in vogue among women of reproductive age. These factors may invariably compromise nutritional status including folate among younger women[4; 17]. Micronutrients deficiencies including folate in overweight/obese people have been reported by some previous studies[18]. Possible mechanisms postulated have been: decrease dietary intake, current cigarette smoking, and possible low serum/plasma concentrations as a result of increased intravascular volume[19]. Consistent with our findings, socio-economic status has been reported to influence folate intake among Japanese workers[20]. Also, educational attainment was reported in Belgium[21], and Australia[22]. In USA, older maternal age, higher education, and higher income status, have been reported to predict the use of FA supplements[23]. In this study, these factors might have favored higher folate status. Other demographic factors associated with suboptimal folate status have been reported from other countries, and these include household size[24], season of the year[25], rural residence[26], and region[27]. We observed that residing in the southern and eastern regions; and seasons of the year were associated with the risk of low folate status.

Traditionally, most Japanese women are full-time house wives. This may explain why the unemployed had lower risk. Working women are likely to skip their meals and may prefer fast foods as reported among children of working women[28]. Of note here is that employment status was broadly classified. Further exploration based on job types may shade more insight on this observation.

Unfavorable lifestyle predictors of folate status

We report self-reported active cigarette smoking and ETS exposure as the major modifiable unfavorable predictors of folate status. Although we could not demonstrate a dose-response pattern in the odds, especially among heavy smokers during pregnancy; this may probably be related to a small subgroup size. Using plasma cotinine biomarker, the risk of low folate status increased in a dose-response pattern. Contrary to this result, another study in Tokyo found no lifestyle habits as risk factors for suboptimal folate status[8]. However, our result is consistent with reports from other developed countries, where lifestyle factors are commonly observed as predictors of folate status. Folate depleting effects of active smoking and ETS exposure have been reported[29; 30; 31; 32; 33; 34; 35]. Possible biologic mechanisms of folate depletion in active and passive smokers include decreased intake[29; 33], inactivating effects of organic nitrites, cyanates, and nitrous oxide on circulating folates[34; 36], and direct effects of oxidative stress or increased folate turnover[31; 37]. We observed lower mean plasma cotinine concentrations among nutritional supplements users. Nutritional

supplements users are more likely to practice healthy lifestyles.

Favorable lifestyle predictors of folate status

FA supplements use is the major modifiable predictor of optimal folate status. This report further confirms the well documented role of FA supplements in improving folate status. Other nutritional supplements used also correlated positively with folate status, probably because most multivitamins also contain FA. Other nutritional supplements used included multivitamins, trace elements, herbs, proteins, ginseng and energy drinks. Over-the-counter (OTC) multivitamins used contained various doses of folic acid in the range of 100μg to 200μg per tablet according to the brand names reported by study participants, majority of whom were recruited between 2002 and 2010. However, lately, folic acid content seems to have been increased by drug makers (up to 480μg/tablet). This may reflect in our findings of increase in mean folate concentrations of participants enrolled from 2010 and beyond, and a reduction in the risk of having low folate status. In this study, majority of folic acid supplements users did not use it because of pregnancy. Those who used it for prenatal purpose started only after confirming they were pregnant. This information may impact on the crucial periconceptional period for prevention of NTDs. Within Japan, some smaller studies outside Hokkaido did report that using FA supplements increased blood folate concentrations more than using dietary sources of folate only. They also observed that Japanese women in their reproductive age do not meet the daily Recommended Dietary Allowance (RDA) of 440μg for folate[5; 7; 8; 38; 39]. Although the Japanese Government has recommended that women of reproductive age or those who plan to become pregnant should take 400μg/day of FA supplements, scholars have reported that the level of awareness and compliance with the recommendations are still low[5; 40]. Furthermore, across the Asian sub-region, prenatal FA supplements use is not a routine prenatal care practice[41]. Our findings are similar to other reports emerging from China, Malaysia and Indonesia. Of these three, mandatory fortification is legislated only in Indonesia [42; 43; 44]. Internationally, studies from other developed countries without food fortification policies are reporting increasing incidence of suboptimal folate concentrations[21; 45; 46]. Our result on the role of alcoholic beverage consumption on folate is consistent with a previous study in Czech Republic, where moderate beer consumption correlated with higher plasma folate[47]. Conversely, chronic heavy alcohol consumption is associated with folate deficiency via numerous mechanisms[48]. We stand with the universal recommendation that pregnant women should abstain from consuming alcoholic beverages, because of adverse fetal effects[49].

Strengths and limitations

This study is the first to utilize a large population of pregnant Japanese women who were recruited early enough within the stage of embryonic neurulation and organogenesis. Epidemiologically, the study identified demographic and lifestyle

determinants of folate status at this critical stage of neural tube formation. Identifying modifiable lifestyle factors as favourable and unfavourable determinants can lay a sound foundation for Public Health intervention policies. All information about the type or brand name of nutritional supplements used, the timing and duration of use were self-reported, hence the risk of bias. However, nutritional supplements use and smoking status were validated by biomarkers to avoid misclassification bias. For instance, the difference observed in folate biomarker concentrations among FA users and non-users was an indication of valid self-reported use. Also, comparable results were obtained with plasma cotinine and self-reported cigarette smoking or ETS exposure. Serum folate was used as an indicator of folate status. Erythrocyte folate signifies tissue folate reserves and is not subject to dietary fluctuations exhibited by serum/plasma folate concentrations, thus making it a more reliable choice. However, because erythrocyte folate assay is more complex, serum folate assay was preferred to conduct this large epidemiological study. Two previous studies have justified its use in epidemiologic studies[50;51]. This study involved only women who presented at the designated health facilities and consented to participate, therefore may not be representative of the general population. Finally, our findings are more of statistical correlations and not in any way signifying causality. Future randomized controlled trials employing erythrocyte folate and known dosages of folic acid supplements may be more informative.

Implications

The implication of active and passive tobacco smoking in the determination of folate status is of public health importance because an increasing prevalence of tobacco smoking among younger Japanese women is being reported[52]. Optimal first trimester folate status is central in this subpopulation. It may be helpful to consider policies that could improve folate status in this group. Mandatory food fortification with FA might be a great precautionary measure. Although, there are emerging controversies about prenatal FA exposure and epigenetic effects[53], however, the folate depleting effects of tobacco smoke may constitute a huge public health challenge in the prevention of NTDs and other birth defects in Japan. Although this Hokkaido cohort data recorded only eight (0.04%) cases of isolated NTDs, the national rate is the second highest in developed countries after Germany.

E. 結論

In conclusion, demographic and lifestyle factors likely predict folate status of Hokkaido women. Active cigarette smoking and ETS exposure are the major modifiable unfavourable predictors of folate status; while the use of FA supplement and FA containing multivitamins are the major favourable predictors. FA supplementation may correct the folate deficits associated with tobacco smoking.

F. 研究発表

1. 論文発表

Under minor revision with British Journal of Nutrition (BJN).

2. 学会発表

I.Manokhina, T.A. Yila, W.P. Robinson. Towards Accurate Quantification of miRNAs for Clinical Use: Evaluation of Technical and Biological Confounders. 4th Annual Canadian Human and Statistical Genetics Meeting, 18 – 21 April, 2015 Vancouver, BC, Canada.

G. 知的財産権の出願・登録状況（予定を含む。）

1.特許取得

なし

2.実用新案登録

なし

3.その他

なし

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Table1: Distributions of maternal characteristics by folate status: The Hokkaido Study on Environment and Children's Health 2002-2012, Japan (N = 15266).

Variables	Categories	n	Folate status (nmol/L)					
			Deficient (<6.80)		Suboptimal (6.80 – 13.59)		Optimal (≥13.60)	
			n	(%)	n	(%)	n	(%)
			79	(0.52)	3916	(25.65)	11271	(73.83)
Age (years)	<20	110	2	(1.82)	53	(48.18)	55	(50.00)
	20-24	1685	16	(0.95)	614	(36.44)	1055	(62.61)
	25-29	4564	25	(0.55)	1249	(27.37)	3290	(72.09)
	30-34	5393	24	(0.45)	1258	(23.33)	4111	(76.23)
	≥35	2827	9	(0.32)	572	(20.23)	2246	(79.45)***
Parity	Nulliparous	5983	27	(0.45)	1549	(25.89)	4407	(73.66)
	Parous	8035	38	(0.47)	2125	(26.45)	5872	(73.08)
BMI (Kg/m^2)	<18.50	2532	8	(0.32)	660	(26.07)	1864	(73.62)
	18.50 -24.99	10576	45	(0.43)	2649	(25.05)	7882	(74.53)
	25.00 -29.00	1225	17	(1.39)	347	(28.33)	861	(70.29)
	≥30.00	313	4	(1.28)	92	(29.39)	217	(69.33)***
Educational level	Junior high school	768	4	(0.52)	283	(36.85)	481	(62.63)
	High school	6573	49	(0.75)	1946	(29.61)	4578	(69.65)
	College	5948	17	(0.29)	1301	(21.87)	4630	(77.84)
	University	1580	7	(0.44)	283	(17.91)	1290	(81.65)***
Annual income (million JPY)	<3	2914	21	(0.72)	915	(31.40)	1978	(67.88)
	3-4,999	5709	23	(0.40)	1462	(25.61)	4224	(73.99)
	5-7,999	3215	13	(0.40)	716	(22.27)	2486	(77.33)
	≥8	889	4	(0.45)	164	(18.45)	721	(81.10)***
Occupation	Unemployed	6464	31	(0.48)	1568	(24.26)	4865	(75.26)
	Employed	8802	48	(0.55)	2348	(26.68)	6406	(72.78)**
Tobacco smoking (cigarette sticks/day)	No	13599	59	(0.44)	3249	(23.96)	10251	(75.60)
	<10	975	8	(0.82)	343	(35.18)	624	(64.00)
	10-19	630	11	(1.75)	290	(46.03)	329	(52.22)
	≥20	102	1	(0.98)	34	(33.33)	67	(65.69)***
[c]ETS at home	No	5763	25	(0.43)	1178	(20.44)	4560	(79.13)
	Yes	9503	54	(0.57)	2738	(28.81)	6711	(70.62)***
[c]ETS at work place	No	1530	11	(0.72)	383	(25.03)	1136	(74.25)
	Yes	13736	68	(0.50)	3533	(25.72)	10135	(73.78)
Combined ETS exposure at home and work place	None	724	4	(0.55)	149	(20.58)	571	(78.87)
	Work place	5039	21	(0.42)	1029	(20.42)	3989	(79.16)
	Home only	806	7	(0.87)	234	(29.03)	565	(70.10)
	Home and work place	8697	47	(0.54)	2504	(28.79)	6146	(70.67)***
Plasma cotinine status (nmol/L)	<1.19	5874	22	(0.37)	1142	(19.44)	4710	(80.18)
	1.19-65.21	7113	35	(0.49)	1905	(26.78)	5173	(72.73)
	>65.21	2279	22	(0.97)	869	(38.13)	1388	(60.90)***

Table1 (continued)

Variables	Categories	n	Folate status (nmol/L)					
			Deficient (<6.80)		Suboptimal (6.80 – 13.59)		Optimal (≥13.60)	
			n	(%)	n	(%)	n	(%)
			79	(0.52)	3916	(25.65)	11271	(73.83)
Alcohol intake (frequency)	No	8084	37	(0.46)	1965	(24.31)	6082	(75.24)
	Monthly	5590	34	(0.61)	1586	(28.37)	3970	(71.02)
	Weekly	723	3	(0.41)	160	(22.13)	560	(77.46)
	Daily	869	5	(0.58)	205	(23.59)	659	(75.83)***
Folic Acid supplements use	No	13559	74	(0.62)	3672	(30.68)	8224	(68.71)
	Yes	1707	5	(0.15)	244	(7.40)	3047	(92.45)***
Other nutritional supplements use	No	13956	74	(0.53)	3660	(26.23)	10222	(73.24)
	Yes	1310	5	(0.38)	256	(19.54)	1049	(80.08)***
Region	Central	6718	27	(0.40)	1522	(22.66)	5169	(76.94)
	South	3589	18	(0.50)	1076	(29.98)	2495	(69.52)
	East	4765	33	(0.69)	1271	(26.67)	3461	(72.63)
	Other regions	194	1	(0.52)	47	(24.23)	146	(75.26)***
Year of enrolment	2002 -2004	4623	15	(0.32)	1290	(27.90)	3318	(71.77)
	2005 - 2007	5651	35	(0.62)	1675	(29.64)	3941	(69.74)
	2008 -2010	4063	22	(0.54)	782	(19.25)	3259	(80.21)
	2011-2012	929	7	(0.75)	169	(18.19)	735	(81.05)***
Season of the year at enrolment	Spring	3850	31	(0.81)	1010	(26.23)	2809	(72.96)
	Summer	3720	17	(0.46)	987	(26.53)	2716	(73.01)
	Autum	2424	13	(0.54)	629	(25.95)	1782	(73.51)
	Winter	5272	18	(0.34)	1290	(24.47)	3964	(75.19)*

SD, Standard deviation ; n, number of participants; BMI, Body mass index; JPY, Japanese Yen; ETS, Environmental Tobacco smoke.

P values were derived from Pearson's chi squared tests and Fisher's exact tests. Means and standard deviations generated from Students t-tests and ANOVA with post-hoc analysis. All percentages are row percentages.

Values may not add up to 100% due to missing values. Levels of significance: *p <0.050; **p<0.010; ***p<0.001.

Table 2: Estimated effects of demographic characteristics and lifestyle factors on folate status: The Hokkaido Study on Environment and Children's Health 2002-2012, Japan (N = 15266)

Variables	Categories	n	Folate status (nmol/L)					
			Deficient (<6.80)		Suboptimal (6.80 – 13.59)		Optimal (≥13.60)	
			n	(%)	n	(%)	n	(%)
			79	(0.52)	3916	(25.65)	11271	(73.83)
Age (years)	<20	110	2	(1.82)	53	(48.18)	55	(50.00)
	20-24	1685	16	(0.95)	614	(36.44)	1055	(62.61)
	25-29	4564	25	(0.55)	1249	(27.37)	3290	(72.09)
	30-34	5393	24	(0.45)	1258	(23.33)	4111	(76.23)
	≥35	2827	9	(0.32)	572	(20.23)	2246	(79.45)***
Parity	Nulliparous	5983	27	(0.45)	1549	(25.89)	4407	(73.66)
	Parous	8035	38	(0.47)	2125	(26.45)	5872	(73.08)
BMI	<18.50	2532	8	(0.32)	660	(26.07)	1864	(73.62)
(Kg/m^2)	18.50 -24.99	10576	45	(0.43)	2649	(25.05)	7882	(74.53)
	25.00 -29.00	1225	17	(1.39)	347	(28.33)	861	(70.29)
	≥30.00	313	4	(1.28)	92	(29.39)	217	(69.33)***
Educational	Junior high school	768	4	(0.52)	283	(36.85)	481	(62.63)
level	High school	6573	49	(0.75)	1946	(29.61)	4578	(69.65)
	College	5948	17	(0.29)	1301	(21.87)	4630	(77.84)
	University	1580	7	(0.44)	283	(17.91)	1290	(81.65)***
Annual income	<3	2914	21	(0.72)	915	(31.40)	1978	(67.88)
(million JPY)	3-4,999	5709	23	(0.40)	1462	(25.61)	4224	(73.99)
	5-7,999	3215	13	(0.40)	716	(22.27)	2486	(77.33)
	≥8	889	4	(0.45)	164	(18.45)	721	(81.10)***
Occupation	Unemployed	6464	31	(0.48)	1568	(24.26)	4865	(75.26)
	Employed	8802	48	(0.55)	2348	(26.68)	6406	(72.78)**
Tobacco smoking	No	13599	59	(0.44)	3249	(23.96)	10251	(75.60)
(cigarette sticks/day)	<10	975	8	(0.82)	343	(35.18)	624	(64.00)
	10-19	630	11	(1.75)	290	(46.03)	329	(52.22)
	≥20	102	1	(0.98)	34	(33.33)	67	(65.69)***
[c]ETS	No	5763	25	(0.43)	1178	(20.44)	4560	(79.13)
at home	Yes	9503	54	(0.57)	2738	(28.81)	6711	(70.62)***
[c]ETS	No	1530	11	(0.72)	383	(25.03)	1136	(74.25)
at work place	Yes	13736	68	(0.50)	3533	(25.72)	10135	(73.78)
Combined ETS	None	724	4	(0.55)	149	(20.58)	571	(78.87)
exposure at home and	Work place	5039	21	(0.42)	1029	(20.42)	3989	(79.16)
work place	Home only	806	7	(0.87)	234	(29.03)	565	(70.10)
	Home and work place	8697	47	(0.54)	2504	(28.79)	6146	(70.67)***
Plasma cotinine status	<1.19	5874	22	(0.37)	1142	(19.44)	4710	(80.18)
(nmol/L)	1.19-65.21	7113	35	(0.49)	1905	(26.78)	5173	(72.73)
	>65.21	2279	22	(0.97)	869	(38.13)	1388	(60.90)***

Table2 (continued)

Variables	Categories	n	Folate status (nmol/L)					
			Deficient (<6.80)		Suboptimal (6.80 – 13.59)		Optimal (≥13.60)	
			n	(%)	n	(%)	n	(%)
			79	(0.52)	3916	(25.65)	11271	(73.83)
Alcohol intake (frequency)	No	8084	37	(0.46)	1965	(24.31)	6082	(75.24)
	Monthly	5590	34	(0.61)	1586	(28.37)	3970	(71.02)
	Weekly	723	3	(0.41)	160	(22.13)	560	(77.46)
	Daily	869	5	(0.58)	205	(23.59)	659	(75.83)***
Folic Acid supplements use	No	13559	74	(0.62)	3672	(30.68)	8224	(68.71)
	Yes	1707	5	(0.15)	244	(7.40)	3047	(92.45)***
Other nutritional supplements use	No	13956	74	(0.53)	3660	(26.23)	10222	(73.24)
	Yes	1310	5	(0.38)	256	(19.54)	1049	(80.08)***
Region	Central	6718	27	(0.40)	1522	(22.66)	5169	(76.94)
	South	3589	18	(0.50)	1076	(29.98)	2495	(69.52)
	East	4765	33	(0.69)	1271	(26.67)	3461	(72.63)
	Other regions	194	1	(0.52)	47	(24.23)	146	(75.26)***
Year of enrolment	2002 -2004	4623	15	(0.32)	1290	(27.90)	3318	(71.77)
	2005 - 2007	5651	35	(0.62)	1675	(29.64)	3941	(69.74)
	2008 -2010	4063	22	(0.54)	782	(19.25)	3259	(80.21)
	2011-2012	929	7	(0.75)	169	(18.19)	735	(81.05)***
Season of the year at enrolment	Spring	3850	31	(0.81)	1010	(26.23)	2809	(72.96)
	Summer	3720	17	(0.46)	987	(26.53)	2716	(73.01)
	Autum	2424	13	(0.54)	629	(25.95)	1782	(73.51)
	Winter	5272	18	(0.34)	1290	(24.47)	3964	(75.19)*

n, number of participants; BMI, Body mass index; JPY, Japanese Yen; ETS, Environmental Tobacco smoke. AOR, Adjusted odds ratio; CI, Confidence interval.

Regression model adjusted for maternal age, parity, BMI, educational level, annual income, occupation, geographical region, year of enrolment into the study, season of the year at enrolment, nutritional supplements use, alcohol intake, and active and passive smoking.

Levels of significance: *p <0.050; **p<0.010; ***p<0.001. McFadden's pseudo-R^2 = 8.69%.

All percentages are row percentages. Values may not add up to 100% due to missing values. NS, not significant.

†Other nutritional supplements used included multivitamins, trace elements, herbs, proteins, ginseng and energy drinks.

Table 3: Estimated effects of active and passive cigarette smoking based on plasma cotinine concentrations on folate status: The Hokkaido Study on Environment and Children's Health 2002-2012, Japan (N = 15266)

Smoking status	Plasma cotinine levels (nmol/L)	Folate status (nmol/L) Suboptimal (<13.60) n	(%)	Optimal (≥13.60) n	(%)	AOR	95%CI	p_{trend}
		3995	(26.17)	11271	(73.83)			
Non smoker	<1.19	1164	(19.82)	4710	(80.18)	1.00	Reference	<0.001
ETS exposed	1.19 – 65.21	1940	(27.27)	5173	(72.73)	1.20	(1.10, 1.31)**	
Active smoker	>65.21	891	(39.10)	1388	(60.90)	1.91	(1.70, 2.14)***	

n, number of participants; BMI, Body mass index; JPY, Japanese Yen; ETS, Environmental Tobacco smoke. AOR, Adjusted odds ratio; CI, Confidence interval.

Regression model adjusted for maternal age, parity, BMI, educational level, annual income, occupation, geographical region, year of enrolment into the study, season of the year at enrolment, nutritional supplements use, alcohol intake, and active and passive smoking.

Levels of significance: *p <0.050; **p<0.010; ***p<0.001.

All percentages are row percentages. Values may not add up to 100% due to missing values. McFadden's pseudo-R^2 = 8.53%.

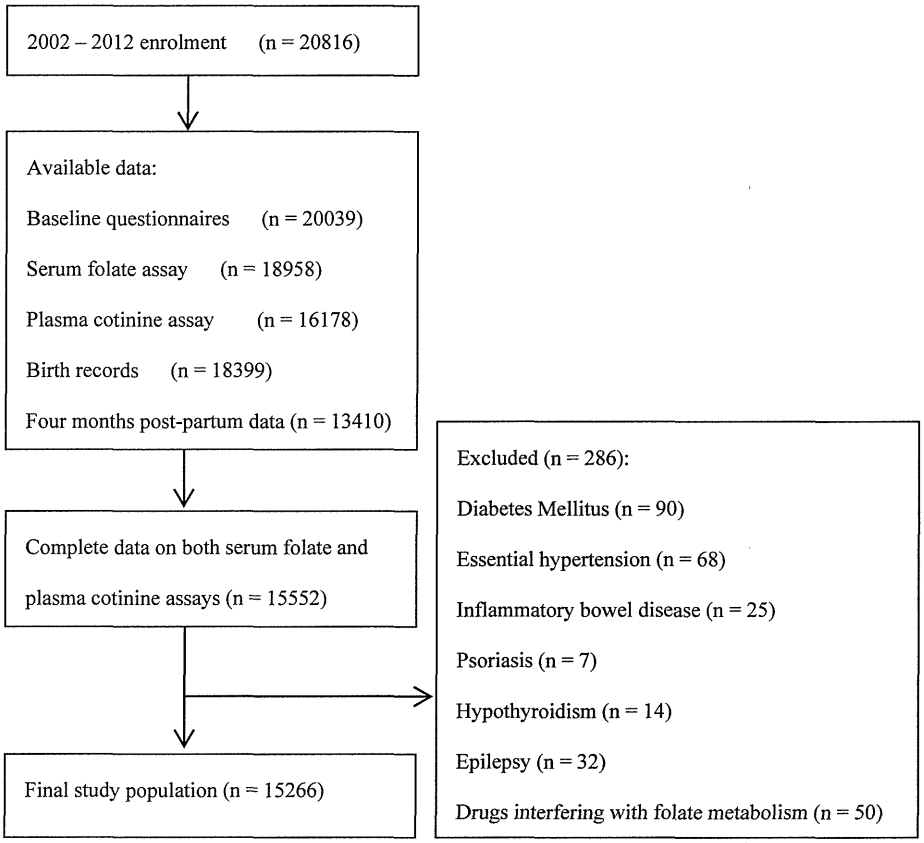

Fig. 1: Study selection chart: The Hokkaido Study on Environment and Children's Health, 2002 – 2012, Japan.

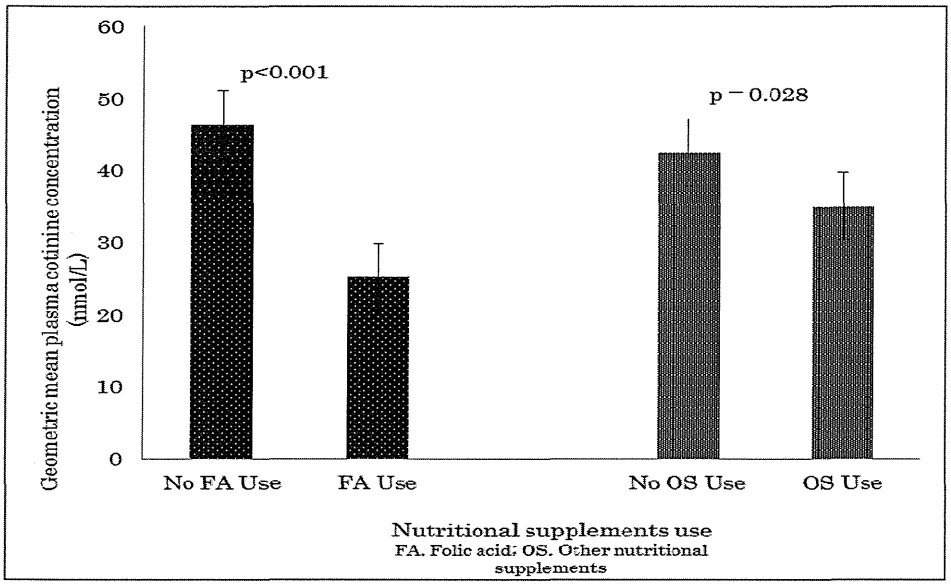

Fig. 2: Mean plasma cotinine concentrations by nutritional supplements use among participants: The Hokkaido Study on Environment and Children's Health, 2002 – 2012, Japan.

厚生労働科学研究費補助金（化学物質リスク研究事業）
分担研究報告書

Effects of prenatal exposure to perfluoroalkyl acids on risk of allergic diseases at 4 years old children

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研究要旨
Perfluoroalkyl acids (PFAAs) as emerged chemicals are extremely resistant chemicals widespread in environment and frequently detected in human blood samples. Animal studies showed exposure to PFAAs cause immunotoxicity. However, the association between PFAAs including long chain PFAAs and allergy in humans are not well understood. We examined whether prenatal exposure to PFAAs is associated with allergic symptoms in 4-year old children in a large-scale prospective birth cohort, Hokkaido, Japan. 1558 mother-child pairs were analyzed in this study and prenatal levels of 11 PFAAs were measured in maternal plasma samples obtained between 28 and 32 weeks of pregnancy by ultra-performance liquid chromatography-tandem mass spectrometry. Information of participants' characteristics were obtained from self-administered pre-, postnatal questionnaires and medical birth records. Infant allergies including eczema, wheezing, and allergic rhinoconjunctivitis were assessed by Japanese version of the International Study of Asthma and Allergies in Childhood (ISAAC) Phase Three questionnaire obtained at 4 years post-delivery. Associations of PFAA quartiles with allergic outcomes were examined using logistic models. Adjusted odds ratios (ORs) in the 4th quartile vs 1st quartile (Q4 vs Q1) for total allergic diseases (including at least one of allergic outcomes) were significantly decreased for PFDoDA (Q4 vs Q1 OR: 0.621; 95% CI: 0.454, 0.847) and PFTrDA (Q4 vs Q1 OR: 0.712; 95% CI: 0.524, 0.966) in all children. We found the same results between PFAAs and eczema. The adjusted OR (Q4 vs Q1) for wheezing in association with higher maternal PFHxS levels was 0.728 (95% CI: 0.497, 1.06) in all children.
Although adjusted OR for allergic outcomes in 2nd to 4th of examined PFAA quartiles reduced compare to first quartile in both sexes, the associations were statistically significant only in boys after sex stratification (p for trend<0.05). In conclusion, prenatal exposure to PFAAs, especially long chain ones, may have immunosuppressive effects on allergic diseases in 4 year old children especially among boys.

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A．研究目的

Perfluoroalkyl acids (PFAAs) are ubiquitous chemicals with widespread contamination in environment, animals and humans. Main route of exposure to PFAAs are contaminated food and water, and house dust (Kato et al., 2009). The most used

PFAAs are perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA). While PFOS and PFOA are being voluntarily phased out by several industries, they are still present in older products. PFAAs are resistant to metabolism with long elimination half-lives for example 3.8, 5.4 and 8.5 years for PFOA, PFOS perfluorohexane sulfonate (PFHxS) in humans, respectively (Olsen et al., 2007). The stability and long half-lives of PFAAs result in continued presence in environment and human exposure.

Exposure to PFOS and PFOA in animals decreased lymphoid organ weights, reduced number of lymphoid cells and antibody production (Yang et al. 2001; Peden-Adams et al., 2007). In animals, PFOS and PFOA inhibit T-cell-dependent Immunoglobulin M (IgM) antibody response (TDAR) which is one of essential and predictor of immune system function.

PFAAs can pass placenta during pregnancy (Inoue et al., 2004); therefore, fetuses and children are continuously exposed to PFAAs. Exposure to PFAAs during these critical window of susceptibility may affect several aspects of health in later life including immune function. Previous epidemiological studies propose immunomodulatory effects of PFAAs indicating that prenatal exposure to PFOS and PFOA were associated with IgE levels of cord blood in different directions (Okada et al., 2012; Wang et al., 2011). Also pre- and post-natal exposure to PFOS and PFOA are associated with reduced antibody levels of tetanus, and diphtheria (Grandjean et al., 2012), and rubella (Granum et al., 2013) in children.

We previously reported declining trend for PFOS and PFOA, however we observed increasing trend for perfluorononanoic acid (PFNA, C9) and perfluorodecanoic acid (PFDA, C10) levels among pregnant women between 2003 and 2011, Hokkaido, Japan (Okada et al. 2013); worthy to note that PFAAs with longer carbon chains including perfluoroundecanoic acid (PFUnDA, C11), perfluorododecanoic acid (PFDoDa, C12), and perfluorotridecanoic acid (PFTrDA, C13) were detectable in more than 90% of maternal plasma samples obtained at 3rd trimester of pregnancy in our cohort. Our group assessed association of prenatal exposure to 11 types of PFAAs and allergic symptoms at 12 to 24 months of age, reported negative association between prenatal exposure to PFTrDA and eczema among female infants (Okada et al., 2014). Although some animal experiments suggest prenatal PFC exposures modified postnatal immune response throughout the period of early childhood (Keil et al. 2008); to this date, effects of PFAAs including long-chain PFAAs on allergic diseases in childhood long observations especially in prospective birth cohorts are not well understood. Therefore, in this study, we followed mother-child pairs in the same cohort of report of Okada et al. (2014) and assessed the association of prenatal PFAAs with allergic disease at 4 year-old children.

B．研究方法

The current work is a part Hokkaido Study on Environment and Children's health, prospective ongoing birth cohort. The details of this study have previously described (Kishi et al. 2011 and 2013). Briefly, pregnant women who had antenatal health care in early pregnancy (>13 weeks of gestational age) at any 37 participating hospitals and clinics in Hokkaido prefecture