

Passive smoking alters circulating naïve/ memory lymphocyte T-cell subpopulations in children

Vardavas CI, Plada M, Tzatzarakis M, Marcos A, Warnberg J, Gomez-Martinez S, Breidenassel C, Gonzalez-Gross M, Tsatsakis AM, Saris WH., Moreno LA, Kafatos AG. Passive smoking alters circulating naïve/memory lymphocyte T-cell subpopulations in children. *Pediatr Allergy Immunol* 2010.

© 2010 John Wiley & Sons A/S

While it has been indicated that exposure to second-hand smoke (SHS) can cause a local *in vivo* response, limited evidence exists on its possible systemic effects from population-based levels of exposure. We investigated into a possible systemic response in the immune parameters and lymphocyte subsets, i.e. B cell (CD19+), T cell (CD4+CD45RO+, CD4+CD45RA+, CD3+CD45RO+, CD3+CD45RA+) and natural killer (CD3+CD16CD56+) lymphocyte subsets relative to exposure to SHS. Blood was drawn from healthy, verified non-smoker, adolescent subjects (n = 68, mean age 14.2) and analysed for cotinine, antioxidants and lymphocyte immunophenotyping. SHS exposure was assessed using serum cotinine. Biomarker quantified exposure to SHS was correlated with a linear dose–response reduction in the percentages of memory CD4+CD45RO+ (p = 0.005) and CD3+CD45RO+ T-cell subsets (p = 0.005 and p = 0.003, respectively) and a linear increase in the percentage of naïve CD4+CD45RA+ and CD3+CD45RA+ T-cell subsets (p = 0.006 and p = 0.003, respectively). Additionally, higher exposure to SHS was associated with a higher CD4+CD45RA+ count (532 vs. 409 cells/ml, p = 0.017). Moreover, after controlling for age, gender, body mass index and plasma antioxidants, SHS exposure was found to be associated with the percentage of circulating naïve and memory CD4+ and CD3+ T-cell subpopulations, as revealed through a linear regression analysis. These findings indicate a systemic immunological response in healthy adolescents exposed to population-based levels of SHS exposure and imply an additional biological pathway for the interaction between exposure to SHS and its adverse effects on human health.

Constantine I. Vardavas^{1,2}, Maria Plada¹, Manolis Tzatzarakis³, Ascension Marcos⁴, Julia Warnberg^{4,5}, Sonia Gomez-Martinez⁴, Christina Breidenassel⁶, Marcela Gonzalez-Gross⁷, Aristeidis M. Tsatsakis³, Wim H. Saris², Luis A. Moreno⁸ and Anthony G. Kafatos¹, on behalf of the HELENA Heraklion Study Group*

¹Department of Social Medicine, Faculty of Medicine, University of Crete, Crete, Greece, ²Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Department of Human Biology, University of Maastricht, Maastricht, The Netherlands, ³Center of Toxicology Science and Research, School of Medicine, University of Crete, Crete, Greece, ⁴Immunonutrition Group, Department of Metabolism and Nutrition, Institute Frio-ICTAN, Spanish Scientific Research Council, Madrid, Spain, ⁵Department of Preventive Medicine and Public Health, School of Medicine, University of Navarra, Navarra Spain, ⁶Department of Nutrition and Food Sciences, Rheinische Friedrich Wilhelms Universität, Bonn, Germany, ⁷Ciencias de la Actividad Física y del Deporte-INEF, Universidad Politécnica de Madrid, Madrid, Spain, ⁸Escuela Universitaria de Ciencias de la Salud, Universidad de Zaragoza, Zaragoza, Spain

Key words: passive smoking; immune response; children; lymphocytes; second-hand smoke

Constantine I. Vardavas, MD, RN, MPH, PhD, Department of Social Medicine, Faculty of Medicine, University of Crete, PO Box 2208, Heraklion 71003, Crete, Greece
Tel.: +30 2810 394599
E-mail: vardavas@edu.med.uoc.gr

*Heraklion HELENA study group members: Anthony Kafatos, Caroline Codrington, Maria Plada, Angeliki Papadaki, Katerina Sarri, Anna Viskadourou, Christos Hatzis, Michael Kiriakakis, George Tsibinos, Constantine I. Vardavas, Manolis Sbokos, Eva Protyeraki and Maria Fasoulaki.

Accepted 2 March 2010

Exposure to second-hand smoke (SHS) is one of the largest threats to global public health and a significant cause of preventable death. These ramifications of SHS exposure on global health are verified by the fact that currently 700 million children are exposed daily to SHS (1). SHS is a potent mixture of carcinogens, volatile toxins and chemicals (2). Substantial scientific evidence has verified the relationship between exposure to SHS and cancer, cardiovascular disease, respiratory illness, asthma exacerbation (1–8). These detrimental effects of SHS exposure on human health are not limited to chronic exposure as even acute exposure to SHS has been proven to modify vascular function and cause a marked increase in circulating inflammatory cytokines (5, 9, 10). Cigarette smoking influences lymphocyte activity resulting in an impaired immune response. There are three major types of lymphocytes, (NK cells, T cells and B cells) each with a significant pathophysiological role in disease development. Among these subsets, T cells are subsequently subdivided according to the existence of a cellular marker into subgroups such as CD4+ T cells, CD3+ T cells and CD8+ T cells, each with a specific role in human immune response. Among the above, circulating CD4+ T-cell lymphocytes have been suggested as the major target cells for smoking. In particular, memory CD4+ T-cell lymphocytes have been found to be selectively and dose-dependently increased by active smoking, which is stimulated through the chronic procedure of habitual smoking (11). Recent evidence indicates that self-reported exposure to SHS can lead to an alteration of CD4+ T-cell lymphocyte subsets, and self-reported measures of exposure to SHS can be subjectively perceived (12, 13). To our knowledge, there is no existing information on the ability of biomarker quantified, exposure to SHS to induce a systemic immunological dose-effect response among healthy adolescents. Thus, the aim of this study was to investigate whether exposure to SHS (using serum cotinine as the main metabolite of nicotine) can induce a systemic dose-effect immunological response in healthy adolescent subjects focusing on their circulating B cell (CD19+), T-cell (CD4+CD45RO+, CD4+CD45RA+, CD3+CD45RO+, CD3+CD45RA+) lymphocyte subsets and natural killer cells (CD16+56+), after controlling for possible confounding factors that could alter their levels, such as age, gender, antioxidant capacity and body weight.

Methods

Study design – setting and participants

The HELENA study is a European Union-funded multi-centre collaborative study conducted among European adolescents and is a randomized multi-centre investigation of the nutritional and lifestyle status of adolescents in 10 European cities. European adolescents of both sexes aged 12.5 up to 17.5 were randomly selected centrally, while adolescents were recruited at schools in a city-based sample. Both the selection of schools and adolescents followed a central randomization procedure with both genders equally distributed over the different grades. In Crete, 400 adolescents were randomly selected and contacted, out of which 341 agreed to participate, of which 311 were within the valid age range (77.8% response rate). Blood samples were collected from a sample of 111 adolescents (of the 142 randomly selected, 78.2%), with complete biochemical, immunological, descriptive and toxicological data available from 68 non-smoking adolescents of the HELENA participants from Heraklion. Further information on the HELENA study procedures and methodology can be found elsewhere (14).

The study was approved by the Research Ethics Committee of the University of Crete, while written informed consent was obtained from the parents of the adolescents and the adolescents themselves (15). The mean age of the participants was 14.3 ± 1.1 yrs, of which 48.9% were men and 51.1% women. Demographic, descriptive, anthropometric and smoking statistics of the study population were collected. Early morning venous blood was drawn from the participants after a 12-h overnight fast, subsequently centrifuged and serum stored at -80°C until analysis.

Assessing SHS exposure

Exposure to SHS was measured using serum cotinine levels. Cotinine, the main metabolite of nicotine in the human body, has a half-life of approximately 16–20 h, depending on age and smoking status and therefore represents recent exposure to SHS, and non-smoker status was cross-evaluated by both self-report (adolescents who had reported that they did not smoke a cigarette in the past month) and biomarker status, with the cut-off cotinine level of smokers and non-smokers designated at 15 ng/ml (16, 17). A detailed elaboration of the analytical approach that was used to

assess the cotinine concentrations can be found elsewhere (18). The population's mean serum cotinine level was calculated at 1.60 ± 2.18 ng/ml and ranged between the level of quantification and the cut-off for active smokers.

Immunoassay preparation

Immunophenotyping was performed to differentiate CD3+ (T-mature cells), CD4+ (T-helper cells), CD8+ (T-cytotoxic/suppressor cells), CD19+ (B cells) and CD16+ 56+ (natural killer cells). The CD3, CD4 and CD8 T-cell subsets examined included the CD45 RA and RO isoforms, representing 'naive' and 'memory' cells, respectively.

Whole blood was collected in EDTA tubes and aliquoted in one Eppendorf, diluted 1:1 with Cytochex™ Reagent (Streck Laboratories, Omaha, NE, USA). All samples were analysed within 7 days after blood withdrawal. The logistics of the sampling, transportation and methodology have been previously described (19). Briefly, blood samples were incubated with monoclonal antibodies purchased from BD Biosciences (San Jose, CA, USA) for 30 min at room temperature. After red blood cells lysis, lymphocytes were gated by forward and side scatter and pan-leucocyte marker expression (CD45) by four staining procedures (CD3+/CD16+ 56+/CD45+/CD19+, CD3+/CD8+/CD45+/CD4+, CD45RA+/CD45RO+/CD4+/CD3+ and CD45RA+/CD45RO+/CD8+/CD3+). All samples were analysed at Consejo Superior de Investigaciones Cientificas in Madrid by flow cytometry (Facsan; BD, Sunnyvale, CA, USA).

Plasma antioxidants

Antioxidative nutrients (vitamins A, E and C) were analysed by high-performance liquid chromatography (Sykam, Gilching, Germany) using ultraviolet detection (UV-Vis 205; Merck, Darmstadt, Germany). For vitamin C analysis, heparin whole blood was centrifuged for 5 min at 1700 g at 4°C. For protein precipitation, 400 µl of heparin plasma was mixed with 400 µl ice cold precipitation reagents [meta-phosphoric acid with 6% (w/w) perchloric acid]. This mixture was centrifuged for 10 min at 3000 g at 4°C. The supernatant was injected in a HPLC system (Sykam), consisting of a S 1100 solvent delivery system, HPLC controller, an autoinjector Triathlon® (Spark, Emmen, Netherlands) with a 20-µl loop and a UV-Vis detector

(Merck). Separation was carried out on a LiChrosper Si 100 RP-18 column (125 mm × 4 mm, 5 µm; Merck) and an isocratic mobile phase. The mobile phase consisted of 0.045 mM sodiumdihydrogen-phosphate1-hydrate buffer adjusted at pH 2.0 with ortho-phosphoric acid (14%, w/w). The flow rate was 1 ml/min, and the detection was carried out at an UV wavelength of 243 nm.

EDTA plasma was used for retinol, alpha-tocopherol and Teac measurements. For retinol and tocopherol analysis, EDTA plasma was mixed with ethanol (1:1) for 2 min; afterwards, 200 µl of n-hexane was added and mixed again for 2 min and centrifuged at 3500 g for 3 min. For the determination on HPLC, 50 µl of the supernatant was used. Separation was carried out on a LiChrosper Si 100 RP-18 column (125 mm × 4 mm, 5 µm; Merck) and with an isocratic mobile phase. The mobile phase consisted of hexane/isopropanol (98/2; wt/wt). The flow rate was 2 ml/min, and the UV wavelength for detection was 325 nm for retinol and 292 nm for tocopherol.

Plasma antioxidant capacity was measured by the Trolox equivalent antioxidant capacity (TEAC). Phosphate buffer saline (PBS) buffer (400 µM, 506 µl), 36 µl metmyoglobin (70 µM) and 300 µl 2,2-azinobis(3-ethylbenzothiazoline-6-sulphonic acid (500 µM) were added to 8.4 µl EDTA plasma, mixed well and incubated inside the photometer (Uvikon Kontron, Zurich, Switzerland) for 3 min. Afterwards, 150 µl H₂O₂ (450 µM) was added and mixed again; after a reaction time of 3 min, the measurement was commenced.

Statistical analysis

All analyses were stratified for non-smoker adolescents. All p-values are based on two-sided tests and a significance level of <5%. The normality of the distribution of cotinine and all other haematological variables was examined with the Kolmogorov–Smirnov test and the use of QQ plots, and cotinine levels were log transformed. During the investigation into the possible correlations between SHS exposure and the immunological response, two-sided Pearson correlations were initially performed, while two-sided *t*-tests were used to investigate the differences in the indicated variables according to low (under 25th percentile) vs. high (over 75th percentile) cotinine levels and thus lower and higher relative exposure. Finally, backward linear regression analyses were performed to investigate the factors that influence CD3+

Table 1. Descriptive immunological, haematological and exposure characteristics of the sample population of healthy, non-smoker* adolescents

	Mean	Standard deviation	Minimum	Maximum
Leucocytes ($10 \times 3/\mu\text{l}$)	6.74	1.37	3.60	10.00†
Lymphocytes ($10 \times 3/\mu\text{l}$)	2.22	0.53	1.10	3.70
Monocytes ($103/\mu\text{l}$)	0.58	0.16	0.30	1.20
Neutrophils ($10 \times 3/\mu\text{l}$)	3.72	1.12	1.3	6.6
Basophils ($10 \times 3/\mu\text{l}$)	0.03	0.04	0.0	0.1
Eosinophils ($10 \times 3/\mu\text{l}$)	0.19	0.23	0.0	1.8
Lymphocyte subsets (%)				
CD3+	69.68	6.02	56.1	81.0
CD3+CD45RA+	57.86	9.72	31.3	77.0
CD3+CD45RO+	41.70	9.99	22.7	68.7
CD4+	39.29	6.35	23.8	61.6
CD4+CD45RA+	55.30	10.64	16.4	76.0
CD4+CD45RO+	44.32	10.78	23.6	83.6
CD8+	25.81	5.25	16.1	39.8
CD8+CD45RA+	67.65	9.67	42.9	83.9
CD8+CD45RO+	32.44	10.67	15.5	67.5
B lymphocytes (CD19+)	12.76	4.28	4.1	25.7
NK cells (CD16+56+)	13.22	5.13	3.1	32.0
CD4+/CD8+ (ratio)	1.59	.45	0.69	2.98
CD3+/CD19+ (ratio)	6.22	2.69	2.52	19.46
Haematological-antioxidants				
Vitamin C (mg/l),	9.63	3.54	1.308	20.19
TEAC (mm),	1.59	0.84	1.38	1.81
Alpha-tocopherol ($\mu\text{g/ml}$),	9.58	2.07	5.66	14.21
Retinol (ng/ml)	311.22	77.29	127.54	549.45
Environmental				
Cotinine‡ (ng/ml)	0.88	0.69–1.13	LOQ	<15

TEAC, Trolox equivalent antioxidant capacity.

*Non-smoking adolescents verified by self-report and cotinine levels <15 ng/ml and over the level of quantification (LOQ) of 0.1 ng/ml.

†Adolescents with a WBC count over 10,000 μl were excluded.

‡Cotinine results in geometric mean and 95% confidence interval, minimum (LOQ) and maximum (cut-off).

and CD4+ lymphocyte subpopulations. The statistical analysis was completed with the statistical package SPSS 16.0 (Social Sciences, SPSS, Inc, IL, USA).

Results

The descriptive immunological characteristics of the study population are shown in Table 1. The mean white blood cell count was 6.78 ($10 \times 3/\mu\text{l}$) after applying the cut-off of 10.0 ($10 \times 3/\mu\text{l}$) white blood cells, so as to exclude any adolescents with an ongoing infection that could possibly alter their lymphocyte subsets, which lead to the exclusion of five adolescents. Furthermore, the absolute number of cells (cells/ml) was as follows: CD3+: 1541 ± 419 ; CD4+: 860 ± 276 ; CD8+: 579 ± 220 ; CD16 CD56+: 291 ± 141 ; CD19+: 281 ± 125 ; CD3+CD45RA+: 899 ± 316 ; CD3+CD45RO+: 632 ± 196 ; CD4+CD45RA+: 480 ± 200 ; CD4+CD45RO+: 374 ± 136 .

Table 2. Correlations between exposure to second-hand smoke (log cotinine levels) and immunological characteristics among healthy non-smoker* adolescents

Immunological variable	N†	Correlation coefficient	p-Value‡
CD3+ (%)	68	0.147	0.232
CD3+CD45RA+ (%)	68	0.350	0.003
CD3+CD45RO+ (%)	68	-0.353	0.003
CD4+ (%)	68	0.173	0.159
CD4+CD45RA+ (%)	68	0.332	0.006
CD4+CD45RO+ (%)	68	-0.337	0.005
CD8+ (%)	68	-0.066	0.595
CD8+CD45RA+ (%)	67	0.178	0.148
CD8+CD45RO* (%)	67	-0.226	0.066
B lymphocytes (CD19+) (%)	68	0.080	0.516
NK cells (CD16+56+) (%)	68	-0.158	0.199
CD4+/CD8+ (ratio)	68	-0.166	0.177

*Non-smoking adolescents verified by self-report and cotinine levels <15 ng/ml.

†Numbers differ to the different subsets for which complete data were available.

‡p-Values based on two-sided Pearson correlation tests.

Bold values indicates the statistically significant differences.

During the analysis, log-transformed serum cotinine values were subsequently correlated with the immunological profile of the adolescents (Table 2). Cotinine levels were significantly correlated with an increase in naïve CD3+ and CD4+ subsets (correlation coefficient of 0.350, $p = 0.003$ and 0.332, $p = 0.005$, respectively) and also with a decrease in the circulating memory CD3+ and CD4+ subsets (correlation coefficient of -0.353, $p = 0.003$ and -0.337, $p = 0.006$, respectively). Furthermore, with regard to the absolute numbers of subsets, the absolute number of CD4+CD45RA+ cells was found to be statistically correlated with serum cotinine levels (correlation coefficient 0.247, $p < 0.05$). No other correlation indicated a statistically significant response.

When we compared (Table 3) the immunological profile of the participants in the first quartile of exposure to SHS (lower exposed/control group) with those in the fourth quartile of exposure (higher exposed), we found that the latter had a higher percentage of CD3+CD45RA+ (62.93% vs. 53.72%, $p = 0.004$) and CD4+CD45RA+ (60.22% vs. 50.37%, $p = 0.004$) and a lower percentage of CD3+CD45RO+ (36.49% vs. 45.98%, $p = 0.004$) and CD4+CD45RO+ T-cell subsets (39.31% vs. 49.41%, $p = 0.004$), indicating a strong dose-response relationship with the noted changes in T-cell lymphocyte subsets as the percentages increased and decreased, respectively (for naïve and memory subsets), in line with the

Table 3. Differences in the immunological response of healthy non-smoker* adolescents in regard to their level of exposure to second-hand smoke

	1st quartile of cotinine levels			4th quartile of cotinine levels			p-Value‡
	N†	Mean	Standard deviation	N†	Mean	Standard deviation	
Leucocytes (10 × 3/μl)	19	6.57	1.44	20	6.50	1.14	0.851
Lymphocytes (10 × 3/μl)	19	2.17	0.52	20	2.18	0.54	0.995
Monocytes (10 × 3/μl)	19	0.55	0.20	20	0.57	0.16	0.769
Neutrophils (10 × 3/μl)	19	3.64	1.30	20	3.52	0.97	0.740
Basophils (10 × 3/μl)	19	0.03	0.05	20	0.04	.049	0.826
Eosinophils (10 × 3/μl)	19	0.16	0.16	20	0.19	0.15	0.670
Lymphocyte subsets (%)							
CD4+ (%)	17	38.88	6.00	19	41.62	5.01	0.145
CD8+ (%)	17	25.72	5.10	19	25.16	4.16	0.719
CD3+ (%)	18	68.32	6.92	19	70.78	5.62	0.240
CD16+56+ %	18	14.28	5.51	19	12.24	3.74	0.195
CD19+ (%)	18	12.41	5.35	19	12.98	3.12	0.694
CD3+CD45RA %	18	53.72	10.45	18	62.93	7.40	0.004
CD3+CD45RO %	18	45.98	10.54	18	36.49	7.58	0.004
CD4+CD45RA %	18	50.37	11.22	18	60.22	7.73	0.004
CD4+CD45RO %	18	49.41	11.31	18	39.31	7.71	0.004
CD8+CD45RA %	18	65.41	10.21	18	68.99	10.66	0.310
CD8+CD45RO %	18	36.17	12.88	18	30.51	10.86	0.163
CD4+/CD8+ (ratio)	17	1.59	0.47	19	1.71	0.39	0.414
CD3+/CD19+ (ratio)	18	6.86	4.02	19	5.82	1.76	0.314

*Non-smoking adolescents verified by both self-report and cotinine levels <15 ng/ml.

†Numbers differ to the different subsets for which complete data were available.

‡p-Values based on two-sided student t-test statistics.

Bold values indicates the statistically significant differences.

increase in serum cotinine levels. No significant differences were noted in CD4+, CD3+, CD8+, CD19+ and CD16CD56+ total percentages nor in CD4+/CD8+ and CD3+/CD19+ ratios. Moreover, the absolute number of CD4+CD45RA+ cells (cells/ml) was found to differ according to quartiles of exposure, with those more heavily exposed found to have a higher absolute CD4+CD45RA+ count (532 vs. 409 cells/ml, p = 0.017). There was also an increase in the absolute number of CD3+CD45RA+ cells (955 vs. 796 cells/ml) and a decrease in the absolute number of CD3+CD45RO+ cells (555 vs. 662 cells/ml) and CD4+CD45RO+ cells (347 vs. 408 cells/ml) but these differences although notable were not statistically significant.

Finally, a backward linear regression model was applied, using the immunological factors that were correlated to cotinine levels in the initial correlations and controlling for the participants' age, gender, BMI and serum antioxidant concentrations (alpha-tocopherol, TEAC, vitamin C, retinol). According to the results of the analysis shown in Table 4, in all instances, serum cotinine levels were found to mediate the naïve and memory circulating CD3+ and CD4+ T-cell subpopulations. Specifically, serum cotinine was found to induce higher

Table 4. Multivariate backward linear regression analysis* of the factors that influence the percentage of circulating naïve and memory CD3+ and CD4+ lymphocyte subpopulations among non-smoking, healthy adolescents†

Immunological characteristics	Variable	Beta coefficient	95% CI		p-Value
			Lower bound	Upper bound	
CD3+CD45RA %	TEAC (mm)	33.798	29.802	37.794	<0.001
	Cotinine	6.596	2.014	11.177	0.005
CD3+CD45RO %	Age	2.861	2.718	3.004	<0.001
	Cotinine	-6.604	-10.660	-2.548	0.002
CD4+CD45RO %	Age	3.433	2.025	4.841	<0.001
	Cotinine	-6.495	-10.625	-2.366	0.003
CD4+CD45RA %	BMI	-0.653	-1.159	-0.146	0.012
	TEAC (mm)	44.874	37.278	52.469	<0.001
	Cotinine	5.989	1.458	10.521	0.010

*Backward linear regression model with the participants age, gender, BMI, alpha-tocopherol (μg/ml), TEAC (Trolox equivalent antioxidant Capacity) (mm), vitamin C (mg/l), retinol (ng/ml) and log-transformed cotinine, entered in step 1.

†Non-smoking adolescents verified by both self-report and cotinine levels <15 ng/ml and over the level of quantification of 0.1 ng/ml.

percentages of CD3+CD45RA+ and CD4+CD45RA+ subsets (p = 0.005 and p = 0.01, respectively) and lower percentages of CD3+CD45RO+ and CD4+CD45RO+ subsets (p = 0.002 and p = 0.003, respectively), after controlling for possible confounding factors.

Discussion

According to our results, acute or elevated exposure to SHS can affect T-cell lymphocyte subsets and is associated with a dose-response systemic immunological reaction among healthy adolescents. This alteration is noted through a reduction in the circulating CD3+ and CD4+ memory cells and an increase in the circulating naïve CD3+ and CD4+ subsets and absolute CD4+CD45RA+ cell count, even after controlling for demographics, anthropometrical characteristics and plasma antioxidants. To our knowledge, this is the first study to indicate such a systemic immunological dose-effect response using biomarker exposure to real-life SHS exposure.

Nakata et al. (12) had hypothesized that exposure to SHS might cause a systemic immunological response among adults and indicated an increase in both naïve and memory CD4+ subsets among those who were self-reportedly exposed to SHS at home or at work. Self-reported measures though, such as the above, provide an indication but not a clear insight into the actual level of exposure as self-reported exposure to SHS is greatly misclassified. In comparison, we have found that serum cotinine levels are correlated with an increase in the circulating naïve T-cell subsets and a decrease in the memory T-cell subsets, with this difference found to be greater between those with limited exposure and among those with higher cotinine levels. Hockertz et al. (20) in 1995 investigated the role of SHS on the immune system by also measuring serum cotinine from subjects who had been experimentally exposed to SHS but did not find any differences in the immunological response, and also they did not investigate whether or not plasma antioxidants may be affected by this exposure. These null results could be because of their study design and the fact that they were not able to investigate different levels of exposure and thus a possible dose-effect response, which we were able to do using a study based on real-life exposure among adolescents drawn from the population.

Previous research has demonstrated that active smoking does cause an increase in the total CD4+ lymphocyte populations, with an emphasis on memory CD4+ subsets, a biological change hypothesized to be promoted by the chronic exposure to the tobacco constituents in the lung (11). It is possible that this acute increase in the percentage of naïve T-cell subpopulations could be a consequence of cellular recruitment or redistribution as part of physio-

logical response against the damaging agents present in the inhaled tobacco smoke. This would lead to the redistribution of T cells from the bronchial lining to the periphery. Similar results have also been found with regard to circulating CD4+ and CD3+ cells among heavy active smokers (21). Passive smoking may induce the increase in naïve T-cell lymphocyte populations as a systemic response to the inhaled toxins, carcinogens and chemicals, which would explain the higher naïve CD4+CD45RA+ and CD3+CD45RA+ lymphocyte populations in those with higher cotinine levels (an indicator of either heavier or more recent exposure). Active smoking on the other hand increases the CD4+ T-cell count, especially the CD4+ memory cells because of its more chronic and heavier character of exposure (11).

Our findings indicate a possible mechanism that could explain how exposure to SHS influences disease predisposition and development. Atherosclerotic lesions in coronary heart disease have been found to accumulate CD4+ T lymphocytes and can contribute to lesion progression and remodelling (22). The rupture of these lesions and the subsequent thrombus formation are the dominant mechanisms for acute coronary events (23). Additional research among mice exposed to side-stream SHS has also provided evidence that long-term exposure to SHS can cause a state of permanent inflammation, influencing pro-inflammatory and cell-mediated responses and produce cytokines that further activate antibody-producing T cells and so activating a cascade of reactions in response to the oxidative stress of SHS (24). As SHS is a potent source of oxidative stress, it is plausible that a higher serum antioxidant status could act protectively and act as a buffer against the increased free radical production, antioxidant depletion and oxidative stress caused by exposure to SHS (25). The implications of the inflammatory response to SHS exposure are not only limited to the systemic response through T-cell production but also can lead to a localized reaction and sequestration of neutrophils in pulmonary microvasculature and cause tissue damage through the release of toxic and chemotactic mediators, and thus predisposition to lung disease and inflammation (26). The finding that serum TEAC concentrations were independently associated with the increase in CD4+CD45RA+ and CD4+CD45RA+ subsets percentages indicates its possible interactive role in mediating the antioxidant capacity and immunological reaction in response to SHS exposure.

While this study has some limitations mainly because of its cross-sectional design, the use of the main metabolite of nicotine, serum cotinine, as a means of measuring SHS exposure allows us to interpret with confidence the noted dose-effect controlling for oxidative stress, a scope of investigation untouched so far in the scientific literature. Furthermore, it is important to highlight that the noted change in the percentage of lymphocyte subsets was also in the same direction of the alteration of the absolute cell count. Further research is warranted to investigate other possible systemic immunological markers that expedite the reaction to SHS and the dietary factors, such as vitamin and antioxidant intake that might attenuate the immunological response to SHS exposure among youth.

Elevated exposure to SHS was found to be associated with a systemic immunological dose-effect response and to alter the naïve/memory CD3+ and CD4+ T-cell subpopulations among health adolescents. This alteration in circulating T-cell subpopulations indicates a possible underlying mechanism of disease initiation and exacerbation among populations exposed to SHS and provides further evidence in support of the necessity for the adoption of smoke-free environments for the protection of children and youth.

Acknowledgments

The authors thank Adelheid Schuch, Anke Berchtold for the antioxidant determinations and Ligia Esperanza Diaz for the immunophenotyping.

Competing interests

We declare that we have no conflict of interest.

Funding

This research was co-funded by a Flight Attendant Medical Research Institute award grant for research into SHS exposure (Author C.I.V) and a European Community Sixth RTD Framework Programme. The HELENA study takes place with the financial support of the European Community Sixth RTD Framework Programme (Contract FOOD-CT-2005-007034). The content of this article reflects the author's views, and the European community is not liable for any use that may be made of the information contained therein.

Authors contributions

Author CIV conceived the idea, had the main role in manuscript preparation and the organization of the current substudy. MP was responsible for data collection and participant organization, authors MNT and AMT performed the cotinine analysis, authors AM and JW performed the immunophenotyping. Authors GMS and CB were responsible for antioxidant analyses, while authors LAM and

AGK provided supervision and were responsible for the HELENA study at a European and regional level, respectively. Author WHS provided support and helped in data interpretation, supervision and manuscript preparation. All authors contributed to manuscript preparation and have approved its content.

References

1. WORLD HEALTH ORGANIZATION (1999) Consultation report. World Health Organization Division of Non communicable Disease, Tobacco Free Initiative. International Consultation on Environmental Tobacco Smoke (ETS) and Child Health, Geneva 1999. http://www.who.int/tobacco/research/en/ets_report. Viewed 3 March 2009.
2. INTERNATIONAL AGENCY FOR RESEARCH ON CANCER. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man. Tobacco Smoke and Involuntary smoking. Vol 83. Lyon France: World Health Organisation, 2002.
3. LEONE A, GIANNINI D, BELLOTTO C, BALBARINI A. Passive smoking and coronary heart disease. *Curr Vasc Pharmacol* 2004; 2: 175–82.
4. XEPAPADAKI P, MANIOS Y, LIARIGKOVINOS T, et al. Association of passive exposure of pregnant women to environmental tobacco smoke with asthma symptoms in children. *Pediatr Allergy Immunol* 2009; 20: 423–9.
5. FLOURIS MD, METSIOS GS, CARRILLO AE, et al. Acute and short-term effects of secondhand smoke on lung function and cytokine production. *Am J Respir Crit Care Med* 2009; 179: 1029–33.
6. KOSECIK M, EREL O, SEVINC E, SELEK S. Increased oxidative stress in children exposed to passive smoking. *Int J Cardiol* 2005; 100: 61–4.
7. BIAGINI JM, LEMASTERS GK, RYAN PH, et al. Environmental risk factors of rhinitis in early infancy. *Pediatr Allergy Immunol* 2006; 17: 278–84.
8. HAN YY, LEE YL, GUO YL. Indoor environmental risk factors and seasonal variation of childhood asthma. *Pediatr Allergy Immunol* 2009; 20(8): 748–56.
9. HEIS C, AMABILE N, LEE AC, et al. Brief secondhand smoke exposure depresses endothelial progenitor cells activity and endothelial function. *J Am Coll Cardiol* 2008; 51: 1760–71.
10. MOFFATT RJ, CHELLAND SA, PECOTT D, STAMFORD BA. Acute exposure to environmental tobacco smoke reduces HDL-C and HDL2-C. *Prev Med* 2004; 38: 637–41.
11. NAKATA A, TAKAHASHI M, IRIE M, FUJIOKA Y, HARATANI T, ARAKI S. Relationship between cumulative effects of smoking and memory CD4+ T lymphocyte subpopulations. *Addict Behav* 2007; 32: 1526–31.
12. NAKATA A, TANIGAWA T, ARAKI S, SAKURAI S, ISO H. Lymphocyte subpopulations among passive smokers. *JAMA* 2004; 291: 1699–700.
13. AL-DELAIMY WK, WILLET WC. Measurement of tobacco smoke exposure: comparison of toenail nicotine biomarkers and self reports. *Cancer Epidemiol Biomarkers Prev* 2008; 17: 1255–61.
14. MORENO L, GONZÁLEZ-GROSS M, KERSTING M, et al. Assessing, understanding and modifying nutritional status, eating habits and physical activity in European adolescents: the HELENA (Healthy Lifestyle in Europe by Nutrition in Adolescence) Study. *Public Health Nutr* 2008; 11: 288–99.

15. BÉGHIN L, CASTERA M, MANIOS Y, et al. Quality assurance of ethical issues and regulatory aspects relating to good clinical practices in the HELENA Cross Sectional Study. *Int J Obes (Lond)* 2008; 32 (Suppl. 5): S12–8.
16. BENOWITZ NL. Cotinine as a biomarker of environmental tobacco smoke exposure. *Epidemiol Rev* 1996; 18: 188–204.
17. BRAMER SL, KALLUNGAL BA. Clinical considerations in study designs that use cotinine as a biomarker. *Biomarkers* 2003; 8: 187–203.
18. VARDAVAS CI, TZATZARAKIS M, TSATSAKIS A, et al. Biomarkers of passive smoking among Greek preschool children. *Eur J Pediatr* 2006; 165: 891–6.
19. GONZALEZ-GROSS M, BREIDENASSEL C, GOMEZ-MARTINEZ S, et al. Sampling and processing of fresh blood samples within a European multicenter nutritional study: evaluation of biomarker stability during transport and storage. *Int J Obes* 2008; 32: S66–75.
20. HOCKERTZ S, EMMENDORFFER A, SCHERER G, et al. Acute effects of smoking and high experimental exposure to environmental tobacco smoke (ETS) on the immune system. *Cell Biol Toxicol* 1994; 10: 177–90.
21. LUPPI P, LAIN KY, JEYABALAN A, DELOIA JA. The effect of cigarette smoking on circulating maternal leukocytes during pregnancy. *J Immunol* 2007; 122: 214–9.
22. ZHOU X. CD4+ T cells in atherosclerosis. *Biomed Pharmacother* 2003; 57: 287–91.
23. HALVORSEN B, OTTERDAL K, DAHL TB, et al. Atherosclerotic plaque stability-what determines the fate of a plaque? *Prog Cardiovasc Dis* 2008; 51: 183–94.
24. YUAN H, WONG LS, BHATTACHARYA M, et al. The effect of secondhand smoke on biological processes important in atherogenesis. *BMC Cardiovasc Disord* 2007; 8: 1.
25. RUBIN RN, NAVON L, CASSANO PA. Relationship of serum antioxidants to asthma prevalence in youth. *Am J Respir Crit Care Med* 2004; 169: 393–8.
26. BHALLA D, HIRATA F, RISHI A, GAIROLA CG. Cigarette smoke, inflammation, and lung injury: a mechanistic perspective. *J Toxicol Environ Health B* 2009; 12: 45–69.