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# The relationship between cotinine concentrations and inflammatory markers among highly secondhand smoke exposed non-smoking adolescents

Yuko Matsunaga [a,*], Constantine I. Vardavas [a,b], Maria Plada [b], Julia Wärnberg [c,d], Sonia Gómez-Martinez [e], Manolis N. Tzatzarakis [f], Aristeidis M. Tsatsakis [f], Esperanza-Ligia Díaz [e], Ascensión Marcos [e], Anthony G. Kafatos [b]

[a] Center for Global Tobacco Control, Department of Social and Behavioral Sciences, Harvard School of Public Health, Boston, USA
[b] Department of Social Medicine, University of Crete, Greece
[c] CIBER Fisiopatología de la Obesidad y Nutrición (CIBERobn), Instituto de Salud Carlos III (ISCIII), Spain
[d] Unit for Nutrition Epidemiology, Department of Preventive Medicine, University of Málaga, Málaga, Spain
[e] Immunonutrition Research Group, Instituto de Ciencia y Tecnología de los alimentos y Nutrición, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain
[f] Centre of Toxicology Science and Research, University of Crete, Greece

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A B S T R A C T

*Background:* Secondhand smoke (SHS) exposure is a risk factor of respiratory, cardiovascular and inflammatory diseases, however its association with inflammatory markers among highly SHS exposed adolescents has not yet been explored.
*Methods:* Participants included in this study were a subset of 68 non-smoking adolescents, aged 12.5–17.5 from the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) study, recruited from Crete Greece. Smoking and SHS exposure was assessed via serum cotinine concentrations. Cytokines (Interleukin-1β, 2, 4, 5 and 6, tumor necrosis factor-α, interferon-γ, tumor growth factor-β1), immunoglobulins IgG, IgA, IgM, complement factors C3, C4, high sensitivity C-reactive protein, and endothelial inflammatory markers [soluble E-selectin, soluble L-selectin, soluble intercellular adhesion molecules (sICAM-1) and soluble vascular cell adhesion molecules-1 (sVCAM-1)] were assessed. Inflammatory markers in the lower 25th percentile and upper 75th percentile groups of cotinine levels were compared and multivariate linear regression analysis was performed controlling for age, sex and BMI.
*Results:* Cotinine concentrations were notably elevated (geometric mean 0.82 ng/ml, 95%CI 0.62–1.07) in this study population. A significant decrease in IL-4 (130.09 vs. 25.77 pg/ml, $p = 0.014$) and IL-6 (19.52 vs. 5.52 pg/ml, $p = 0.008$) concentrations between the upper 75th percentile cotinine level group and lower 25th percentile cotinine level group was observed. In a multivariate linear regression analysis, cotinine concentrations had a weak inverse association with IL-4 and IL-6 ($p = 0.028$ and $p = 0.06$) which was not statistically significant when adjusted for multiple comparisons (modified Bonferroni, $p > 0.016$). No differences in the other variables was noted.
*Conclusions:* Among highly SHS exposed adolescents, cotinine levels had weak inverse association with IL-4 and IL-6, which did not achieve statistical significance. However, our results potentially indicate an immunosuppressive role of SHS. Further research is warranted to explore this hypothesis.
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## 1. Introduction

The burden of smoking related diseases is substantial and includes conditions associated with both direct and secondhand smoke (SHS) exposure [1]. SHS in particular has a considerable impact on health status of exposed children. Previous research has shown a number of detrimental SHS related effects on children such as asthma, infection, cardiovascular effects and increased cancer risk later in life [2]. SHS exposure among young adolescents has also been identified to lead to an impaired endothelial function and suggested to increase the risk of atherosclerosis [3]. Early atherosclerotic processes may be initiated by the activation of circulating inflammatory cells and their migration into the endothelium. Cell adhesion molecules including; E-selectin (expressed on

*Abbreviations:* SHS, secondhand smoke; HELENA, Healthy Lifestyle in Europe by Nutrition in Adolescence.

* Corresponding author. Address: Center for Global Tobacco Control, Department of Social and Behavioral Sciences, Harvard School of Public Health, 401 Park Drive, Landmark Center, 4th Floor, Boston, MA 02215, USA. Tel.: +1 (617) 9988809.
*E-mail address:* yum892@mail.harvard.edu (Y. Matsunaga).

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endothelial cells), P-selectin (expressed on platelet), L-selectin (expressed on leukocyte), intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecules (VCAM-1) are responsible for cell migration and adhesion to the vascular wall, and are suggested as early markers of atherosclerosis [4]. Thus, these cell adhesion molecules are potent biomarkers for the early detection of atherosclerotic process.

It is well acknowledged that inflammatory responses play a pivotal role in the development of chronic obstructive pulmonary disease (COPD) and cardiovascular disease. While the immune response to SHS exposure appears to be altered, the precise nature of the alteration is unclear, as human and animal studies have demonstrated inconsistent and sometimes contradictory results. Following acute SHS exposure for 1 h, Interleukin-5 (IL-5), IL-6 and interferon-γ (IFN-γ) have been identified to be increased among both genders, while IL-4 and tumor necrosis factor-α (TNF-α) were found to increase only among males [5]. Research on chronic SHS exposure has indicated increased IL-6 concentrations among SHS exposed adults [6]. On the other hand, other studies have reported no elevation in serum IL-6 or other any acute inflammatory cytokine such as IL-1α, IL-1β and TNF-α in SHS exposed populations. [7] [8] [6]. While among 1–6 year-old children exposed to SHS, Wilson et al. showed decreased serum IL-1β, IL-4, IL-5 and IFN-γ levels, suggesting an immunosuppressive effect of SHS exposure [9]. These studies indicate equivocal results which may be attributable to different SHS exposure levels, and may be related to SHS exposure in a dose response relationship. Thus, to address the issue, we assessed the relationship between inflammatory markers and cotinine concentrations, among a unique population of highly SHS exposed adolescents.

## 2. Methods

### 2.1. Study design and participants

This report is based on the Healthy Lifestyle in Europe by Nutrition in Adolescence cross-sectional study (HELENA-CSS) conducted throughout Europe from October 2006 to December 2007 [10]. European adolescents of both sexes aged 12.5 up to 17.5 years 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 sexes equally distributed over the different grades. In Crete, 400 adolescents were randomly selected and 341 agreed to participate, of which 311 were within the valid age range. Demographic and smoking characteristics of the study participants were collected. All participants were Caucasians.

Blood was collected from a random sample of 106 adolescents of the HELENA participants; however, as the cotinine analysis was an ad-hoc analysis, it was only performed on 83 blood samples for which surplus serum was available. Previous analysis between the HELENA-CSS participants from which blood samples were derived and those who did not give a blood sample were investigated into, and revealed no differences between their age category (>15 vs. <15, $p = 0.352$) nor gender (males vs. females, $p = 0.223$) [11]. Adolescents were excluded if they had: self-reported symptoms of acute infection within 1 week of recruitment or a white blood cell count greater than or equal to $10.0 \times 10^3/\mu l$ ($n = 5$); incomplete questionnaires ($n = 7$); or cotinine levels over 15 ng/ml (the cut-off applied for active smoking) or any self-reported smoking in the past month ($n = 11$). Furthermore, subjects with cotinine levels below the level of quantification (LOQ) of 0.1 ng/ml were very few ($n = 2$), therefore also excluded. After applying the above exclusion criteria, complete biochemical, immunological, descriptive, and toxicological data for 68 non-smoking adolescents from HELENA participants in Heraklion was compiled for analysis.

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 [11].

### 2.2. 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 and represents recent exposure to SHS [11]. For cotinine analysis, whole blood was centrifuged and the serum samples were stored at −20 °C until analysis. Cotinine concentrations were determined by gas chromatography–mass spectrometry chromatograms (Shimadzu, Kyoto, Japan). Further detailed cotinine measurement was described elsewhere [11].

### 2.3. Blood sampling and laboratory measures

Early morning (0800–1000 h) venous blood was drawn from the participants after a 12-h overnight fast. The logistics of the sampling, transportation, methodology and stability of samples during transport and storage have been previously described [10]. White blood cells (WBC) and differential (neutrophils and lymphocytes) were analyzed at local automated cell counter.

Serum cytokines IL-1β, IL-4, IL-5, IL-6, IFN-γ and TNF-α were determined using the High Sensitivity Human Cytokine MILLIPLEX™ MAP kit (Millipore Corp., Billerica, MA, USA) and collected by flow cytometry (Luminex-100 v.2.3, Luminex Corporation, Austin, TX, USA). C3 and C4 serum complement was analyzed by nephelometry (Behring Diagnostics). The total plasma C3 values in our study represented C3, C3b, and C3c production. Serum adhesion molecule sL-selectin and sE-selectin (g/L) were analyzed using commercial ELISA kits (Diaclone) on the Universal Microplate spectrophotometer (Power WaveTM XS, Biotek Instruments). The sensitivity was 1.0 g/L. Measurement of serum soluble sE-selectin, sVCAM-1, and sICAM-1 (all g/L) was performed with Luminex-100 IS (Integrated System: Luminex) technology by using the multiplex assay kit Linco Human Cardiovascular Disease (CVD) Panel 1 Lincoplex, 96 Well Plate Assay (HCVD1-67AK), manufactured by Linco Research. Multianalyte profiling calibration microspheres for classification and reporter readings, as well as sheath fluid, were also purchased from Luminex Corporation. Acquired fluorescence data were analyzed by the Luminex 2.3 version software. All analyses were performed according to the manufacturer's protocols. The intra- and inter-assay precision CVs were: 3.5% and 4.5% respectively, for IL-6; and 3.5% and 3.8%, respectively, for TNF-α, 11.2% and 13.4%, respectively, for sE-selectin; 4.5% and 8.5%, respectively, for VCAM-1; and 7.9% and 9.7%, respectively, for ICAM-1, 6.7% and 8.5%, respectively, for TGF-β1. Detection limits (sensitivity) for all the analyses were 0.007 mg/L for CRP, 0.01 g/l for C3, 0.002 g/l for C4, 0.1 pg/ml for IL-6, and 0.05 pg/ml for TNF-α, 79.0 ng/L for sE-selectin, 16.0 ng/L for sVCAM-1, and 9.0 ng/L for sICAM-1. The intra- and inter-assay precision CV were: Serum TGF-β1 levels were measured using commercial ELISA kits (Diaclone) and analyzed by the Universal Microplate spectrophotometer (Power WaveTM XS, Biotek Instruments).

All samples were analyzed at Consejo Superior de Investigaciones Cientificas in Madrid.

### 2.4. Statistical analysis

IL-1β, IL-4, IL-5, IL-6, and IFN-γ concentrations were the a priori primary interest endpoints. The normality of the distribution of

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