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Biomarker evaluation of Greek adolescents' exposure to secondhand smoke

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Abstract

Exposure to secondhand smoke (SHS) is a significant threat to public health, and represents a danger for both the development and health status of children and adolescents. Taking the above into account, our aim was to quantify Greek adolescents' exposure to SHS using serum cotinine levels. During 2006, 341 adolescents aged 13-17 were randomly selected from high schools in Heraklion and agreed to participate as part of the European Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) study. Blood samples were drawn from a random sample of 106 adolescents, while serum cotinine/nicotine concentrations were measured by Gas Chromatography–Mass Spectrometry (GC-MS). The mean levels of serum cotinine and nicotine were calculated at 1.60 $\pm$ 2.18 ng/mL and 4.48 $\pm$ 4.00 ng/mL, respectively, while 97.7% of the non-smoker adolescents were found to have measureable levels of serum cotinine indicating exposure to SHS. The analysis revealed that their paternal ($p = .001$) and maternal smoking habits ($p = .018$) as also the existence of a younger brother or sister ($p = .008$) were the main modifiers of SHS exposure during adolescence. Conclusively, almost all of the measured Greek adolescents were exposed to SHS, even when their parents were non-smokers. This finding indicates the need for both community and school-based educational programmes as also the implementation of a comprehensive ban on smoking in public places.

Keywords

passive smoking, exposure, cotinine, adolescents, health effects, environmental tobacco smoke

Introduction

Exposure to secondhand smoke (SHS) is a prominent threat to public health.[1] SHS exposure during childhood and adolescence has been associated with a plethora of negative health outcomes such as asthma, allergies, respiratory infections and cognitive disabilities, as also the predisposition for future chronic disease, through molecular and genetic damage, lung and vascular endothelium dysfunction.[2-8] SHS itself is a documented human carcinogen, containing over 2000 chemical components, 200 of which are volatile and poisonous and 50 known or suspected cancer-causing agents.[9]

A problem in evaluating SHS exposure is its accurate quantification, which can ideally be achieved by directly assessing tobacco smoke constituents or their metabolites in biological samples. Among the biomarkers used for the specific assessment of SHS exposure, cotinine (the major metabolite of nicotine) is most often used due to its availability in a number

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of biological fluids, its stability, as well as its half-life of 16-20 hours among smoker adults, which can be even longer among children and non-smokers.[10,11]

Air monitoring in Greece has revealed that the Greek population is heavily exposed to SHS in hospitality venues, educational and health provision institutes and public places.[12,13] Although biomarker-quantified exposure to SHS has been measured among Greek preschool children, to date biomarkers of SHS exposure have never been analysed and quantified for Greek adolescents.[14] Taking the above into account, the aim of this study was to document and quantify the extent of SHS exposure, among a representative sample of Greek adolescents, using their serum cotinine and nicotine levels.

Methods

Study design – setting and participants

The HELENA study (Healthy Lifestyle in Europe by Nutrition in Adolescence) is an European Union–funded multi-centre collaborative study conducted among European adolescents. The core of the HELENA project study material is an overall European cohort of 3000 adolescents, equally recruited in ten cities from nine countries. The basic objective of the HELENA project is to obtain reliable and comparable data from a random sample of European adolescents on a broad battery of relevant nutrition and health-related parameters: dietary intake, food choices and preferences, anthropometry, serum indicators of lipid metabolism and glucose metabolism, vitamin and mineral status, immunological markers, physical activity, fitness and genetic markers. Furthermore, sub-studies were performed such as the HELENA-CSS (HELENA-cross-sectional) sub-study on which the results presented in this paper were obtained from. Further information on the HELENA study procedures and methodology can be found elsewhere.[15,16]

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 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).

The study was approved by the Research Ethics Committees of each city involved, while written informed consent was obtained from the parents of the adolescents and the adolescents themselves. Demographic and descriptive statistics of the study participants, such as age, gender, nationality, family status, parental educational level and economical status were collected from the participating adolescents as part of the HELENA-CSS study procedure. Information on current smoking status, habits and experimentation were also collected, while blood samples were collected from a random sample of 111 adolescents (of the 142 randomly selected, 78.2%) of the HELENA participants from Heraklion. Early morning venous blood was drawn from the participants after a 12-hour overnight fast, subsequently centrifuged and stored at –45°C until taken to the Centre of Toxicology Science and Research, of the University of Crete, for nicotine and cotinine analysis.

Non-smokers were defined as those who had answered that they have not smoked a cigarette in the past month and whose cotinine levels were below the cut-off level of 15 ng/mL. Initially, 106 cotinine/nicotine analyses were performed of which 6 were classified as smokers (due to cut-off for smokers of cotinine>15 ng/mL), and an additional 5 were also classified as smokers due to their self-report that they smoke occasionally, leaving 95 samples. Questionnaire data was incomplete for seven classified non-smokers, while only two non-smokers had cotinine levels below the level of quantification (LOQ) of 0.1 ng/mL. Differences 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, $p = .352$) nor gender (males vs. females, $p = .223$).

Standards and reagents

The reagents used in the toxicological analysis were the isomers (-)-nicotine and (-)-cotinine, which were obtained from Sigma (Sigma-Aldrich Co., St. Louis MO). Ammonia 25%, isoamylalcohol, dichloromethane and buffer solution ready for use pH 6.88 were obtained from Merck (Merck, D-6100 Darmstadt, FR Germany). The solid phase extraction (SPE) columns Discovery DSC-18, 1 mL tubes, 100 mg, were provided by Supelco (Supelco, Bellefonte, PA). Isopropanol and methanol (analytical grade) were obtained from Labscan (Labscan limited,

Dulbin, Ireland) and Scharlau (Scharlau hemie S.A. La Jota, Barcelona, Spain), respectively.

Preparation of standard curves

Nicotine and cotinine stock solutions at a concentration of 100 ppm were prepared in methanol, while dilutions in methanol (working solutions) were prepared weekly in order to cover the range of nicotine and cotinine concentrations expected in serum. All stocks were stored at –20°C, while working solutions were stored at 0°C. The sample analysis methodological approach was the same used in previous research protocols by the research team and can be found in detail elsewhere.[14]

Sample preparation

An aliquot (1 mL) of each serum sample was mixed with 1 mL of buffer solution pH 6.88, respectively. The SPE columns were preconditioned with 2 mL of methanol and 2 mL of buffer pH 6.88. The mixture of biological sample and buffer spiked with 100 μL of ketamine (IS; 0.1 ppm) was passed through the conditioned SPE columns at a flow rate of 1 mL/min by applying vacuum. The columns were rinsed with 2 mL high-pressure liquid chromatography (HPLC) water and dried under full vacuum for 5 min. The analytes were eluted with 2 mL dichloromethane-isopropanol-isoamyalcohol-ammonia (96/2/2/0.4% v/v). The organic phase was dried under steam of N_2 and reconstituted in 50 μL of methanol.

Apparatus

Electron ionization mass spectrometric confirmatory analysis was performed on a Shimadzu QP2010 system equipped with a BPX5 (30 m × 0.25 mm ID × 0.25 μm; SGE forte) capillary column and with an autosampler (AOC-5000, Shimadzu, Kyoto, Japan). Pure helium (99.999%) was used as a carrier gas with a column flow 1.5 mL/min. Two microliter of each sample was injected into the system in the splitless mode. Analysis conditions were as follows: the column temperature programme started from 70°C for 2 minutes and was raised to 300°C at the rate of 20°C/min.

Full scan gas chromatography–mass spectrometry (GC-MS) chromatograms were obtained by scanning from m/z 40 to 600 with a scan time of 0.5 s. Quantitative analysis was accomplished in selected ion monitoring mode with a scan time of 0.2 s, using the following fragment ions m/z = 84, 162 for nicotine, m/z = 98, 176 for cotinine and m/z = 180, 209 for ketamine. Under these conditions, nicotine eluted at 7.65 min, cotinine at 10.04 min and ketamine at 10.98 min.

Statistical analysis

All analyses were stratified for non-smokers. All p values are based on two-sided tests and a significance level of 5% was acknowledged. The normality of the distribution of cotinine and nicotine levels was examined with the Kolmogorov-Smirnov test and the use of QQ plots, and subsequently log transformed. Continuous variables are presented as mean ± standard deviation, while qualitative variables were depicted with the use of frequencies. During the investigation into the possible factors that influence the adolescents' level of SHS exposure initially, two-sided Student's t-tests were performed, while bivariate correlations and two linear regression analyses followed. Specifically, two regression models were developed due to the difference in available data. Model 1 ($n = 86$) included the participants gender and age and parity, as also the parents nationality and educational status. Model 2 ($n = 50$) additionally included data on maternal and paternal smoking habits as also the existence of a younger or elder brother. Additionally, analysis of variance (ANOVA) tests were also applied so as to investigate the relationship between educational status and SHS exposure. The statistical analysis was completed with the statistical package SPSS 16.0 (Statistical Package for Social Sciences, SPSS, Inc, IL).

Results

Greek adolescents' exposure to SHS

In addition to the eight adolescents who were self-reported current smokers (at least one cigarette during the last month), the cotinine analysis revealed another four participants with cotinine levels above that of the cut-off, with serum cotinine levels of 28.86 ng/mL, 38.54 ng/mL, 132.61 ng/mL and 290.97 ng/mL.

Taking self-reported smoking status into account, and after excluding the participants whose cotinine levels were above those of the cut-off between non-smokers and smokers, 97.7% (86/88) of non-smoking Greek adolescents had measurable levels of exposure to SHS, revealing the extent of youth exposure to SHS. The participants' mean cotinine and

Table 1. Characteristics of serum cotinine and nicotine levels among non smoking Greek adolescents[a]

		Gender		
		Boys	Girls	Total
Cotinine (ng/mL)	N	43	45	88
	Mean (±SD)	1.70 (2.36)	1.51 (2.02)	1.60 (2.18)
	Minimum	LOQ	LOQ	LOQ
	Maximum	14.32	9.91	14.32
	25th percentile	0.52	0.25	0.34
	50th percentile	0.99	0.69	0.88
	75th percentile	2.09	1.91	2.04
Nicotine (ng/ml)	N	43	45	88
	Mean (±SD)	3.87 (2.95)	5.07 (4.76)	4.48 (4.00)
	Minimum	LOQ	LOQ	LOQ
	Maximum	11.42	24.06	24.06
	25th percentile	1.52	2.56	1.89
	50th percentile	3.07	3.53	3.52
	75th percentile	5.52	5.68	5.54

LOQ, level of quantification (0.1 ng/mL); SD, standard deviation.
[a] Non-smoking classified by both self report and cotinine levels <15 ng/mL.

nicotine levels are shown in Table 1. The mean serum cotinine levels were 1.70 ± 2.36 ng/mL for boys and 1.51 ± 2.02 ng/mL for girls ($p = .349$), averaging 1.60 ± 2.18 ng/mL and ranging from the LOQ (below the LOQ $n = 2$ samples) to 14.32 ng/mL for non-smoker adolescents, when taking the 15 ng/mL cut-off into account. Mean serum nicotine levels were found to average 4.48 ± 4.00 ng/mL (3.87 ± 2.95 ng/mL for boys and 5.07 ± 4.76 ng/mL for girls) and ranged between the LOQ and 24.06 ng/mL.

Levels of SHS exposure according to demographic, descriptive and social factors

Due to the non-normal distribution of the serum cotinine and nicotine levels, the log10-transformed values and geometrical means were calculated. The geometric mean (GM) cotinine level of all participants was 0.88 ng/mL, while the geometric mean nicotine level was estimated at 3.34 ng/mL. Applying a univariate analysis on the calculated geometrical means, the role of different demographic, family and housing characteristics on the biomarker quantified level of SHS exposure was investigated into (Table 2). Cotinine levels were related to a number of characteristics, such as paternal and maternal smoking habits, which were found to be the strongest determinants of SHS exposure (Figure 1). Specifically, children with both parents active smokers had GM cotinine levels four times higher than those whose parents did not smoke (1.61 ng/mL vs 0.44 ng/mL). Paternal and maternal smoking habits separately also had a strong effect on the adolescents' level of exposure to SHS, with higher levels of exposure found among those with fathers (1.28 ng/mL vs 0.47 ng/mL, $p < .001$) and mothers that smoke (1.23 ng/mL vs 0.62 ng/mL, $p = .019$). Adolescent serum cotinine levels were also positively correlated with both the number of maternal and paternal cigarettes smoked per day with a stronger relationship identified for the later ($R^2 = .443$, $p = .001$ and $R^2 = .305$, $p = .031$, respectively)

Geometric mean levels of cotinine were not found to differ significantly according to adolescent gender (male: 0.99 ng/ml vs female: 0.79 ng/mL), age group (>15 years: 1.01 ng/mL vs <15 years: 0.64 ng/mL), maternal or paternal educational status (high school vs university education) or maternal or paternal nationality (Greek vs immigrant). As for the housing characteristics, no statistically significant differences were noted in regard to having their own bedroom or not. Investigating into parity however, having a younger brother or sister was strongly associated with lower SHS exposure (p=0.008), while the number of brothers or sisters did indicate a trend in geometric mean cotinine levels ($p = 0.74$ for no brothers vs >1 brother).

Following the above univariate analysis, two linear multivariate regression analyses were performed so as to identify the factors that cumulatively affect

Table 2. Non-smoking adolescents' serum concentrations of nicotine and cotinine in relation to various demographic, socioeconomic and housing characteristics

		Nicotine			Cotinine		
	N	GM	SD	*p* Value[a]	GM	SD	*p* Value[a]
Total[b]	86	3.34	2.38	–	0.88	3.13	
Gender							
Boys	42	2.87	2.57	.116	0.99	2.97	.349
Girls	44	3.86	2.16		0.79	3.30	
Age group							
<15	60	3.44	2.56	.625	1.01	3.19	.89
>15	26	3.12	1.99		0.64	2.85	
Maternal Education							
School education	66	3.41	2.49	.887	0.88	3.17	.998
Higher education	20	3.30	2.01		0.88	3.19	
Paternal Education							
School education	66	3.30	2.51	.638	0.94	3.00	.271
University education	18	3.68	1.96		0.67	3.76	
Maternal Nationality							
Greek	79	3.25	2.43	.343	0.92	3.10	.299
Non Greek	6	4.63	1.84		0.56	3.78	
Paternal Nationality							
Greek	78	3.23	2.44	.336	0.91	3.14	.348
Non Greek	6	4.63	1.84		0.57	3.72	
Housing characteristics							
Shared bedroom	38	3.29	2.01	.810	0.81	2.96	.367
Own bedroom	44	3.45	2.77		1.02	3.27	
Paternal Smoking[c]							
No	23	2.72	2.43	.291	0.47	2.47	<.001
Yes	28	3.67	3.01		1.28	2.55	
Maternal Smoking[c]							
No	31	3.47	2.40	.523	0.62	2.82	.019
Yes	20	2.88	3.39		1.23	2.51	
Parental Smoking							
None	18	2.70	2.56	.874	0.44	2.44	<.001
Both parents	20	2.88	3.86		1.61	2.05	
Other smoker in house[c]							
No	43	3.16	2.88	.878	0.83	2.88	.676
Yes	6	3.31	1.82		0.65	3.24	
Other children in the family[c]							
Elder brother/sister	20	3.09	2.09	.795	0.63	2.88	.300
None	24	3.39	3.55		0.89	2.88	
Younger brother/sister	21	2.14	3.16	.018	0.51	2.69	.008
None	20	4.47	2.04		1.12	2.24	
Number of brothers/sisters							
0	14	4.47	2.23	.239	1.48	2.71	.74
>1 in total	68	3.31	2.39		0.82	3.14	
1	37	3.40	2.72	.482	0.78	2.93	.188
2+	31	3.20	2.01		0.87	3.46	

GM, geometric mean; SD, standard deviation.

[a] *p* Values based on two-sided Student's *t*-tests.

[b] Non smoking adolescents only verified by self report and cotinine levels <15 ng/mL and over the level of quantification 0.1 ng/mL.

[c] Lower number of respondents due to ad hoc information collected.

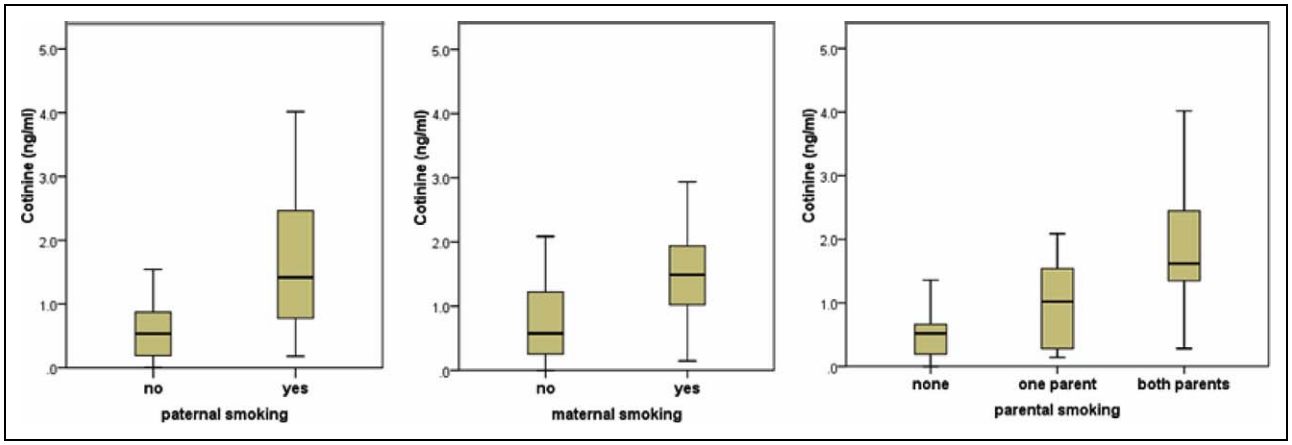

Figure 1. Serum cotinine levels in relation to paternal[1], maternal[2] and parental[3] smoking status.[4]
1, statistically significant difference ($p < .001$); 2, statistically significant difference ($p = .019$); 3, statistically significant difference ($p < .001$); 4, presented as actual serum concentrations but statistically tested using geometrical means and two-sided Student's *t*-tests.

adolescent exposure to SHS. In Model 1 ($n = 86$), the regression analysis revealed that the participants age (standardised B coefficient = –0.266; $p = .019$), and the existence of other brothers and sisters were the main determinants of SHS exposure during adolescence (standardised B coefficient = –0.260, $p = .025$). A trend for their mothers nationality to play a role in SHS exposure was also noted (standardised B coefficient = –0.215; $p = .064$), but did not reach a level of statistical significance. In Model 2, the fathers educational status and smoking habits were noted as significant factors ($p < .001$ and $p = .037$, respectively).

Discussion

The majority of the non-smoking adolescents of our sample (97.7%) had detectable levels of cotinine indicating that the majority of Greek adolescents are exposed to SHS, with the parental smoking habits, the adolescents' age and the existence of a younger brother or sister found to be significant modifiers of exposure.

According to the Greek Global Youth Tobacco Survey (GYTS) data (representative of all Greek adolescents), 89.8% of 13-15 year olds are exposed to SHS at home, while 94.1% are exposed to SHS in public places, numbers that our results verify with the use of biomarkers. The sample selection of both the Heraklion HELENA participants and the sub-cohort from which blood samples were collected allow us to confidently generalize our results to adolescents in Greece in general and are in line with the representative results of the GYTS Greek study.[17]

In comparison with Greek preschool children of Crete, Greek adolescents were found on average to have three times higher serum cotinine levels than those of preschool children of non-smoking parents but almost half the level found in preschool children of smoking parents. Although Greek girls of preschool age have been found to have much higher levels of SHS exposure in comparison to preschool boys, gender difference were not noticed among Greek adolescents.[14] In comparison to NHANES III data among adolescents of the same age group, geometric mean levels of cotinine were not found not to differ greatly (0.88 ng/mL for Greek adolescents vs 0.80 ng/mL for US adolescents). On the contrary though, the NHANES III data indicated a number of other factors that did influence adolescent serum cotinine levels, such as parental education and the number of rooms in the house, two factors that were not duplicated in our study. These above discrepancies we attribute to the different social determinants of smoking in the United States and Greece and the fact that a significant source of SHS exposure in Greece could possibly be due to smoking in public places, which during the time of the study was still permitted.[18] Other factors that have been correlated with childhood cotinine levels include their gender, maternal smoking characteristics, social status, the presence of a smoker in the household, socio-economical deprivation and the child's age.[19,20] Our results that indicated that serum cotinine and not serum nicotine levels were correlated to a number of parental and family characteristics (with the exclusion of the existence of a younger brother or sister) is a fact that we attribute to the differences in half-life between the two compounds.

Pharmacokinetic models have indicated that the long half-life of cotinine provides much less fluctuation in its concentrations throughout the day when compared with serum nicotine concentrations.[21]

The multivariate analysis revealed that younger adolescents and adolescents with younger brothers/sisters were found to have significantly lower levels of SHS exposure in comparison to their peers, while children with no brothers or sisters had higher cotinine levels in comparison to children who had either 1 or more siblings, with lower cotinine levels found in families with a greater number of children.

Protecting children and adolescents from SHS exposure is a complex and sensitive issue, especially in home environments. Research into the determinants of household smoking restrictions have indicated the existence of children in the home (none vs at least one) as also the age of the youngest children are central predictors of restricting smoking in the household.[22,23] Community counselling, either through parental/health care service meetings or mass media campaigns can play an important role in educating both the family and the adolescent of the dangers of exposure to SHS.[24,25] For instance, smoke-free legislations not only change smoking habits but also lead to a significant reduction in children's level of exposure to SHS, as documented after the implementation of a smoke-free legislation in Scotland, where geometric mean salivary cotinine levels dropped by 39%.[26] Household smoking restrictions are an imperative part of the elimination of SHS exposure, and adolescents' exposure to SHS can be drastically affected by their implementation.[27] In addition to the above, forbidding smoking in the home also can contribute towards a reduced risk of adolescent smoking, even when the parents are active smokers.[28] Conclusively, implementing restrictions on household smoking within Greek communities would possibly be able to play a significant role in minimising the adverse health effects of SHS exposure found among Greek adolescents and also may contribute to reducing the percentage of adolescents that experiment smoking by promoting antismoking attitudes.[3,29]

The central sampling framework of the HELENA study allows us with a certain level of certainty to generalize our results to Greek adolescents of that age group who live in urban centres like the city of Heraklion. In addition to the above, the use of the tobacco-specific biomarker cotinine allows us to confidently quantify SHS exposure among Greek adolescents. Despite the above, further research is warranted so as to further investigate into additional socio-demographic and lifestyle habits that influence SHS exposure among Greek adolescents, among larger population-based samples. For example, air monitoring results in Greece have indicated that public places are heavily polluted with SHS and therefore it would be of interest to investigate into the relationship between the adolescents SHS exposure in public venues and their serum cotinine levels.

Conclusively, almost all Greek adolescents are exposed to SHS daily, as detected by our biomarker analysis, with younger adolescents found to have higher levels of exposure in comparison to their elder peers. Taking the above biomarker assessments and existing scientific literature into account, comprehensive smoke-free laws are an imperative step towards eradicating exposure to SHS among Greek adolescents.

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Declaration of Conflicting Interests

There is no conflict of interest regarding the content of this paper, nor among the authors contributing to it.

Appendix

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

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