Environmental Research 143 (2015) 170–176

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

# Outdoor air pollution exposures and micronuclei frequencies in lymphocytes from pregnant women and newborns in Crete, Greece (Rhea cohort)

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ARTICLE INFO

*Article history:*
Received 22 June 2015
Received in revised form
13 October 2015
Accepted 13 October 2015
Available online 21 October 2015

*Keywords:*
Micronucleus assay
Air pollution
Mother–child cohorts

ABSTRACT

*Background:* Micronuclei (MN) are biomarkers of early genetic effects that have been used to investigate the association between environmental exposures and cancer. However, few studies have examined the association between environmental exposures during pregnancy and MN in mothers and newborns.
*Objectives:* We examined MN frequency in maternal blood and in cord blood, in relation to maternal air pollution exposure, and the potential interaction with maternal vitamin C intake and maternal smoking.
*Methods:* We used the cytokinesis-block micronucleus assay to assess MN frequency per 1000 bi-nucleated T-lymphocytes from 181 mothers and 183 newborns born in 2007–2008 in Heraklion (Crete, Greece). The ESCAPE land-use regression methods were used to estimate annual mean exposure to outdoor air pollution [particulate matter (PM), black carbon, nitrogen dioxide ($NO_2$) and nitrogen oxides ($NO_x$)] at maternal home addresses. Food frequency questionnaires were used to estimate maternal dietary vitamin C intake during pregnancy. Smoking habits were self-reported using questionnaires which were checked by measuring maternal urinary cotinine levels.
*Results:* Exposure to $PM_{2.5}$ was associated with increased MN frequencies in pregnant women [rate ratio [RR (95%CI)] per 5 $\mu g/m^3$ = 1.53 (1.02, 2.29)]. This increase was considerably higher among women who did not fulfill the recommended vitamin C dietary allowances [RR = 9.35 (2.77, 31.61); $n$ = 20]. Exposure to $PM_{2.5-10}$, $PM_{10}$, $NO_2$ and $NO_x$ were also associated with a higher incidence of MN frequencies in smoker women ($n$ = 56). No associations were found for newborns.
*Conclusions:* We found an association between air pollution, particularly $PM_{2.5}$, and MN frequency in mothers but not in newborns. This association was more pronounced among women with a lower dietary intake of vitamin C during pregnancy and among women who smoked during pregnancy. While

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http://dx.doi.org/10.1016/j.envres.2015.10.011
0013-9351/

results are clear in mothers, the association between maternal carcinogenic exposures during pregnancy and biomarkers of early biologic effect in the newborn remains poorly understood.


## 1. Introduction

Outdoor air pollution has been evaluated by the International Agency for Research on Cancer (IARC) Monographs Programme as carcinogenic to humans (Group 1). The IARC evaluation showed an increasing risk of lung cancer with increasing levels of exposure to particulate matter and air pollution (Loomis et al., 2013). Whether air pollution may affect other cancers or cancer in susceptible populations is unknown. Cancer incidence among European children has been steadily increasing in the last decades (Kaatsch, 2010) but causes of this increase are unknown. Maternal exposures before and during pregnancy may modulate gene expression in the fetus, which might lead to long-lasting changes that influence health outcomes during childhood and adulthood (Sly and Carpenter, 2012).

The NewGeneris research project (Newborns and Genotoxic exposure Risk; https://www.newgeneris.org/) was conducted to test the hypothesis that maternal intake of dietary carcinogens results in in utero exposure and early biological effects in the unborn child, possibly leading to increased risk of cancer in later childhood (Merlo et al., 2009). Results from the NewGeneris analysis showed that newborns from Crete, Greece (Rhea Mother–Child cohort), presented the highest mean frequencies of micronuclei (MN) in cord blood compared with newborns from other European countries (Merlo et al., 2014). MN are small extra-nuclear bodies that result from acentric chromosome/chromatid fragments or whole chromosomes/chromatids that lag behind in anaphase stage and are not included in the daughter nuclei in telophase stage (Kirsch-Volders et al., 2014). MN indicate genome damage and MN frequency in peripheral blood lymphocytes had been associated with increased risk of cancer in adults (Bonassi et al., 2007). We have previously reported association of water disinfection by-products and reproductive factors in relation to MN frequency in the Rhea Mother–Child cohort (Stayner et al., 2014; Vande Loock et al., 2011). Here we analyze the association between maternal outdoor air pollution exposure during pregnancy and MN frequency, considering potential interactions with maternal vitamin C intake and smoking.

## 2. Methods

### 2.1. Study population

The present study included mothers and newborns from the Rhea Mother–Child cohort (Heraklion, Crete, Greece) described elsewhere (Chatzi et al., 2011). The first contact with potential participants was made at around 10th–13th weeks of pregnancy, at the time of the first ultrasound examination. Briefly, women who became pregnant during February 2007–February 2008 in the prefecture of Heraklion were asked to participate in the study. Women were eligible to participate in the study if they were residents of the study area, other than 16 years of age, visiting a participating hospital or private clinic during the 10th–13th week of gestation, and did not have communication limitations. Participating women were contacted again during the 14th–18th, 28th–32nd weeks of pregnancy and at birth. During recruitment, 1610 eligible women agreed to participate, and 1459 (91%) were followed through delivery. A random subset of 408 participants, among pregnancies with no complications such as preterm births, donated maternal and/or cord blood for a wide range of biomarkers measurement as part of the NewGeneris study. 232 samples were randomly selected for MN measurement (Merlo et al., 2009). Mothers and newborns with MN analysis in maternal and cord blood lymphocytes, from singleton pregnancies and data on outdoor air pollution at maternal home addresses were considered for this study. Overall, 181 mothers and 183 newborns were included. Of these, 136 were mother–child pairs.

The Ethics Committee of the University Hospital in Heraklion, Greece, approved the study and all participants provided written informed consent.

### 2.2. Exposures assessment

We used face-to-face structured questionnaires, self-administered questionnaires and medical records to obtain demographical information on mothers and newborns (Chatzi et al., 2011). Maternal home address was recorded around 10th–13th weeks of gestation (during first visit). Around 14th–18th weeks of gestation (during second visit), validated food frequency questionnaires (FFQ) were administered to collect information on diet during pregnancy (Chatzi et al., 2011). Vitamin C intake was derived from information recorded in FFQ using food composition tables (McCance and Widdowson's, 1991). During the same visit, self-reported information on active smoking (nonsmoker/ex-smoker/active smoker) and passive smoking (at home and work place) was collected. We also collected urine samples to measure cotinine levels and we used the 100 ng/ml cut-off value, previously defined for this study population (Vardavas et al., 2011), to differentiate smokers from passive-smokers/non-smokers. Exposure to second hand smoke in Crete is much higher than in other populations and therefore a specific cut-off value had to be defined for the Rhea cohort. Distribution of cotinine in the Rhea cohort led to two very distinct groups, with a large gap between them. The 100 ng/ml cut-off differentiated well these two groups.

Outdoor air concentrations of particulate matter (PM) with an aerodynamic diameter below 2.5 μm ($PM_{2.5}$), between 2.5 μm and 10 μm ($PM_{2.5-10}$, coarse PM), below 10 μm ($PM_{10}$), $PM_{2.5}$ absorbance (a measure of black carbon), nitrogen dioxide ($NO_2$) and nitrogen oxides ($NO_x$) were measured between February 2009 and 2010 in Heraklion. Measures were collected in 40 different sites during two weeks period in three different seasons (cold, warm and intermediate)(Cyrys et al., 2012). Measured average concentrations were combined with geographic predictors to develop land use regression (LUR) models following the protocols developed as part of the European Study of Cohorts for Air Pollution Effects (ESCAPE: http://www.escapeproject.eu) (Beelen et al., 2013; Eeftens et al., 2012). Annual mean concentrations between February 2009 and 2010 were estimated at maternal home address assuming that annual mean estimations are stable from year to year (Cesaroni et al., 2012; Eeftens et al., 2011; Madsen et al., 2011).

### 2.3. Outcome assessment

We collected cord blood immediately after delivery and maternal peripheral blood no later than 24 h after delivery for MN quantification. We carried out the cytokinesis-block micronuclei (CBMN) assay using whole blood according to the standardized protocol for the semi-automated image analysis system (Decordier et al., 2009; Stayner et al., 2014; Vande Loock et al., 2011). We recorded the total numbers of bi-nucleated T lymphocytes (i.e. T lymphocytes that have divided once) with MN and the total number of T lymphocytes counted for each participant, and we calculated MN frequency per 1000 bi-nucleated T lymphocytes. We scored a minimum of 2500 T lymphocytes per slide.

### 2.4. Statistical analyses

We generated descriptive analysis of the study population characteristics, exposure and outcome variables. Negative binomial regression models were used to assess the association between air pollution exposures and MN frequency. A natural log link was used and the model included the total number of cells scored as an offset. We estimated rate ratios and 95% confidence intervals [RR (95%CI)] using robust standard errors (Stayner et al., 2014).

To control for potential confounding effects we included in the regression models *a priori* covariates selected using Directed Acyclic Graphs (DAGs). We adjusted maternal models for maternal age at delivery (years), residence in an urban area (no/yes), education (low: $\leq 9$ years of education; medium: 10–14 years of education; high: University degree or higher), origin (Greek/non Greek) and season of delivery (winter: December–February; spring: March–May; summer: June–August; autumn: September–November). We adjusted newborns models for gestational age (completed weeks), maternal education, maternal residence and season of birth. All estimates presented correspond to adjusted models. Statistical interaction was assessed by fitting models with cross-product terms and testing their significance using the Wald test.

All potential confounders except sex, gestational age and season of birth had missing values: the percentage of missing values ranged from 0.4 (birth weight and pre-pregnancy BMI) to 2.6 (maternal active smoking). In order to increase efficiency and minimize selection bias, we applied multiple imputation methods as previously described (Stayner et al., 2014). We conducted all adjusted analyses using imputed covariates. Analyses using non-imputed data gave similar results (not shown).

Statistical analyses were done using Stata version 12.1 (StataCorp, College Station, TX, USA).

## 3. Results

### 3.1. Study population characteristics

Participating mothers had a mean ($\pm$SD) age of $29.7 \pm 5.0$ years, were mainly of Greek origin (87%), lived in urban areas (72%) and about half of them had medium educational level (54%). 8% of women moved home during pregnancy. Median (interquartile range – IQR) fruit and vegetable intake during pregnancy was 422.4 (339.6) and 202.9 (135.7) g/day respectively, and median vitamin C dietary intake 172.2 (136.5) mg/day. 16% of women did not reach the 85 mg vitamin C/day recommended during pregnancy (Institute of Medicine, Food and Nutrition Board, 2001). 84% of women took folate supplements and reached the 5 μg/day recommended during pregnancy (Institute of Medicine, Food and Nutrition Board, 2001). 30% of women self-reported active smoking during pregnancy. For 59% of the women we had urinary cotinine levels available which allowed us to check the consistency of self-reported smoking: 2% of women reporting non-smoking during pregnancy had cotinine levels $>100$ ng/ml. Half of newborns were boys (53%), mean gestational age was $38.4 \pm 1.3$ weeks and mean birth weight $3.225 \pm 412$ g. The prevalence of preterm birth was 6%. 4% of children were born with low birth weight ($<2.5$ kg) and 3% with high birth weight ($>4$ kg). In our study population there was a higher percentage of non-Greek mothers (13% versus 8% respectively, $p$-value$=0.037$) and higher percentage of mothers living in rural areas (28% versus 21%, $p$-value$=0.037$) than in the overall Rhea cohort. Distribution of season on deliveries/births was different in the population included in this analysis that in the overall cohort ($p$-value $<0.001$). There was a higher proportion of deliveries/births during winter and spring in the study population that in the overall cohort. The percentage of preterm births was lower than in the overall cohort (6% versus 12%, $p$-value$=0.006$), as mothers with complications during labor could not be approached to collect biological samples because the research protocol was too demanding. No other differences were detected between our study population and the entire cohort.

### 3.2. MN frequency in maternal and cord blood

The median MN frequency was 2.63‰ (2.53) in the 181 maternal blood samples and 1.48‰ (1.83) in the 183 cord blood samples. There was no correlation between mother and newborn MN frequency based on the 136 paired samples (Spearman correlation coefficient$=0.14$, $p$-value$=0.104$). There were differences in MN frequency median levels among mothers by maternal age, educational level, origin, season of delivery and sex of the child. In newborns differences were observed by season of birth (Table 1).

### 3.3. Exposure to outdoor air pollution and MN frequency

The estimated median concentrations of $PM_{2.5}$, $PM_{2.5-10}$, $PM_{10}$, $PM_{2.5}$ absorbance, $NO_2$ and $NO_x$ were 14.4 (1.3) $\mu g/m^3$, 22.5 (3.0) $\mu g/m^3$, 37.0 (3.0) $\mu g/m^3$, 1.1 $(0.3) \times 10^{-5}$ per m, 12.2 (4.1) $\mu g/m^3$ and 19.5 (9.6) $\mu g/m^3$, respectively.

Higher exposure to $PM_{2.5}$ was associated with elevated MN frequency in maternal blood. A 53% increase in maternal MN frequency was detected for each 5 $\mu g/m^3$ increment of $PM_{2.5}$ levels (Table 2). No other statistically significant associations between air pollution and MN frequency in maternal blood were found (Table 2). $PM_{2.5}$ was associated with MN frequency only among women with vitamin C intake below recommended values during pregnancy ($<85$ mg/day). Statistically significant interaction with vitamin C was found for $PM_{2.5}$ but not for other air pollutants (Table 3). Air pollutants were not associated with MN frequency among non-smoker women but were strongly associated in women who smoked during pregnancy. Statistically significant interactions were found for $PM_{2.5-10}$, $PM_{10}$, $NO_2$ and $NO_x$ (Table 4). Association between smoking and MN frequency did not change after excluding non-smoker women (self-reported) with cotinine levels $>100$ ng/ml.

We did not find statistically significant associations between air pollutants and MN frequency in cord blood (Table 2). Interaction with vitamin C intake was observed for $PM_{10}$ (Table 3): decreased MN frequency was observed at increasing exposure to $PM_{10}$ among newborns from mothers with the lowest levels vitamin C intake. A similar pattern was observed for $PM_{2.5}$ absorbance but results did not reach statistical significance values (Table 3). No interaction with smoking was observed in MN frequency in cord blood (Table 4).

**Table 1**
Characteristics of study subjects and median (IQR) MN frequency (MN per 1000 bi-nucleated T lymphocytes) in maternal and cord blood samples.

| Characteristics of mothers and newborns | Maternal blood ($n=181$) | | | Cord blood ($n=183$) | | |
|---|---|---|---|---|---|---|
| | *n* (%) | Median | IQR | *n* (%) | Median | IQR |
| Maternal age (years) | | | | | | |
| < 35 | 149 (82) | 2.2* | 2.5 | 158 (86) | 1.5 | 1.9 |
| ≥ 35 | 30 (17) | 3.5 | 3.3 | 25 (14) | 1.8 | 1.6 |
| Missing | 2 (1) | 2.2 | 2.5 | | | |
| Residence | | | | | | |
| Urban | 128 (71) | 2.9 | 2.5 | 129 (70) | 1.5 | 1.8 |
| Rural | 48 (27) | 2.1 | 2.5 | 51(28) | 1.5 | 1.8 |
| Missing | 5 (3) | 2.0 | 1.4 | 3 (2) | 0.8 | 2.0 |
| Maternal education | | | | | | |
| Low ( ≤ 9 years) | 43 (4) | 1.6* | 2.5 | 43(23) | 1.7 | 1.9 |
| Medium (10–14 years) | 99 (55) | 2.4 | 2.7 | 98 (54) | 1.4 | 1.9 |
| High (University degree or higher) | 38 (21) | 3.5 | 1.8 | 41(22) | 1.6 | 1.0 |
| Missing | 1 (0) | 1.8 | 0.0 | 1 (1) | 0.0 | 0.0 |
| Ethnic origin | | | | | | |
| Greek | 153 (85) | 2.9* | 2.5 | 161 (88) | 1.5 | 1.9 |
| Others | 26 (14) | 1.8 | 2.6 | 21 (11) | 1.6 | 0.8 |
| Missing | 2 (1) | 3.1 | 4.8 | 1 (1) | 0.7 | 0.0 |
| Maternal active smoking | | | | | | |
| No | 116 (64) | 2.7 | 2.5 | 126 (69) | 1.5 | 1.8 |
| Yes | 56 (31) | 2.5 | 2.8 | 49 (27) | 1.4 | 1.9 |
| Missing | 9 (5) | 1.6 | 1.8 | 8 (4) | 1.4 | 1.3 |
| Maternal passive smoking[a] | | | | | | |
| No | 17 (15) | 3.0 | 2.2 | 13 (10) | 1.5 | 0.7 |
| Yes | 97 (83) | 2.4 | 2.5 | 111 (88) | 1.5 | 1.9 |
| Missing | 2 (2) | 4.0 | 0.5 | 2 (2) | 2.2 | 4.3 |
| Cotinine levels (ng/ml) | | | | | | |
| ≤ 100 | 82 (45) | 3.2 | 2.9 | 94 (51) | 1.5 | 1.9 |
| > 100 | 16 (9) | 2.0 | 2.9 | 14 (8) | 1.1 | 1.2 |
| Missing | 83 (46) | 2.2 | 2.4 | 75 (41) | 1.5 | 1.8 |
| Pre-pregnancy BMI ($kg/m^2$) | | | | | | |
| Normal ( ≥ 18.5 to ≤ 25) | 115 (64) | 2.4 | 2.5 | 106 (58) | 1.5 | 1.7 |
| Underweight ( < 18.5) | 13 (7) | 3.0 | 3.2 | 13 (7) | 1.5 | 1.9 |
| Overweight ( < 25) | 35 (19) | 2.9 | 2.5 | 37 (20) | 1.4 | 2.1 |
| Obese ( < 30) | 17 (9) | 2.9 | 2.4 | 26 (14) | 1.4 | 1.8 |
| Missing | 1(1) | 1.8 | 0.0 | 1 (1) | 0.0 | 0.0 |
| Vegetables intake (g/day) | | | | | | |
| < 16–150 | 40 (22) | 2.2 | 2.8 | 41 (22) | 1.3 | 1.3 |
| ≥ 150–248 | 48 (27) | 2.6 | 3.0 | 39 (21) | 1.5 | 2.0 |
| > 248 | 42 (23) | 2.9 | 2.2 | 47 (26) | 1.4 | 1.3 |
| Missing | 51 (28) | 3.0 | 2.5 | 56 (31) | 1.8 | 2.2 |
| Fruit intake (g/day) | | | | | | |
| < 0–315 | 40 (22) | 3.0 | 2.5 | 46 (25) | 1.1 | 1.1 |
| ≥ 315–545 | 49 (27) | 2.2 | 2.5 | 44 (24) | 1.3 | 1.7 |
| > 545 | 41 (23) | 2.9 | 2.8 | 37 (20) | 1.5 | 1.1 |
| Missing | 51 (28) | 3.0 | 2.5 | 56 (31) | 1.8 | 2.2 |
| Vitamin C intake (mg/day) | | | | | | |
| < 85 | 20 (11) | 2.4 | 2.4 | 22 (12) | 1.1 | 1.3 |
| ≥ 85 to < 170 | 41(23) | 3.1 | 3.2 | 43 (24) | 1.4 | 2.0 |
| ≥ 170 | 69 (38) | 2.4 | 2.6 | 62 (33) | 1.4 | 1.3 |
| Missing | 51 (28) | 3.0 | 2.5 | 56 (31) | 1.8 | 2.2 |
| Folate supplements (μg/day) | | | | | | |
| < 5 | 23 (13) | 2.4 | 1.8 | 23 (13) | 1.5 | 1.5 |
| 5 | 114 (63) | 2.4 | 2.9 | 110 (60) | 1.5 | 1.8 |
| > 5 | 37 (20) | 3.1 | 2.4 | 41 (22) | 1.2 | 1.5 |
| Missing | 7 (4) | 1.7 | 2.2 | 9 (5) | 2.8 | 3.5 |
| Sex | | | | | | |
| Male | 95 (52) | 2.9* | 3.1 | 98 (54) | 1.3 | 1.4 |
| Female | 86 (48) | 2.3 | 2.3 | 85 (46) | 1.7 | 1.8 |
| Gestational age (weeks) | | | | | | |
| < 37 | 12 (7) | 1.5 | 2.1 | 10 (5) | 1.3 | 1.2 |
| ≥ 37 | 169 (93) | 2.7 | 2.5 | 173 (95) | 1.5 | 1.8 |
| Birth weight (kg) | | | | | | |
| Normal ( > 2.5 to < 4) | 167 (92) | 2.7 | 2.6 | 172 (94) | 1.5 | 1.8 |
| Low ( ≤ 2.5) | 8 (5) | 2.2 | 1.7 | 6 (3) | 1.4 | 1.6 |
| High ( ≥ 4) | 6 (3) | 1.2 | 2.5 | 4 (2) | 1.7 | 0.6 |
| Missing | | | | 1 (1) | 0.6 | 0.0 |
| Season of birth (months) | | | | | | |
| Winter (Dec–Feb) | 69 (38) | 2.9* | 2.2 | 64 (35) | 1.4* | 1.3 |
| Spring (Mar–May) | 57 (31) | 3.1 | 2.4 | 72 (39) | 1.6 | 1.8 |
| Summer (Jun–Aug) | 23 (13) | 4.0 | 2.7 | 26 (14) | 3.0 | 4.0 |
| Autumn (Sep–Nov) | 32 (18) | 1.5 | 1.5 | 21 (12) | 0.8 | 1.0 |

* *p*-Values based on Kruskal–Wallis test < 0.05.
[a] Only non-smoker women considered.

**Table 2**
Association between air pollution exposure during pregnancy and rate ratio [RR (95%CI)] of MN frequency in maternal and cord blood samples.

| Air pollutants | Maternal blood ($n=181$)[a] | Cord blood ($n=183$)[b] |
|---|---|---|
| | RR (95%CI) | RR (95%CI) |
| $PM_{2.5}$ (5 μg/m$^3$) | 1.53 (1.02, 2.29) | 0.97 (0.63, 1.50) |
| $PM_{2.5-10}$ (5 μg/m$^3$) | 1.14 (0.94, 1.38) | 0.96 (0.79, 1.17) |
| $PM_{10}$ (10 μg/m$^3$) | 1.16 (0.82, 1.64) | 1.14 (0.79, 1.65) |
| $PM_{2.5}$ absorbance ($10^{-5}$ per m) | 0.85 (0.58, 1.25) | 0.85 (0.55, 1.34) |
| $NO_2$ (10 μg/m$^3$) | 1.08 (0.84, 1.40) | 1.07 (0.79, 1.45) |
| $NO_x$ (20 μg/m$^3$) | 1.09 (0.88, 1.34) | 1.11 (0.83, 1.48) |

[a] Maternal models adjusted for maternal age at delivery, maternal education, maternal residence, maternal origin and season of delivery.
[b] Cord models adjusted for gestational age, maternal education, maternal residence and season of birth.

## 4. Discussion

This is the largest single country cohort study that has assessed air pollution exposure during pregnancy in association with MN frequency in both mothers and newborns. We found an association between $PM_{2.5}$ and MN frequency in mothers, particularly among those that did not fulfill the 85 mg/day recommended vitamin C allowances during pregnancy (Institute of Medicine, Food and Nutrition Board, 2001). Among women smoking during pregnancy, increased MN frequency was also associated with $PM_{10}$, $NO_2$ and $NO_x$. No association was detected for newborns.

Long-term exposure to ambient air pollution and PM is associated with lung cancer in humans (Loomis et al., 2013). The association between ambient air pollution exposure and MN is also quite clear in adults (DeMarini, 2013), but results in children are less conclusive (Demircigil et al., 2014; Huen et al., 2006; Neri et al., 2006; Pedersen et al., 2006). There are few studies that have focused on maternal exposure to air pollution during pregnancy and MN in the newborn (Pedersen et al., 2009; Rossnerova et al., 2011). In contrast to our results, these studies found some association between air pollution and MN in newborns but not in mothers. We used individual-level air pollution estimates, whereas previous studies estimated exposure at area level (comparing cities and areas with different traffic density). Individual estimates may take into account spatial variation better than ecological comparisons.

Our results suggest that vitamin C intake modified the effect of $PM_{2.5}$ on MN frequency: only women who did not reach vitamin C daily recommended allowances during pregnancy ($<85$ ng/day) had increased MN frequency at higher levels of exposure to $PM_{2.5}$. Number of mothers with vitamin C intake lower than recommended in our study population was low ($n=20$) and these results have to be interpreted with caution. However, there is a strong biological rationale that supports the protective effect of vitamin C on MN formation. Some studies have shown that vitamin C, an important natural antioxidant, is associated with reduced MN formation after exposure to mutagens, although other studies have not found such differences (reviewed by Sram et al., 2012). DNA damage associated with exposure to air pollution particles occurs via inflammation and oxidative damage (i.e. production of reactive oxygen species – ROS)(Møller et al., 2014), which is one of the mechanisms known to induce MN formation (Kirsch-Volders et al., 2014). Our results support the hypothesis that vitamin C neutralizes ROS produced after exposure to air pollution particles. Therefore, formation of MN is higher in women exposed to $PM_{2.5}$ with insufficient vitamin C intake. Associations with other air pollutants were not detected. These findings are also in concordance with the fact that $PM_{2.5}$ is the PM fraction most strongly associated with several adverse health effects (WHO, 2013). In vitro studies have also shown increased MN after $PM_{2.5}$ exposure (Lepers et al., 2014). Contrary as we observed in mothers, newborns from mothers who did not reach vitamin C daily recommended allowances during pregnancy ($<85$ ng/day) had lower MN frequency at higher levels of exposure to $PM_{10}$. Number of newborns whose mothers did not reach recommended levels of vitamin C intake during pregnancy was also very low ($n=22$). This might be considered a chance finding, as there is no biological rationale to support the positive association between vitamin C and MN formation.

Among women who smoked during pregnancy, higher levels of $PM_{10}$, $PM_{2.5-10}$ $NO_2$ and $NO_x$ were also associated with increased MN frequency, suggesting a higher vulnerability of this group to the effect of air pollution. Higher vitamin C daily allowances are recommended for smokers (35 mg above recommended values (Institute of Medicine, Food and Nutrition Board, 2001)). Differences in MN frequencies at same levels of exposure to air pollution between smokers and non-smokers could be driven by insufficient vitamin C intake among smokers. A smaller percentage of smoker women (72%) than non-smoker women (85%, $p$-value=0.059) fulfilled the recommended dose of vitamin C, although this difference was not large. Due to the sample size of the study, we were

**Table 3**
Association between air pollution exposure during pregnancy and rate ratio [RR (95%CI)] of MN frequency in maternal and cord blood samples according to daily levels of vitamin C intake (ng/day) during pregnancy.[a]

| Air pollutants | Maternal blood ($n=130$)[b] | | | | Cord blood ($n=134$)[c] | | | |
|---|---|---|---|---|---|---|---|---|
| | $<85$ ($n=20$) | $\geq 85$ to $<170$ ($n=41$) | $\geq 170$ ($n=69$) | $p$-Value* | $<85$ ($n=22$) | $\geq 85$ to $<170$ ($n=43$) | $\geq 170$ ($n=69$) | $p$-Value* |
| | RR (95%CI) | RR (95%CI) | RR (95%CI) | | RR (95%CI) | RR (95%CI) | RR (95%CI) | |
| $PM_{2.5}$ (5 μg/m$^3$) | 5.57 (1.96,15.81) | 1.67 (0.88, 3.17) | 1.02 (0.62, 1.69) | 0.044 | 0.84 (0.18, 3.88) | 0.82 (0.35, 1.89) | 1.55 (0.90, 2.65) | 0.350 |
| $PM_{2.5-10}$ (5 μg/m$^3$) | 1.56 (0.97, 2.5) | 0.97 (0.69,1.37) | 1.05 (0.85, 1.31) | 0.545 | 0.87 (0.52, 1.46) | 1.00 ((0.72, 1.37) | 1.24 (0.95, 1.63) | 0.336 |
| $PM_{10}$ (10 μg/m$^3$) | 0.51 (0.06, 4.36) | 0.9 (0.39, 2.09) | 0.95 (0.62, 1.47) | 0.823 | 0.30 (0.11, 0.83) | 1.36 (0.68, 2.71) | 1.48 (0.84, 2.61) | 0.018 |
| $PM_{2.5}$ absorbance ($10^{-5}$ per m) | 2.08 (0.53, 8.09) | 0.91 (0.49, 1.68) | 0.9 (0.46, 1.78) | 0.844 | 0.68 (0.27, 1.72) | 0.83 (0.26, 2.64) | 2.13 (1.23, 3.69) | 0.064 |
| $NO_2$ (10 μg/m$^3$) | 2.07 (0.65, 6,57) | 0.95 (0.62, 1.45) | 0.9 (0.69, 1.19) | 0.482 | 1.10 (0.35, 3.49) | 1.22 (0.80, 1.84) | 1.58 (1.00, 2.51) | 0.403 |
| $NO_x$ (20 μg/m$^3$) | 1.64 (0.52, 5,15) | 1.05 (0.8, 1.39) | 0.89 (0.68, 1.16) | 0.277 | 1.07 (0.39, 2.92) | 1.23 (0.83, 1.83) | 1.57 (1.11, 2.21) | 0.180 |

* $p$-Value for interaction (Wald test).
[a] Given the smaller sample size in some of the groups, we adjusted these models only for those co-variables with a significant regression coefficient. Unadjusted estimates were also calculated and results were similar.
[b] Maternal models adjusted for maternal age at delivery and season of delivery.
[c] Cord models adjusted for season of birth.

**Table 4**
Association between air pollution exposure during pregnancy and rate ratio [RR (95%CI)] of MN frequency in maternal and cord blood samples according to active smoking during pregnancy.

| Air pollutants | Maternal blood ($n=172$)[a] | | | Cord blood ($n=175$)[b] | | |
|---|---|---|---|---|---|---|
| | Non-smokers ($n=116$) | Smokers ($n=56$) | $p$-Value* | Non-smokers ($n=126$) | Smokers ($n=49$) | $p$-Value* |
| | RR (95%CI) | RR (95%CI) | | RR (95%CI) | RR (95%CI) | |
| $PM_{2.5}$ (5 μg/m$^3$) | 1.42 (0.80, 2.51) | 1.71 (0.95, 3.1) | 0.190 | 0.81 (0.47, 1.41) | 1.32 (0.62, 2.78) | 0.283 |
| $PM_{2.5-10}$ (5 μg/m$^3$) | 1.05 (0.86, 1.29) | 1.39 (0.94, 2.05) | 0.036 | 0.94 (0.75, 1.17) | 1.11 (0.71, 1.75) | 0.384 |
| $PM_{10}$ (10 μg/m$^3$) | 0.93 (0.66, 1.29) | 2.33 (1.16, 4.67) | 0.017 | 1.1 (0.73, 1.66) | 1.27 (0.5, 3.25) | 0.734 |
| $PM_{2.5}$ absorbance ($10^{-5}$ per m) | 0.94 (0.60, 1.48) | 0.75 (0.38, 1.46) | 0.853 | 0.72 (0.4, 1.27) | 1.16 (0.61, 2.21) | 0.249 |
| $NO_2$ (10 μg/m$^3$) | 0.87 (0.69, 1.10) | 1.62 (1.06, 2.47) | 0.001 | 0.95 (0.67, 1.35) | 1.58 (0.83, 2.99) | 0.176 |
| $NO_x$ (20 μg/m$^3$) | 0.86 (0.69, 1.08) | 1.46 (1.08, 1.96) | 0.001 | 0.95 (0.67, 1.35) | 1.43 (0.96, 2.15) | 0.125 |

* $p$-Value for interaction (Wald test).
[a] Maternal models adjusted for maternal age at delivery, maternal education, maternal residence, maternal origin and season of delivery.
[b] Cord models adjusted for gestational age, maternal education, maternal residence and season of birth.

unable to formally assess the effect of vitamin C intake levels on the association between air pollutants and MN frequency in smokers and non-smokers.

The main limitation of our study is that we could not calculate temporally adjusted air pollution estimates as the background station information in Heraklion was insufficient (Pedersen et al., 2015). Estimation of annual mean concentrations might have lead to some exposure misclassification as air pollution levels vary within a year. However, we adjusted all our models for season of delivery/birth, and this might have help to control some of the variability in air pollution estimates. Exposure misclassification might have also affected women who moved during pregnancy as we used maternal home address reported at study enrollment (10th–13th weeks of pregnancy). This misclassification might have had higher effects in newborns, as generation of cord blood lymphocytes occurs mostly at the third trimester (Blackburn, 2007). However, results of sensitivity analysis excluding women who moved during pregnancy indicate that potential misclassification did not bias our results (data not shown). In fact, in mothers, the association between $PM_{2.5}$ and MN was stronger in mothers who did not move during pregnancy than among all study mothers. In cord blood, no association was observed between exposure to air pollution and MN in newborns whose mothers did not move during pregnancy, as observed when all newborns were included. Another limitation of our paper is the lack ozone estimates, as ozone levels have been associated with increased MN (Huen et al., 2006). Ozone measurements were not included in the ESCAPE protocols.

Our study has several strengths and among the main ones are the technique used to measure MN, the quality of exposure assessment and the evaluation of several potential confounders and effect modifiers. MN frequency was available from a relatively large and well characterized sample of maternal blood and cord blood; samples were processed using a pre-defined protocol (Merlo et al., 2009) and fixed cells were examined using a semi-automated image based scoring system that reduced potential variation and subjectivity of manual scoring (Decordier et al., 2009). Air pollution exposures were measured using the ESCAPE models for air pollution that have been previously established and used in large European studies. Information on diet (used to estimate vitamin C intake) was based on 250 food items and for each food item frequency of consumption and average portion was also recorded (Chatzi et al., 2011). Our models were adjusted for several *a priori* and possible confounders, although we cannot completely rule out the effect of residual confounding in our results.

In conclusion, we found an association of $PM_{2.5}$ air pollution with MN frequency in pregnant mothers while no association was detected between pregnancy exposure and MN frequency in newborns. The association of $PM_{2.5}$ with MN was more pronounced in women with insufficient vitamin C intake during pregnancy, while associations between $PM_{10}$, $NO_2$ and $NO_x$ were only detected among women who smoked during pregnancy.

## A competing financial interests declaration

The authors declare they have no actual or potential competing financial interests.

## Acknowledgments

We thank all of the participants of the Rhea study and the interviewers, statisticians and the hospital personnel for their cooperation and contribution to this study. We want to thank Constantine Vardavas for input on the cotinine analysis. This study was supported by the European Union-funded projects NewGeneris (Contract no. FOOD-CT-2005-016320), ESCAPE (FP7-2007-211250), HiWATE (Contract no FOOD-CT-2006-036224) and FAMRI grant (Clinical Investigator Award 072058). C.O.G holds a Sara Borrell postdoctoral fellowship awarded from the Carlos III National Institute of Health (CD13/00072). M.P. held a Juan de la Cierva postdoctoral fellowship awarded from the Spanish Ministry of Ministry of Economy and Competitiveness (JCI-2011-09479).

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