

Association between long-term air pollution exposure and DNA methylation: the REGICOR study.

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ABSTRACT

Introduction: Limited evidence suggests that epigenetic mechanisms may partially mediate the adverse effects of air pollution on health. Our aims were to identify new genomic loci showing differential DNA methylation associated with long-term exposure to air pollution and to replicate loci previously identified in other studies.

Methods: A two-stage epigenome-wide association study was designed: 630 individuals from the REGICOR study were included in the discovery and 454 participants of the EPIC-Italy study in the validation stage. DNA methylation was assessed using the Infinium HumanMethylation450 BeadChip. NOX, NO₂, PM₁₀, PM_{2.5}, PM_{coarse}, traffic intensity and traffic load exposure were measured according to the ESCAPE protocol. A systematic review was undertaken to identify those cytosine-phosphate-guanine (CpGs) associated with air pollution in previous studies and we screened for them in the discovery study.

Results: In the discovery stage of the epigenome-wide association study, 81 unique CpGs were associated with air pollution (p-value <10⁻⁵) but none of them were validated in the replication sample. Furthermore, we identified 15 CpGs in the systematic review showing differential methylation with a p-value fulfilling the Bonferroni criteria and 1673 CpGs fulfilling the false discovery rate criteria, all of which were related to PM_{2.5} or NO₂. None of them was replicated in the discovery study, in which the top hits were located in an intergenic region on chromosome 1 (cg10893043, p-value=6.79·10⁻⁵) and in the *LRRC45* and *PXK* genes (cg05088605, p-value=2.15·10⁻⁰⁴; cg16560256, p-value= 2.23·10⁻⁰⁴).

Conclusions: Neither new genomic loci associated with long-term air pollution were identified, nor previously identified loci were replicated. Continued efforts to test this potential association are warranted.

Keywords: Air pollution, DNA methylation, Epigenome-wide association study.

LIST OF ABBREVIATIONS

WHO: World Health Organization.

REGICOR: REgistre Gironí del COR.

EPIC-Italy: The Italy center of European Prospective Investigation into Cancer and Nutrition.

450K: Infinium Human Methylation450 BeadChip.

CpG: Cytosine-phosphate-guanine.

PM₁₀: Particulate matter with an aerodynamic diameter of <10µm.

PM_{2.5}: Particulate matter with an aerodynamic diameter of <2.5µm.

PM_{coarse}: The difference between PM₁₀ and PM_{2.5}.

NO_x: Nitrogen oxides.

NO₂: Nitrogen dioxide

LUR: Land use regression.

FDR: False Discovery Rate.

1. INTRODUCTION

Exposure to air pollution remains a global threat with more than 90% of the world's population now exceeding the exposure limits proposed for particulate matter by the World Health Organization (WHO) (WHO, 2018). At the same time, a growing body of evidence consistently supports the adverse health effects of air pollution, which the same WHO report estimates to be related with 3 million premature deaths worldwide each year. However, the mechanisms by which air pollution induces these deleterious effects are not completely understood.

Epigenetics encompasses mechanisms that regulate gene expression without changing the DNA sequence, and may contribute to the relation between air pollution and health. The most studied epigenetic mechanism is DNA methylation, which is heritable but can also be modified by life-style and environmental factors. Recently, several studies have analyzed the association between air pollution and DNA methylation using a genome-wide approach, and have reported numerous loci showing differential methylation related to this exposure (Breton et al., 2016; Chi et al., 2016; de F.C. Lichtenfels et al., 2018; Goodrich et al., 2016; Gruzieva et al., 2017; Jiang et al., 2014; Panni et al., 2016; Plusquin et al., 2017; Zhong et al., 2017).

The aims of this study were both to identify new genomic loci showing differential methylation associated with long-term exposure to air pollution in a population-based study in Spain and to replicate loci previously reported in other studies.

2. METHODS

2.1. Identification of new genomic loci showing differential methylation related to long-term air pollution exposure (Aim 1)

2.1.1. Study design and population

We designed a cross-sectional epigenome-wide association study in two stages. We used the REGICOR (REgistre GIroní del COR) cohort as the discovery study and the Italy center of the

European Prospective Investigation into Cancer and Nutrition (EPIC-Italy) as the replication study, followed by a meta-analysis of the results observed in both studies (REGICOR + EPIC-Italy).

As previously described (Grau et al., 2007), the REGICOR discovery sample included 648 participants randomly selected from the second wave of the REGICOR study in 2008-2013. The initial survey, performed during 2003-2005, included participants aged between 35 and 79 years, not institutionalized, and residing in Girona province (Catalonia, Spain) (Grau et al., 2007).

The EPIC-Italy replication study included 47,749 individuals in a multicenter prospective cohort recruited during 1993-1998 (Beulens et al., 2010; Palli et al., 2003). The samples selected for the present study were from two case-control studies on breast cancer (van Veldhoven et al., 2015) and colorectal cancer in Varese and Turin.

2.1.2. HumanMethylation450 BeadChip

DNA was extracted using standardized methods from peripheral blood (Puregen TM; Gentra Systems) and buffy coats (QIAGEN QIAasympyony DNA Midi Kit) in the REGICOR and EPIC-Italy studies, respectively. DNA was bisulphite-converted and the epigenome-wide methylation profiles were obtained using the Infinium HumanMethylation450 BeadChip (Illumina) (450K) to assess methylation on 485,577 cytosine-phosphate-guanine (CpGs) throughout the genome, following the Illumina Infinium HD Methylation protocol (Bibikova et al., 2011; Sandoval et al., 2011). The REGICOR samples were processed in two centers of the Spanish National Genotyping Center: the Center for Genomic Regulation in Barcelona (n=188 samples) and the Centro Nacional de Investigaciones Oncológicas in Madrid (n=460 samples). All the processed batches contained two duplicate samples used as an internal quality control. The EPIC-Italy samples were analyzed at the Human Genetics Foundation in Turin.

The same well-defined pipeline was used in both studies to assess the quality control of the methylation data (Sayols-Baixeras et al., 2016).

We used the M-value as the main DNA methylation measurement (Equation 1). An M-value=0 indicates that the CpG is half methylated, a positive M-value that the CpG is more methylated than unmethylated, and a negative M-value the inverse result. We standardized the M-value by batch (Equation 2) to reduce the batch effect and other potential technical sources of variation.

Equation 1: $M_{value} = \log_2 \left(\frac{M_i + \alpha}{U_i + \alpha} \right)$
 M_i = intensity of methylated probes.
 U_i = intensity of unmethylated probes.
 $\alpha= 1$.

Equation 2: $Z = \frac{(X - \bar{X})}{\sqrt{\frac{\sum (X - \bar{X})^2}{(n-1)}}}$
 X = M-value for a specific individual.
 \bar{X} = mean of M-value for a specific batch.
 n = sample size.

2.1.3. Air pollution exposure

Both the REGICOR data and the Turin component of EPIC-Italy contained particulate matter exposure [aerodynamic diameter of <10 μ m (PM₁₀), <2.5 μ m (PM_{2.5}), and PM_{coarse} (the difference between PM₁₀ and PM_{2.5})], nitrogen oxides (NO_x) and nitrogen dioxide (NO₂) measurements. For the Varese component of EPIC-Italy, only NO_x and NO₂ were available.

Both studies used the ESCAPE protocol to assess long-term exposure to air pollution (Beelen et al., 2013; Eeftens et al., 2012). As previously described, address histories for the past 10 years were collected by questionnaire, and each address was geocoded at the front-door level (Rivera et al., 2013). Using land use regression (LUR) models, 10-year weighted average exposure to NO_x and to nitrogen dioxide NO₂ for each participant were estimated (Basagaña et al., 2013). The model's coefficient of determination for NO₂ was 0.63 for REGICOR and 70% for Turin, and for NO_x was 66% and 72%, respectively (Rivera et al., 2013).

PM₁₀, PM_{2.5} and PM_{coarse} were also assessed using LUR models. The R² of the models for REGICOR and Turin was 71% and 69% for PM₁₀, 51% and 59% for PM_{2.5}, and 71% and 58% for PM_{coarse}, respectively (Eeftens et al., 2012).

We also used traffic proximity markers as surrogates of air pollution exposure in independent analyses. For each address, we calculated the traffic intensity at the nearest street and the traffic load (sum of traffic intensity multiplied by length of road segment) for all segments in a 100 meters buffer and derived 10-year average values for each participant.

2.1.4. Other covariates

REGICOR's trained team of nurses collected relevant sociodemographic, lifestyle, and cardiovascular risk factors using standardized and validated questionnaires (Baena-Díez et al., 2010; WHO, 2000). Smoking exposure was grouped in four categories: current smoker (smoked ≥ 1 cigarette/day at the time of the visit, on average, or gave up smoking within the year of the visit); former smoker, 1-5 years (gave up smoking up to 5 years before the visit); former smokers >5 years; and never smokers. The EPIC-Italy study collected the same variables, as well as the participating center and patient's diagnostic status.

We estimated cell concentration using Houseman algorithm by means of *minfi* R package (Aryee et al., 2014; Houseman et al., 2012). In both cohorts, we also calculated surrogate variables to control for potential residual confounding, using the *sva* R package (Leek et al., 2014). These variables are directly constructed from high-throughput data, and identify and remove potential and non-measured sources of variation due to technical and biological confounders.

2.1.5. Statistical analysis

We assessed the association between air pollution and DNA methylation using robust linear regression model to reduce the effect of outliers. We used the differing air pollution exposures as independent variables and DNA methylation as the dependent variable. The models were adjusted for age, sex, smoking exposure, and cell composition (Model 1). Moreover, a second model adjusted for surrogate variables was also fitted (Model 2).

In the REGICOR discovery study, we selected for validation those CpGs with a p-value of the association below an arbitrary threshold (p-value $<10^{-5}$) for each specific exposure, as previously described (Sayols-Baixeras et al., 2015; Jovanova et al., 2018). The results were replicated in the EPIC-Italy study using the same method and models, including also disease status and study center as additional covariates. The results for each CpG were then meta-analyzed using a random effects model. We applied Bonferroni criteria ($0.05/427,948$ CpGs; p-value $<1.17 \cdot 10^{-07}$) and false discovery rate (FDR), using the Benjamini-Hochberg procedure, to assess the statistical significance of the associations of interest.

2.2. Replication of previously published CpGs associated with air pollution (Aim 2)

We performed a systematic review to identify relevant epigenome-wide association studies indexed in Pubmed (<https://www.ncbi.nlm.nih.gov/pubmed/>) from its inception to March 2019. We used the following search terms strategy: (methylation* [Title/Abstract]) AND (epigenome-wide [Title/Abstract] OR genome-wide [Title/Abstract] OR 450K [Title/Abstract] OR 450 [Title/Abstract] OR HumanMethylation450 [Title/Abstract]) AND ("Air pollution" [Title/Abstract]). The articles identified were manually screened by 1 reviewer (SS-B), focusing first on the title and abstract and then on the complete manuscripts to assess their appropriateness for inclusion in the review. The same author extracted the CpGs that were significantly associated with air pollution and replicated in at least one external population or

experimental studies. In case of doubts the article was evaluated by a second reviewer (RE) to achieve a consensus. The identified CpGs were then screened in the selected REGICOR cohort.

2.2.1. Statistical analysis

The same analysis strategy and methodology described above was followed for the replication. We selected as distinctive the CpGs reported as statistically significant in the original studies, based on both Bonferroni corrected p-values and false discovery rate (FDR) p-values < 0.05. We considered as replicated those CpGs that fulfilled both the Bonferroni criteria according to the number of CpGs previously discovered and the FDR p-value.

3. RESULTS

3.1. Identification of new genomic loci showing differential methylation related to air pollution (Aim 1)

3.1.1. Discovery Stage

After applying the quality control of the 450K array, we excluded 3 individuals and 57,629 CpG probes. Moreover, we removed those individuals without information on air pollution exposure (n=15). Finally, 630 individuals and 427,948 probes were included in the analysis.

The main sociodemographic and clinical characteristics and the air pollution exposures of the study participants are shown in Table 1. The Manhattan plots and q-q plots of the associations between air pollution exposures and DNA methylation are shown in Supplementary Figure 1.

We identified 81 unique CpGs associated with air pollution exposures with a p-value < 10^{-5} . In model 1, 6 CpGs were associated with PM₁₀, 0 related to PM_{2.5}, 5 to PM_{coarse}, 2 to NO_x, 4 to NO₂, and 2 to traffic at the nearest street (Supplementary Table 1). In model 2, 7 CpGs were associated with PM₁₀, 18 related to PM_{2.5}, 6 to PM_{coarse}, 28 to NO_x, 32 to NO₂, and 9 to traffic at the nearest street (Supplementary Table 2).

3.1.2. Validation Stage and Meta-analysis

After applying a similar quality control of the 450K array, we included all 81 CpGs selected for replication and we excluded 5 individuals. Moreover, we removed those individuals without information on confounder variables (n=15) and air pollution exposure (n=61). Finally, 454 individuals were included in the analysis of NO traits (Turin and Varese) and 297 in the analysis of PM traits (Turin). The main characteristics and air pollution exposures of the EPIC-Italy participants are shown in Table 1. The associations between air pollution and the selected CpGs in this analysis are shown in Supplementary Table 1 and 2.

The results of the meta-analysis of the REGICOR and the EPIC-Italy studies are shown in Supplementary Table 1 and 2. None of the selected CpGs was validated in the joint analysis.

3.1.3. Statistical power

The regression coefficient values (effect size) that we could detect as statistically significant in the meta-analysis, accepting an alpha risk of $1.17 \cdot 10^{-07}$ in a two-sided test and with an 80% power, are shown in Supplementary Table 1 and 2.

3.2. Replication of the previously published CpGs associated with air pollution (Aim 2)

We initially identified 31 manuscripts (Abraham et al., 2018; Anto et al., 2017; Breton et al., 2016; Chi et al., 2016; Christensen and Marsit, 2011; Commodore et al., 2019; de F.C. Lichtenfels et al., 2018; Gao et al., 2019; Goodrich et al., 2016; Gref et al., 2017; Gruzieva et al., 2017; Holloway et al., 2012; Hong and Wang, 2014; Janssen et al., 2013; Jeong et al., 2019; Jiang et al., 2017, 2014; Ladd-Acosta et al., 2019; Lee et al., 2019; Li et al., 2018; Mostafavi et al., 2017; Nwanaji-Enwerem et al., 2018; Panni et al., 2016; Plusquin et al., 2017; Shukla et al.,

2019; Sominen et al., 2016; van Dongen et al., 2018; Wang et al., 2012; Zhang et al., 2017, 2018; Zhong et al., 2017) based on the search terms and after reading the full manuscripts we selected 17 studies (Abraham et al., 2018; Breton et al., 2016; Chi et al., 2016; Commodore et al., 2019; de F.C. Lichtenfels et al., 2018; Gao et al., 2019; Goodrich et al., 2016; Gruzieva et al., 2017; Jiang et al., 2014; Ladd-Acosta et al., 2019; Lee et al., 2019; Li et al., 2018; Mostafavi et al., 2018; Panni et al., 2016; Plusquin et al., 2017; Zhang et al., 2018; Zhong et al., 2017). Finally, we selected CpGs from two manuscripts. A total of 15 CpGs showing differential methylation in relation to PM_{2.5} from two manuscripts after correcting for Bonferroni criteria (Li et al., 2018; Panni et al., 2016). Three of these CpGs could not be analyzed in the REGICOR study as they did not pass the quality control, and none of the others was replicated (Supplementary Table 3).

In a secondary analysis, we included 1,673 CpGs from three manuscripts (Gruzieva et al., 2017; Li et al., 2018; Panni et al., 2016) fulfilling a FDR p-value<0.05. Among all the selected FDR results, 195 CpGs could not be analyzed in our study as they did not pass the quality control and 14 because they weren't available in the 450k array. We did not replicate any of the 1,464 CpGs analyzed (Table 2 and Supplementary Table 3). In the REGICOR study, the top hits were located in an intergenic region on chromosome 1 (cg10893043, p-value=6.79·10⁻⁵) and in the *LRRC45* and *PXK* genes (cg05088605, p-value=2.15·10⁻⁰⁴; cg16560256, p-value= 2.23·10⁻⁰⁴) for PM_{2.5}.

4. DISCUSSION

This population-based and cross-sectional study did not identify new loci or replicate loci previously identified as showing differential methylation related to long-term exposure to air pollution.

The lack of positive results in our study should be interpreted with caution and some methodological issues must be considered. First, our study is underpowered to detect small effect size associations. The statistical power of our study was estimated (Supplementary Table 1 and 2) and is similar to previous studies. Second, we defined strict criteria to consider an association as statistically significant based on the Bonferroni or the false discovery rate multiple comparisons correction. Third, the exposure to air pollution and its variability is lower than that observed in other studies, limiting our capability to identify real associations. Fourth, the exposure assessment was estimated using land use regression models. Although this methodology is commonly used and we followed the ESCAPE protocol (Beelen et al., 2013; Eeftens et al., 2012), using validated exposure estimations (Basagaña et al., 2013), some exposure misclassification could still be present, reducing the power to detect the association of interest in our study. Fifth, the replication was carried in a case-control study that may have a different methylation pattern. Sixth, we estimated long-term exposure to air pollution whereas other studies have analyzed the association between short-term exposure and DNA methylation, thus, comparison may be limited given that response patterns may indeed vary across time domains. Gruzieva et al analyzed gestational NO₂ exposure and DNA methylation at birth (cord blood), 4 years and 8 years of age (Gruzieva et al., 2017). Li et al designed a crossover experimental studies to assess the effect of 9 days of controlled exposure to high and low PM exposure (Li et al., 2018). However, in studies that analyzed several time exposures, the longer the exposure the higher the number of loci showing differential methylation (Panni et al., 2016). Seventh, the great heterogeneity between the three studies and ours in terms of sample size, age of the participants (children, young individuals, and adult population), design (experimental vs observational), and air pollution exposure of interest (PM, NO₂) could also explain the poor replication of previous findings. Eighth, we have also to consider the cross-sectional design of our study that limits the analysis of changes in air pollution exposure and changes in DNA

methylation over time. Finally, we could not adjust for potential relevant variables such as indoor air pollution and second-hand smoking.

Despite these limitations that should be considered, we would highlight some of the results observed in this study. In the discovery effort, we identified 7 CpGs associated with PM₁₀, 18 related to PM_{2.5}, 6 with PM_{coarse}, 28 with NO_x, 32 with NO₂, and 9 related to traffic at the nearest street in the REGICOR study. However, none of them were validated in the EPIC-Italy study. In our effort to replicate previous findings, we identified one locus located in an intergenic region on chromosome 1 (cg10893043, p-value=6.79·10⁻⁵) as potentially associated with PM_{2.5}. The cg10893043 is close to the *CDKN2C* gene. The protein encoded by this gene is a member of the INK4 family of cyclin-dependent kinase inhibitors and regulates cell growth by controlling cell-cycle G1 progression (Cánepa et al., 2007). Some studies have shown that the expression of this gene inhibits the growth of human cells in animal models and have suggested a potential role in tumorigenesis (van Veelen et al., 2009).

Among the strengths of our study, we would mention the standardized methodology following the ESCAPE protocol that was consistently used to assess air pollution exposure. This methodology was validated in our population and was used to assess long-term air pollution exposure. Moreover, we applied a commonly used methodology to assess DNA methylation at the genome-wide level and a standardized methodology with both a discovery and an independent validation population.

5. CONCLUSIONS

The results of our study are negative as we did not identify any new genomic loci associated with long-term air pollution and we did not replicate any previously identified loci. However, these negative results should be interpreted with caution. New joint efforts, increasing the statistical power of the analysis and the variability of the exposure to air pollution, and

considering both short- and long-term exposure, are warranted to assess the potential association between air pollution and DNA methylation.

DECLARATIONS

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Ethics approval and consent to participate

All participants in both studies (REGICOR and EPIC-Italy) signed an informed consent; the studies were approved by the local ethic committees (PSMAR CEIC- 2012/4729/I) and followed national legislation and the Declaration of Helsinki criteria.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author [RE] on request.

Competing interests

The authors declare that they have no competing interests.

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