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Research Article

Oxidative Stress and Inflammation Mediate the Effect of Air Pollution on Cardio- and Cerebrovascular Disease: A Prospective Study in Nonsmokers

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Air pollution is associated with a broad range of adverse health effects, including mortality and morbidity due to cardio- and cerebrovascular diseases (CCVD), but the molecular mechanisms involved are not entirely understood. This study aims to investigate the involvement of oxidative stress and inflammation in the causal chain, and to identify intermediate biomarkers that are associated retrospectively with the exposure and prospectively with the disease.

Additional Supporting Information may be found in the online version of this article.

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We designed a case-control study on CCVD nested in a cohort of 18,982 individuals from the EPIC-Italy study. We measured air pollution, inflammatory biomarkers, and whole-genome DNA methylation in blood collected up to 17 years before the diagnosis. The study sample includes all the incident CCVD cases among former- and never-smokers, with available stored blood sample, that arose in the cohort during the follow-up. We identified enrichment of altered DNA methylation in "ROS/Glutathione/Cytotoxic granules" and "Cytokine signaling" pathways related genes, associated with both air pollution (multiple comparisons adjusted p for enrichment ranging from 0.01 to 0.03 depending on pollutant) and with CCVD risk ($P = 0.04$ and $P = 0.03$, respectively). Also, Interleukin-17 was associated with higher exposure to NO_2 ($P = 0.0004$), NO_x ($P = 0.0005$), and CCVD risk (OR = 1.79; CI 1.04–3.11; $P = 0.04$ comparing extreme tertiles). Our findings indicate that chronic exposure to air pollution can lead to oxidative stress, which in turn activates a cascade of inflammatory responses mainly involving the "Cytokine signaling" pathway, leading to increased risk of CCVD. Inflammatory proteins and DNA methylation alterations can be detected several years before CCVD diagnosis in blood samples, being promising preclinical biomarkers. Environ. Mol. Mutagen. 59:234–246, 2018. © 2017 Wiley Periodicals, Inc.

Key words: air pollution; oxidative stress; inflammation; cytokine signaling; cardiovascular diseases; cerebrovascular diseases

INTRODUCTION

Cardio- and cerebrovascular diseases (CCVD) are among the leading causes of death and disability worldwide (Guilbert, 2003; Vos et al., 2016; Wang et al., 2016). Exposure to ambient air pollution, particularly fine coarse ($PM_{2.5}$) and thoracic (PM_{10}) particulate matters, nitrogen oxide (NO_x), nitrogen dioxide (NO_2), and elemental carbon, has been linked to a wide range of adverse health effects. Epidemiological studies have increasingly shown that air pollution is associated with not only respiratory diseases (Chung et al., 2011, 2016; Li et al., 2016; Cox, 2017) but also coronary artery disease (Wolf et al., 2015; McGuinn et al., 2016), cardiovascular diseases (Brook et al., 2010; Franklin et al., 2015), and cerebrovascular diseases (Stafoggia et al., 2014) including ischemic stroke (Chung et al., 2017; Cox, 2017).

In a recent meta-analysis, including eleven European cohorts from the "European Study of Cohorts for Air Pollution Effects" (ESCAPE) project, in which 5,157 individuals experienced incident coronary events, a 5 $\mu g/m^3$ increase in estimated annual mean $PM_{2.5}$ was associated with a 13% increased risk of coronary fatal events, and a 10 $\mu g/m^3$ increase in estimated annual mean PM_{10} was associated with a 12% increased risk of coronary events (Cesaroni et al., 2014). An independent study conducted within the same cohorts reported 26% and 4% increased risk for cerebrovascular diseases for each 5 $\mu g/m^3$ increase in $PM_{2.5}$ and 10 $\mu g/m^3$ increase in NO_2 respectively (Stafoggia et al., 2014). Interestingly, an increased risk was also detected considering exposures below the current annual European limit value of 25 $\mu g/m^3$ for $PM_{2.5}$ and 40 $\mu g/m^3$ for PM_{10} (Cesaroni et al., 2014; Stafoggia et al., 2014).

Several mechanistic explanations have been put forward to explain these associations, particularly oxidative stress and inflammation (Uzoigwe et al., 2013; Newby et al., 2015). There is, in fact, evidence that air pollution can induce both patterns, and both patterns have been associated with CCVD (Cosselman et al., 2015; Munzel et al., 2016a,b). In an ESCAPE cross-sectional study, living close to busy traffic was associated with increased C-reactive protein concentrations, a known inflammatory biomarker and risk indicator for CCVD (Lanki et al., 2015). Also, long-term exposure to NO_x was associated with decreased levels of circulating Interleukin 8, another inflammation marker (Mostafavi et al., 2015). DNA methylation (DNAm) dysregulation, in turn, was described in relation to long-term air pollution exposure. Air pollution has been hypothesized to be related to CCVD through deregulation of genes coding for both pro-inflammatory and anti-inflammatory cytokines (Chi et al., 2016). Also, reduced methylation of mitochondrial DNA (that is one of the primary targets of oxidative stress) was associated with exposure to fine particulate $PM_{2.5}$ and modified the adverse relationships between $PM_{2.5}$ exposure and heart rate variability outcomes (Byun et al., 2016). Associations of DNAm levels of single CpG sites with CCVD biomarkers including homocysteine (Ingrosso et al., 2003) and C-reactive protein (Fu et al., 2007), or with CCVD risk factors like smoking (Guida et al., 2015) and obesity (Dick et al., 2014) were recently described. Other studies focused on specific pathways (Fiorito et al., 2014) or showed decreased methylation in Long Interspersed Nuclear Elements 1 (LINE 1) in blood, in association with CCVD (Baccarelli et al., 2010; Guarrera et al., 2015).

No study to date has assessed mediation of the relationship between air pollution and CCVD by intermediate biomarkers with a longitudinal design. This study aimed to investigate the hypothesis of the involvement of inflammatory pathways and oxidative stress in the causal chain and to identify intermediate biomarkers that are associated retrospectively with the exposure and prospectively with the disease in the same subjects. We measured a set of inflammatory proteins and whole-genome DNA methylation in a case-control study nested in the Turin and Varese EPIC cohorts. The study sample includes

nonsmoking CCVD incident cases (and matched controls) only, since it has been shown that smoking habits could confound the association of air pollution with CCVD (Sheppard et al., 2012) and the association of air pollution with inflammation (Essouma and Noubiap, 2015).

METHODS

Study Population

Study participants were part of the Italian component (Turin and Varese centers) of the EPICOR study (Bendinelli et al., 2011), that is the cardiovascular section of the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort (Palli et al., 2003). In the period 1993–1998, EPIC Italy completed the recruitment of 47,749 volunteers (15,171 men and 32,578 women). The Turin and Varese cohorts include 10,604 and 12,083 participants respectively, all aged 35–65 years (men and women). From hospital discharge records, we have identified all newly diagnosed cases of CCVD and revascularization, which arose in the cohort during 12.2 years of follow-up on average. Subjects with suspected CCVD were identified according to the criteria of the International Classification of Diseases (ICD-10) codes I21, I46, I63, Z95 or the procedure codes for coronary revascularization. Suspected CCVD events were confirmed when acute myocardial infarction, acute coronary syndrome, coronary revascularization or ischemic stroke were present, supported by information on onset symptoms, levels of cardiac enzymes and troponins, imaging, and electrocardiographic data coded according to the Minnesota Code. Cases were cross-checked with mortality files to identify fatal and nonfatal cases (the latter defined as alive 28 days after diagnosis). Study participants with CCVD at or before cohort entry were identified from the baseline questionnaire, from linkage with hospital discharge records, or by direct examination of clinical records, and were excluded from this study. Details on covariate acquisition are reported in Supporting Information methods.

This study complies with the Declaration of Helsinki principles and conforms to ethical requirements. All volunteers signed an informed consent form at enrollment. The EPIC study protocol was approved by Ethics Committees at the International Agency for Research on Cancer (Lyon, France) and the Human Genetics Foundation (Turin, Italy).

Case-Control Study Nested in the Cohort

We designed a case-control study nested in the cohort including 386 samples (193 matched case-control pairs) for biomarker analyses, using the incident density sampling method (Richardson, 2004). We selected all the incident CCVD cases which arose in the cohort during the follow-up period (up to 17 years) fulfilling the following conditions:—never smokers or former smokers for at least one year;—archived blood samples (serum and buffy coat collected at recruitment) available and stored in liquid nitrogen;—existence of at least one matched control. Criteria for case-control matching based on baseline characteristics were: one-to-one matching by smoking (never/time since quitting), gender, age (no more than 2.5 years difference at recruitment), season and year of recruitment in the cohort.

Exposure Assessment

We have estimated exposures to NO_2, NO_x, and $PM_{2.5}$. The first two measures were developed in the context of the ESCAPE study and extensively described elsewhere (Eeftens et al., 2012; Beelen et al., 2013). Briefly, in ESCAPE Land Use Regression (LUR) models were developed and used to estimate air pollution concentrations at the home address of all study participants, using GIS (Geographic Information System) procedures. The LURs describe the spatial distribution of the annual mean concentrations taken as a proxy for the long-term averages for all exposure indicators. For $PM_{2.5}$ we used a bespoke Western European model based on Satellite-derived measurements and chemical transport models nested within LUR models (de Hoogh et al., 2016; Nunen et al., 2017). For the latter, estimates were provided for 100*100 m grids, whereas for the ESCAPE models, estimates were provided for points (address coordinates).

Back Extrapolated Measures

Since the EPIC cohort was recruited between 1993 and 1998, baseline clinical measurements, blood drawing, and interviews occurred up to 17 years before the ESCAPE measurement campaigns performed in 2010 in Turin and Varese. In light of the substantial changes in air pollution during these decades, estimated exposure values were back-extrapolated to the year of recruitment for each individual, to correct for time trends of pollution. Back extrapolation was conducted by assuming constant within-city spatial patterns. Individual estimates of ambient concentrations were adjusted (calibrated) for the long-term trends using a procedure developed during the ESCAPE project (Stafoggia et al., 2014). We obtained annual extrapolated exposures based on the comparison of the concentration measured at the routine background monitor in each year of the recruitment period (1993–1998) with the yearly average during 2010 (year of sampling campaign). A detailed description of the back-extrapolation procedure can be found at "www.escapeproject.eu". Back extrapolation procedure was not possible for $PM_{2.5}$ due to the absence of historical data.

Biomarker Measurements

DNA Methylation

Details for DNA extraction, array design, bisulfite conversion, and methylation analyses are reported in Supporting Information Methods. Raw fluorescence intensities data were extracted from "*idat*" files using the "*minfi*" package in R statistical environment (Aryee et al., 2014). Background subtraction, color bias adjustment, and fluorescence intensities normalization were performed using the Subset-quantile Within Array Normalization (SWAN) procedure described by Maksimovic et al. (Maksimovic et al., 2012). Samples were excluded if the bisulfite conversion fluorescence intensity was less than 10,000 for both type I and type II probes. Methylation measures were set to missing if the detection *P*-value was higher than 0.01. Additionally, the set of cross-reactive or polymorphic (with minor allele frequency greater than 0.01 in Europeans) CpGs ($N = 39{,}238$) described by Chen et al. (Chen et al., 2013) was excluded due to the low reliability of methylation measures. CpGs and samples were excluded if the total call rate was less than 95%. Control samples whose matched CCVD case was excluded were also removed from the analyses.

DNAm levels were expressed as the ratio of the intensities of methylated cytosines over the total intensities (β values). For statistical analyses, a logarithmic transformation of β values was used $M = \log_2 \frac{\beta}{1-\beta}$ as recommended by Du et al. (Du et al., 2010). Probe design bias was corrected using the BMIQ function in the "*wateRmelon*" package in R statistical environment (Teschendorff et al., 2013). Known batch effect by plate and position on the Illumina Beadchip was removed before statistical analyses using the ComBat algorithm described by Johnson et al. (Johnson et al., 2007). In Supporting Information Figure S1, the average beta values by plate and position on the Beadchip before and after ComBat normalization are reported. Finally, we have analyzed DNAm data for 320 individuals (160 matched CCVD case-control pairs).

Inflammatory Proteins

Targeted proteomics has been performed by the Luminex Multianalyte Profiling platform for plasmatic inflammation-related proteins for a total of 23 signals per sample. Samples and inflammatory biomarkers

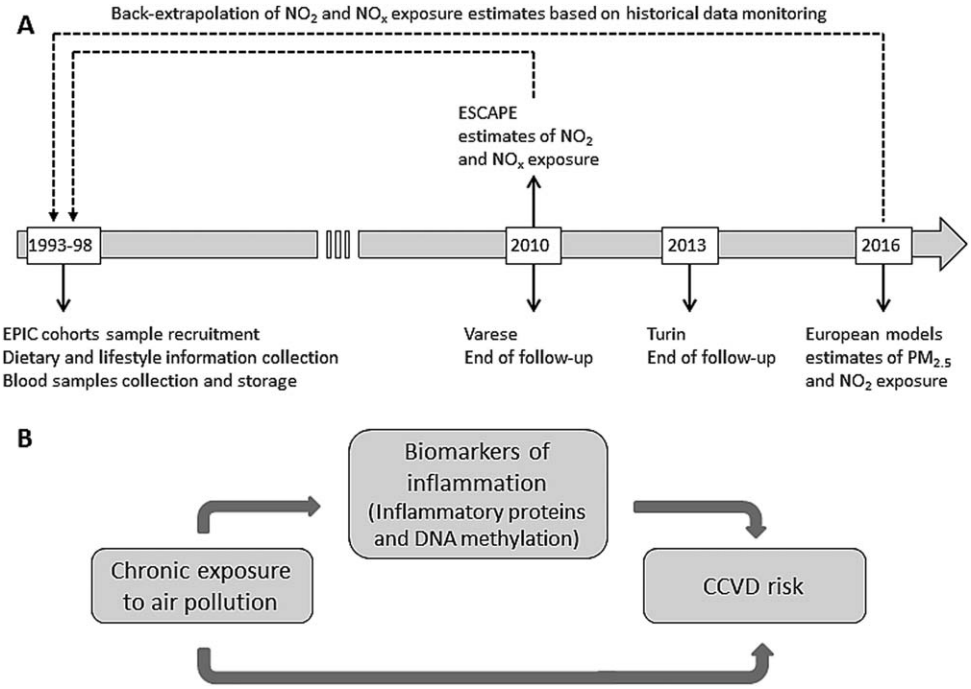

Fig. 1. Timeline for collection of the relevant variables and exposure estimates (**A**), and study hypothesis (**B**). We aimed to identify biological pathways associated with both chronic exposure to air pollution and incident CCVD according to a meet-in-the-middle (MITM) design.

with low call rate (less than 40%) were excluded from the analysis. Imputation of missing values has been applied for inflammatory markers that had at least 40% detectable samples measurement per study group, based on maximum likelihood estimation procedure (Lubin et al., 2004). To allow for plate to plate variation we imputed values based on each plate-specific limit of quantification and included the plate as one of the predictor variables in the imputation model. After quality controls, biomarkers and sample filtering, we have analyzed data for 372 samples (186 CCVD cases and one-to-one matched controls), and thirteen inflammatory biomarkers: C-reactive protein (CRP), Epidermal Growth Factor (EGF), Eotaxin, Interleukin 17 (IL17), Interleukin 1 receptor Antagonist (IL-1rA), Interleukin (IL-8), Interferon-γ–inducible protein 10 (IP-10), Monocyte Chemoattractant protein 1 (MCP-1), Human Macrophage–derived Chemokine (MDC), Macrophage Inflammatory protein 1 (MIP1), Myeloperoxidase (MPO), Periostin, and Vascular Endothelial Growth Factor (VEGF).

Figure 1 reports a summary of the timeline for the collection of relevant variables (Fig. 1A) and a schematic description of the study hypothesis (Fig. 1B). Descriptive statistics of the study sample are reported in Table I. The present study complies with international established STROBE guidelines for observational studies (Little et al., 2009).

Statistical Analyses

Workflow

We performed the statistical analysis in two steps. Initially, we have estimated the risk for CCVD conferred by chronic exposure to air pollution (NO_2, NO_x, and $PM_{2.5}$) in the full cohort ($N = 18{,}982$; 948 CCVD events), and performed a sensitivity analysis in former- and never-smokers ($N = 14{,}712$; 661 CCVD events). Then, we examined the association of biomarkers with air pollution exposure and, independently, with CCVD risk within the case-control study nested in the cohort. For DNAm biomarkers analysis, instead of the classical epigenome-wide association analysis, we have investigated enrichment of altered DNAm levels in 17 *a priori* defined inflammatory pathways (DNAmIPs). The relationships between protein and DNAmIPs was also investigated to understand whether DNAm in inflammatory genes could regulate protein expressions. Below, we describe in detail the statistical methods used in each step.

Association of Air Pollution with CCVD Risk

In the full cohort, Cox proportional hazard regression models with age as the time variable were used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) for the association of chronic exposure to air pollution (NO_2, NO_x, and $PM_{2.5}$) with the risk of CCVD. Hazard ratios were expressed for each 10 $\mu g/m^3$ increase in NO_2 and NO_x, and for each 5 $\mu g/m^3$ increase in $PM_{2.5}$. Covariates included in the models were gender, center of recruitment, season of pollutant measurements (as a proxy for the weather conditions), BMI (continuous), smoking habits (categorical: never, former, current smokers), alcohol intake (categorical: no-moderate: less than 28 g/day, habitual drinker: more than 28 g/day), Mediterranean diet score (ordinal categorical score from 0 to 10), physical activity (ordinal categorical: inactive, low, medium, high), educational level (as proxy for the socio-economic status, categorical: low, medium, high), prevalent diabetes, hypertension, and hyperlipidemia. In the subset selected for biomarker analysis (case-control study nested in the cohort), we used logistic regression models adjusting for the same set of confounders.

Biomarker Analysis: Inflammatory Proteins

Protein concentration values were transformed to correct for skewness in the data, using the Box-Cox nonlinear transformation (Han and Kronmal, 2004). Linear regression models were used to investigate the association of protein concentrations in blood with air pollution exposure; logistic regression models were used to examine the association of proteins with CCVD risk. Covariates included in the models were age,

TABLE I. Descriptive Statistics of Study Participants after Quality Controls and Sample Filtering

	Overall cohort		Selected subset for biomarker analyses	
Variable	Controls ($N = 18,034$)	CCVD cases ($N = 948$)	Controls ($N = 193$)	CCVD cases ($N = 193$)
Gender (men)[a]	6,468	498	80	80
Age at recruitment (years)[a]	50.59 (7.8)	55.7 (7.6)	54.9 (7.1)	54.8 (7.4)
Centre of recruitment				
Varese	9,699	530	98	98
Turin	8,335	418	95	95
BMI (Kg/m^2)[a]	25.5 (3.9)	26.6 (3.9)	26.4 (3.9)	27.1 (4.3)
Smoking status[a]				
Current	3,983	287	0	0
Former	4,811	260	68	68
Never	9,240	401	125	125
Alcohol (habitual drinkers)[a]	2,689	211	32	28
Physical activity[a]				
Inactive	3,380	219	39	38
Moderately inactive	7,002	353	68	71
Moderately active	4,230	195	31	45
Active	3,422	181	28	28
Mediterranean diet score	3.9 (1.8)	3.8 (1.8)	4.1 (1.2)	3.9 (1.8)
Education[a]				
Low	9,329	581	92	112
Medium	6,810	298	48	57
High	1,877	66	26	13
Prevalent diabetes[a]	282	58	4	16
Prevalent hypertension[a]	3,885	346	47	82
Prevalent hyperlipidemia[a]	4,457	365	47	80
CCVD event				
AMI/ACS	-	200	-	37
Coronary angioplasty	-	267	-	36
AMI/ACS + coronary angioplasty	-	302	-	52
Ischemic stroke	-	112	-	49
Carotid angioplasty	-	33	-	10
Fatal coronary event	-	34	-	9
Air pollution ($\mu g/m^3$)				
NO_2[a]	48.3 (16.0)	50.2 (15.1)	49.5 (16)	50.1 (15.6)
NO_x[a]	92.0 (36.3)	96.5 (34.8)	93.6 (35.6)	96.1 (36.2)
$PM_{2.5}$[a]	21.7 (1.9)	22.0 (1.9)	21.7 (1.9)	21.9 (1.8)

Mean and standard deviation are reported for continuous variables, the number of individuals in each group are reported for categorical variables.
[a]*t*-test (for continuous variables)/Chi-squared test (for categorical variables) *P*-value < 0.001.

gender, smoking habits, center, season, and year of recruitment. Additionally, for the proteins that resulted significantly associated with CCVD risk, we have investigated the association of biomarkers with the time lag between recruitment and the CCVD event, named "time to disease" (TTD). In this analysis, the differences between case and the matched control biomarker values were the predictors and TTD the outcome in linear regression models adjusting for the same set of confounders as previously described. To take into account correction for multiple comparisons, we have estimated the number of independent tests through a principal component analysis (PCA) based procedure described in Supporting Information Material.

Biomarker Analysis: DNAmIPs

For DNAm we focused on genes pertaining to 17 inflammation-related pathways described by Loza and colleagues (Loza et al., 2007). For each pathway, we tested for overrepresentation of significant signals in association with air pollution and with CCVD risk, with a weighted Kolmogorov-Smirnov (WKS) enrichment test. The complete list of all the genes included in each pathway, according to the authors, is reported in Supporting Information Table SI. In this classification, no genes are annotated in multiple pathways, avoiding bias due to redundancy in the enrichment analysis. The algorithm for enrichment analysis is described in detail in Supporting Information material and by Charmpi and Ycart (Charmpi and Ycart, 2015). Briefly, it is composed of two main steps: 1) a genome-wide scan to evaluate the association of each CpG with the exposure (or with the disease) using linear regression models adjusting for matching variables and white blood cell (WBC) percentages, the latter estimated using the Houseman algorithm (Houseman et al., 2012); 2) a comparison of the observed distribution of *P*-values (or equivalently the Z statistics), for the set of probes of interest, with the empirical distribution expected under the null hypothesis of no enrichment, the latter being estimated from genome-wide results. The Kolmogorov-Smirnov test rejects the null hypothesis (i.e., no enrichment) when the estimated effects for CpGs in the pathway are lower than (or equal to) those expected by chance. The overall procedure includes a stringent permutation-based correction for multiple testing.

TABLE II. Association of Exposure to Air Pollutants with the Risk of CCVD

	Overall cohort ($N = 18,982$)		Nonsmokers ($N = 14,712$)		Subset selected for biomarker analyses ($N = 386$)	
Exposure	HR (95% CI)	*P*	HR (95% CI)	*P*	OR (95% CI)	*P*
NO_2	1.04 (1.01–1.08)	0.0032	1.04 (1.00–1.07)	0.0374	1.03 (0.94–1.14)	0.4972
NO_x	1.01 (1.00–1.03)	0.0225	1.01 (0.99–1.03)	0.0844	1.01 (0.97–1.06)	0.4892
$PM_{2.5}$	1.29 (1.08–1.55)	0.0061	1.29 (1.05–1.60)	0.0173	1.33 (0.72–2.50)	0.3685

Hazard ratios (HRs) and confidence intervals (CIs) were estimated in the overall cohort (948 CCVD events), in non-smokers (661 CCVD events), and in the subset of subjects selected for biomarker analyses (193 CCVD events). Covariates included in the models were gender, center of recruitment, season of pollutant measurements (as a proxy for the weather conditions), BMI (continuous), smoking habits (categorical: never, former, current smokers), alcohol intake (categorical: no-moderate: less than 28 g/day, habitual drinker: more than 28 g/day), Mediterranean diet score (ordinal categorical score from 0 to 10), physical activity (ordinal categorical: inactive, low, medium, high), educational level (as proxy for the socioeconomic status, categorical: low, medium, high), prevalent diabetes, hypertension, and hyperlipidemia.

The WKS enrichment analyses were performed to investigate the association between protein and DNAmIPs. That is, we performed 13 additional genome-wide analyses (one for each inflammatory proteins), and then we ran the WKS enrichment algorithm to investigate whether DNAm in inflammation-related genes could regulate protein expressions.

All the statistical analyses were conducted using the R software v.3.3.2 (R-Core-Team, 2016).

RESULTS

Association of Air Pollution with CCVD Risk

In the overall cohort study ($N = 18,982$; 948 CCVD cases), exposure to NO_2, NO_x, and $PM_{2.5}$ were significantly associated with an increased risk of future CCVD events. The estimated risks were comparable when evaluated in nonsmokers only, and statistically significant for NO_2 and $PM_{2.5}$ (Table II).

For NO_2, the HRs were 1.04 (95% CI 1.01–1.08, $P = 0.003$) in the overall cohort and 1.04 (95% CI 1.00–1.07, $P = 0.04$) in nonsmokers, for each increase of 10 $\mu g/m^3$. For NO_x, the HRs were 1.01 (95% CI 1.00–1.03, $P = 0.02$) in the overall cohort and 1.01 (95% CI 0.99–1.03, $P = 0.08$) in nonsmokers, for each increase of 10 $\mu g/m^3$. Finally, For $PM_{2.5}$, the HRs were 1.29 (95% CI 1.08–1.55, $P = 0.006$) in the overall cohort and 1.29 (95% CI 1.05–1.60, $P = 0.02$) in nonsmokers, for each increase of 5 $\mu g/m^3$. The ORs estimated in the subset of individuals selected for biomarker analysis (193 CCVD cases and matched controls) were comparable to those estimated in the whole cohort, though associations were not significant due to reduced statistical power (Table II).

DNAm in Inflammatory Pathways Related Genes

In the probe-by-probe analyses none of the CpGs was significantly associated with NO_2, NO_x, $PM_{2.5}$ nor with case-control status after false discovery rate (FDR) for multiple testing. Supporting Information Figure S2 shows a summary of the genome-wide results using *volcano plots*. The top 100 CpGs for each EWAS analysis are listed in Supporting Information material.

P-values for the WKS enrichment tests are reported in Table III. Four out of the seventeen DNAm inflammatory pathways showed enrichment of significant signals in association with the risk of future CCVD events. Specifically, the "Cytokine signaling" pathway ($P = 0.03$), "Innate pathogen detection" pathway ($P = 0.02$), "Phagocytosis-Antigen presentation" pathway ($P = 0.02$), and the "Reactive Oxygen Species (ROS)/Glutathione/Cytotoxic granules" pathway ($P = 0.04$). Interestingly, "Cytokine signaling" pathway was associated with NO_2 and $PM_{2.5}$ also ($P = 0.02$ and $P = 0.03$, respectively), whereas "ROS/Glutathione/Cytotoxic granules" pathway was associated with $PM_{2.5}$ also ($P = 0.04$). No pathways were associated with the exposure to NO_x. In Supporting Information Figures S3–S19 the comparisons of the observed vs. expected (estimated from genome-wide results) distributions of *P*-values for each inflammatory pathway are reported.

Inflammatory Proteins

Association with Air Pollution

Seven out of thirteen inflammatory proteins (Eotaxin, IL17, EGF, IL-8, MIP1, MPO, and VEGF) were associated with at least one air pollutant (NO_2, NO_x, $PM_{2.5}$) with nominal *P*-values ranging from 0.004 to 0.04. Among those, Eotaxin, IL17, IL-8, and EGF were significantly associated with air pollution after correction for multiple testing (FDR threshold of significance = 0.006). Higher concentrations of Eotaxin and IL17 were observed with higher exposures whereas, lower concentrations of EGF and IL-8 were associated to higher exposure to pollutants (Table IV).

Association with CCVD Risk

Higher concentrations of CRP, IL17, IL-1rA, and IP-10 were nominally associated with increased risk of CCVD. No significant associations were found after FDR correction for multiple testing.

TABLE III. Weighted Kolmogorov-Smirnov (WKS) Pathway Enrichment Analyses for 17 Pathways Involved in Inflammation (from reference: Loza et al. 2007)

Inflammatory pathway	# Genes	# CpGs	NO_2	NO_x	$PM_{2.5}$	Case-control status
Adhesion-Extravasation-Migration	142	1,045	0.27	0.62	0.07	0.57
Apoptosis signaling	68	504	0.88	0.08	0.65	0.96
Calcium signaling	14	267	0.46	0.32	0.75	0.58
Complement Cascade	40	423	0.18	0.44	0.70	0.26
Cytokine signaling	172	1,120	**0.02**	0.13	**0.03**	**0.03**
Eicosanoid signaling	39	276	0.69	0.34	0.85	0.32
Glucocorticoid/PPAR signaling	21	255	0.29	0.46	0.19	0.45
G-Protein Coupled Receptor signaling	42	716	0.20	0.53	0.77	0.84
Innate pathogen detection	50	305	0.69	0.61	0.66	**0.02**
Leukocyte signaling	121	1,342	0.56	0.82	0.93	0.51
MAPK signaling	118	1,667	0.97	0.83	0.25	0.30
Natural Killer Cell signaling	31	198	0.47	0.22	0.60	0.55
NF-kB signaling	33	455	0.33	0.14	0.69	0.41
Phagocytosis-Antigen presentation	39	434	0.54	0.99	0.84	**0.02**
PI3K/AKT signaling	37	478	0.93	0.87	0.88	0.37
ROS/Glutathione/Cytotoxic granules	22	90	0.45	0.37	**0.01**	**0.04**
TNF Superfamily signaling	38	390	0.96	0.96	1.00	0.57

The table shows *P*-values for enrichment adjusted for multiple comparisons using a 10,000 permutations based procedure. Significant enrichments are highlighted in bold. # Genes = number of genes in the pathway; # CpGs = number of CpG sites in the pathway.

TABLE IV. Association of Inflammatory Proteins with Air Pollutants and Case-Control Status

	NO_2		NO_x		$PM_{2.5}$		Case - control status		
	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*		OR (95% CI)	*P*
CRP	−0.08 (–0.18; 0.02)	0.1343	−0.03 (–0.08; 0.01)	0.1494	−0.35 (–1.02; 0.32)	0.3091	2nd tertile	**1.72 (1.03; 2.87)**	**0.038**
							3rd tertile	**1.96 (1.18; 3.29)**	**0.0099**
EGF	**–0.93 (–1.51; –0.34)**	**0.0021**	**–0.39 (–0.65; –0.14)**	**0.003**	−2.82 (–6.63; 0.98)	0.1471	2nd tertile	0.96 (0.57; 1.61)	0.8766
							3rd tertile	0.84 (0.50; 1.41)	0.5185
Eotaxin	**0.09 (0.03; 0.14)**	**0.0012**	**0.03 (0.01; 0.05)**	**0.0094**	**0.49 (0.16; 0.82)**	**0.0041**	2nd tertile	1.04 (0.62; 1.74)	0.8769
							3rd tertile	1.08 (0.65; 1.80)	0.7754
IL17	**0.08 (0.03; 0.12)**	**0.0004**	**0.03 (0.01; 0.05)**	**0.0005**	0.16 (–0.12; 0.43)	0.2618	2nd tertile	1.24 (0.75; 2.06)	0.4062
							3rd tertile	**1.79 (1.04; 3.11)**	**0.0374**
IL-1rA	**–0.01 (–0.02; 0.00)**	**0.0379**	**0.00 (–0.01; 0.00)**	**0.036**	−0.05 (–0.10; 0.01)	0.0845	2nd tertile	1.52 (0.91; 2.55)	0.109
							3rd tertile	**1.70 (1.01; 2.89)**	**0.0464**
IL-8	**–0.08 (–0.13; –0.03)**	**0.0024**	**–0.03 (–0.06; –0.01)**	**0.0032**	−0.32 (–0.66; 0.02)	0.0626	2nd tertile	0.70 (0.42; 1.18)	0.1804
							3rd tertile	0.66 (0.38; 1.13)	0.1344
IP-10	0.01 (–0.01; 0.02)	0.3526	0.00 (0.00; 0.01)	0.3423	0.04 (–0.03; 0.11)	0.2699	2nd tertile	1.94 (1.16; 3.29)	0.0124
							3rd tertile	1.52 (0.90; 2.59)	0.1222
MCP-1	0.01 (–0.06; 0.08)	0.7407	0.00 (–0.03; 0.03)	0.8425	0.09 (–0.36; 0.54)	0.6883	2nd tertile	1.61 (0.95; 2.76)	0.0804
							3rd tertile	1.26 (0.71; 2.24)	0.4402
MDC	0.00 (–0.16; 0.16)	0.9853	0.01 (–0.06; 0.08)	0.8442	−0.18 (–1.20; 0.84)	0.7284	2nd tertile	1.26 (0.76; 2.10)	0.3628
							3rd tertile	1.19 (0.71; 2.03)	0.507
MIP1	**–0.21 (–0.37; –0.06)**	**0.0082**	**–0.10 (–0.16; –0.03)**	**0.0068**	**–1.24 (–2.25; –0.23)**	**0.0162**	2nd tertile	0.85 (0.51; 1.41)	0.5237
							3rd tertile	0.97 (0.58; 1.61)	0.901
MPO	−0.54 (–1.24; 0.16)	0.1336	−0.26 (–0.57; 0.05)	0.1016	−3.81 (–8.32; 0.70)	0.0991	2nd tertile	1.63 (0.99; 2.70)	0.0574
							3rd tertile	1.35 (0.80; 2.28)	0.2622
Periostin	0.24 (–0.34; 0.82)	0.4117	0.03 (–0.22; 0.29)	0.7923	−0.15 (–3.86; 3.57)	0.9376	2nd tertile	1.03 (0.62; 1.71)	0.9155
							3rd tertile	0.87 (0.52; 1.46)	0.5993
VEGF	0.09 (0.00; 0.18)	0.0616	**0.04 (0.00; 0.08)**	**0.033**	0.11 (–0.49; 0.70)	0.7259	2nd tertile	1.04 (0.63; 1.72)	0.8763
							3rd tertile	1.32 (0.79; 2.20)	0.2907

Significant results (*P*-value < 0.05) are highlighted in bold. β (regression coefficients) indicates to the increase/decrease in Box-Cox transformed protein levels per unit increase in air pollution; OR (odds ratio) refers to the first tertile used as the reference category; CI indicates the 95% confidence interval

For CRP, the ORs were 1.72 (95% CI 1.03; 2.87, $P = 0.04$) and 1.96 (95% CI 1.18; 3.29, $P = 0.001$) comparing the 2nd tertile and the 3rd tertile with the reference (1st tertile), respectively.

For IL17, the corresponding ORs were 1.24 (95% CI 0.75; 2.06, $P = 0.4$) and 1.79 (95% CI 1.04; 3.11, $P = 0.04$). For IL-1rA, ORs were 1.52 (95% CI 0.91; 2.55,

$P = 0.1$) and 1.70 (95% CI 1.01; 2.89, $P = 0.05$). Finally, for IP-10, ORs were 1.94 (95% CI 1.16; 3.29, $P = 0.01$) and 1.52 (95% CI 0.90; 2.59, $P = 0.1$) (Table IV).

Among the four proteins related with increased CCVD risk, IL17 had a significant association with the TTD also: $\beta = -0.60$ (95% CI -1.13; -0.06, $P = 0.03$).

Identification of Intermediate Biomarkers

Based on the results described above, we identified one inflammatory protein (IL17) and two DNAmIPs ("Cytokine signaling" and "ROS/Glutathione/Cytotoxic granules") that were independently associated with at least one pollutant and the risk of CCVD. For intermediate biomarkers, we have evaluated the reduction in the estimated effect on CCVD risk due to the inclusion of air pollution measure as a covariate in the statistical model.

The inclusion of NO_2 as a covariate in the logistic regression model only partially reduced the magnitude (and significance) of the association of IL17 with CCVD risk. The ORs after including exposure to NO_2 in the model were 1.19 (95% CI 0.68; 1.70, $P = 0.60$) comparing the 2nd tertile with the 1st tertile and 1.70 (95% CI 1.00; 2.91, $P = 0.05$) comparing the 3rd tertile with the 1st tertile.

Similarly, for the DNAmIP enrichment analysis, we repeated the enrichment analysis using the WKS method. The first step of the algorithm (the genome-wide scan) was modified by including NO_2 as a covariate in the logistic regression models. The procedure resulted in a slight increase of the enrichment *P*-values that were still significant after a permutation-based correction for multiple testing. *P*-values for enrichment were 0.05 for "ROS/Glutathione/Cytotoxic granules" and 0.04 for "Cytokine signaling" pathways respectively. We repeated the described procedures using NO_x and $PM_{2.5}$ instead of NO_2 as relevant exposure, obtaining comparable results due to the high correlation among air pollution measures (Supporting Information Table SII).

Association of Protein Biomarkers with DNAm in Inflammation-Related Genes

To investigate whether DNAm could regulate inflammatory protein concentrations in blood, we repeated the WKS enrichment algorithm using the 13 inflammatory proteins as the outcome. All but two protein biomarkers (Eotaxin and MIP1) resulted significantly associated with at least one DNAmIP (Supporting Information Table SIII). Particularly, IL17 was independently associated with 11 DNAmIPs (p range from 0.03 to 0.0004, Supporting Information Table SIII) including "Cytokine signaling" ($P = 0.0004$) and "ROS/Glutathione/Cytotoxic granules" pathways ($P = 0.001$), whereas MPO was independently associated with 10 DNAmIPs (*P* range from 0.048 to 0.0004, Supporting Information Table SIII).

Details on further sensitivity analyses using other estimates of air pollution and considering cardiovascular outcomes only (i.e., excluding ischemic strokes) are reported in Supporting Information material.

DISCUSSION

We have investigated the relationships between exposure to air pollution, inflammatory biomarkers, and incident coronary and cerebrovascular diseases longitudinally in the same set of data. Previous studies investigated the associations of biomarkers either with air pollutants or with CCVD separately, thus limiting causal interpretation.

As a first step, we have replicated previous observations of the positive association between chronic exposure to air pollution (NO_2, NO_x, both back-extrapolated, and $PM_{2.5}$) and CCVD risk (Cesaroni et al., 2014; Peng et al., 2016). We used an update of the ESCAPE estimates, based on Europe-wide models (Nunen et al., 2017) in addition to the previously developed LUR models.

The main aim of this study was to elucidate the biological mechanisms linking exposure to air pollution to CCVD risk and to identify intermediate biomarkers. With these goals, we designed a case-control study nested in the EPIC Turin and Varese cohort. Since it has been shown that smoking habits confound the association of air pollution with CCVD (Sheppard et al., 2012), our study sample includes nonsmoking CCVD incident cases (and matched controls) only. Specifically, we selected all the nonsmokers (including never and former smokers for at least one year) experiencing a CCVD event during the follow-up period (up to 17 years), for whom archived blood sample was available, and at least one matched control existed in the cohort. We measured a set of inflammatory proteins and whole-genome DNA methylation in blood collected several years (between 3 months and 17 years; 12 years on average) before CCVD diagnosis. We have applied a longitudinal design, previously defined as "meet-in-the-middle" approach (Vineis et al., 2013) to investigate the relationship between air pollution, inflammatory biomarkers and CCVD onset. The "meet-in-the-middle" analysis aims to identify biomarkers associated with both air pollution measures (retrospectively) and the risk of CCVD (prospectively), the latter association being adjusted for air pollution measures.

DNA Methylation Biomarkers

For epigenetic biomarkers analysis, we focused on 17 *a priori* defined inflammatory pathways (DNAmIPs) and we tested for overrepresentation (enrichment) of altered DNAm levels, compared with those expected by chance. We have chosen this approach because we had limited statistical power to identify single methylation probes associated to CCVD and air pollutants in a genome-wide

study. According to the study by Tsai and Bell (Tsai and Bell, 2015), our sample size (less than 200 case-control matched pairs) allowed us to reach a statistical power of at least 80% (that is 20% probability of type II error), only with differences in DNAm β values higher than 10%, considering the genome-wide significance level. Such difference is highly unlikely to be observed in a prospective study (all the individuals were healthy at the time of sample collection) conducted on blood samples. As an example, a systematic review on the role of epigenetic modifications in cardiovascular disease by Muka et al. (Muka et al., 2016) indicates the association between hypo-methylation in *F2RL3* gene and the risk of CVD mortality as the most consistent epigenetic association (related to CVD) found in the recent literature so far. However, the average differences between cases and healthy controls were lower than 10%. Further, hypomethylation at *F2RL3* gene was strongly associated with smoking habits (Zhang et al., 2014; Allione et al., 2015; Fasanelli et al., 2015), a known risk factor for CCVD, suggesting *F2RL3* gene as a potential mediator in the association of smoking with CCVD (Breitling et al., 2012). The evidence above suggested us to consider the results of the single CpG analyses carefully. Instead, we hypothesized that a general dysregulation of DNAm levels in genes related to oxidative stress and inflammation occurs as a consequence of exposure to pollutants and that, in turn, such dysregulation could be associated with an increased risk of CCVD. To test this hypothesis, we selected candidate genes based on a list of the main inflammation-related genes proposed by Loza et al. (Loza et al., 2007). They reviewed various phases of inflammation responses, including the development of immune cells, sensing of danger, influx of cells to sites of insult, activation and functional responses of immune and non-immune cells, and resolution of the immune response, and identified 17 functional pathways that are involved in one or multiple stages. In the case our hypothesis is correct, although we have not the power to detect a single differentially methylated CpG, we expected to find an overrepresentation of nominally significant CpGs among those pertaining to the genes described by Loza et al., compared with the CpGs distributed in the rest of the genome. Given the above, a suitable way to test our *a priori* hypothesis was to run enrichment analyses on the set of candidate pathways. Recently, Geeleher and colleagues reported strong bias for gene set analyses when applied to methylation data (Geeleher et al., 2013). The most popular available tools for gene set enrichment (Subramanian et al., 2007; Huang da et al., 2009) were built for the analysis of gene expression experiments and are severely biased when applied to methylation data, as a result of differences in the numbers of CpG sites associated with different classes of genes and gene promoters. Moreover not all the changes in DNA methylation are clearly associated with changes in gene expression of the same transcript, but the relationship is much more complex (van Eijk et al., 2012). An alternative method to test for over-representation of significant signals in a set of probes of interest was proposed by Charmpi and Ycart (Charmpi and Ycart, 2015). This method compares the distribution of the test statistics for a set of probes of interest with the empirical distribution estimated under the null hypothesis of no association, the latter being estimated from genome-wide results. We chose this approach because it takes into account correlation patterns among probes, it is not biased when used for DNA methylation data, and above all, allowed us to test for enrichment of user-defined pathways.

Results of enrichment analysis highlighted two DNAmIPs associated with both air pollution and CCVD, specifically "ROS/Glutathione/Cytotoxic granules" and "Cytokine signaling" pathways. Both pathways are strongly related to oxidative damage and are relevant in the light of previously proposed mechanisms (Reuter et al., 2010; Muralidharan and Mandrekar, 2013). According to the review by Newby and colleagues (Newby et al., 2015), during exposure to airborne pollutants the normal phagocytes of the lung surfaces and, to an extent, the epithelial cells generate oxygen radicals and can become oxidatively stressed. We showed that not only these pathways were associated in a statistically significant manner to exposure to air pollutants, but they were also associated with the onset of CCVD longitudinally.

Protein Biomarkers

Among the inflammatory proteins we have investigated, Interleukin 17 (IL17) in circulating plasma was identified as an intermediate biomarker, being higher concentrations associated with both CCVD risk and exposure to pollutants. Interestingly, IL17 was also significantly inversely associated with time to disease (TTD), indicating an increasing difference in circulating levels of IL17 between cases and matched controls with decreasing time between the date of blood sampling and the date of the CCVD event (in cases). IL17 belongs to the "Cytokine signaling" pathway according to Loza and colleagues (Loza et al., 2007), confirming the results obtained using DNAm data. The involvement of IL17 in the pathogenesis of CCVD was recently explained via amplification of the inflammation induced by other cytokines (Ding et al., 2012). It was also showed that IL17 plays a role in the atherosclerotic process (Chen et al., 2010; Gong et al., 2015), that is in turn related to CCVD onset.

Association between Protein and DNAm Biomarkers

Finally, we have investigated the association of protein and DNAm biomarkers, using the same statistical approach described to study the relationship of DNAm

with air pollution exposure and CCVD risk (i.e., the WKS enrichment algorithm). We found several statistically significant associations, being all but two out of 13 protein biomarkers associated with at least one DNAmIP (Supporting Information Table SIII). Our results reinforce previous knowledge about DNAm regulation of inflammatory biomarkers (Sabunciyan et al., 2015; Ligthart et al., 2016; van Otterdijk et al., 2017), including cytokines (Verschoor et al., 2017) and interleukins (Takahashi et al., 2015), and support the usefulness of omic profiling in peripheral blood, for the early identification of disease-related perturbations caused by toxic exposures (Georgiadis et al., 2016).

Strengths and Limitations

This study has limitations: the small sample size limited the standard EWAS analysis looking for single CpGs associated with air pollution. This investigation is ongoing in the context of the EXPOsOMICS project in which we have the appropriate sample size to identify robust DNA methylation signals associated with air pollution exposure. Further, knowing the role of inflammation in allergic/respiratory diseases (Galli et al., 2008; Murdoch and Lloyd, 2010), and the co-morbidity with CCVD (Triggiani et al., 2008; Iribarren et al., 2012; Bellocchia et al., 2013), we cannot exclude that our findings could be partly related to allergic- and respiratory-related epigenetic alterations.

The study also has strengths, particularly the inclusion of all incident cases (with available archived blood sample) arising in 17 years of follow-up of the EPIC study among never smokers or former smokers, and the use of prediagnostic blood samples for biomarker analysis helping causal interpretation of the results.

CONCLUSIONS

We focused on the *a priori* hypothesis that oxidative stress-induced inflammation is one of the principal mechanisms involved in the association of air pollution with CCVD. Our findings help in the understanding of the causal chain linking chronic exposure to air pollution with CCVD risk, suggesting oxidative stress as the primary pathway, that in turn activates a series of inflammatory responses, mainly involving the "Cytokine signaling" pathway. DNA methylation dysregulation induced by chronic exposure to pollutants contribute to inflammatory proteins (above all cytokines and interleukins) alterations.

This study results contribute to disentangle the relationship between exposure to air pollution and increased risk for CCVD, and provide evidence that altered levels of cytokine inflammatory proteins and changes in DNAm of key inflammatory genes can be detected several years before CCVD diagnosis in blood samples, being promising preclinical biomarkers for CCVD.

AUTHOR CONTRIBUTION

GF, JV, RV, ZH, PV, AG, and AN contributed to the study design, data analysis and drafted the manuscript. SP, CG, AR, VK, SG, CA, CS, SP, MYT, contributed to data collection and analysis. JG, NPH, GH contributed to draft and revise the manuscript. All authors approved the final manuscript.

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DECLARATION OF COMPETING FOR FINANCIAL INTERESTS

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

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