Inflammatory markers in relation to long-term air pollution

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Abstract

Long-term exposure to ambient air pollution can lead to chronic health effects such as cancer, cardiovascular and respiratory disease. Systemic inflammation has been hypothesized as a putative biological mechanism contributing to these adverse health effects. We evaluated the effect of long-term exposure to air pollution on blood markers of systemic inflammation.

We measured a panel of 28 inflammatory markers in peripheral blood samples from 587 individuals that were biobanked as part of a prospective study. Participants were from Varese and Turin (Italy) and Umea (Sweden). Long-term air pollution estimates of nitrogen oxides (NO_x) were available from the European Study of Cohorts for Air Pollution Effects (ESCAPE). Linear mixed models adjusted for potential confounders were applied to assess the association between NO_x and the markers of inflammation.

Long-term exposure to NO_x was associated with decreased levels of interleukin (IL)-2, IL-8, IL-10 and tumor necrosis factor- α in Italy, but not in Sweden. NO_x exposure levels were considerably lower in Sweden than in Italy (Sweden: median (5th, 95th percentile) $6.65\mu\text{g}/\text{m}^3$; (4.8, 19.7); Italy: median (5th, 95th percentile) $94.2\mu\text{g}/\text{m}^3$ (7.8, 124.5)). Combining data from Italy and Sweden we only observed a significant association between long-term exposure to NO_x and decreased levels of circulating IL-8.

We observed some indication for perturbations in the inflammatory markers due to long-term exposure to NO_x . Effects were stronger in Italy than in Sweden, potentially reflecting the difference in air pollution levels between the two cohorts.

Keywords: Air pollution, inflammatory markers, gene expression, chronic health effects

1. Introduction

Epidemiologic studies have consistently shown an association between long-term exposure to ambient air pollution and cardiovascular mortality and morbidity, non-malignant respiratory diseases (Brunekreef and Holgate 2002; Hoek et al. 2013), and lung cancer (Demetriou et al. 2012). Although the exact mechanisms behind the observed associations are not clear, long-term pulmonary oxidative stress and inflammation induced by chronic exposure to inhaled pollutants has been hypothesized to result in a systemic inflammatory state capable of activating hemostatic pathways, impairing vascular function, and accelerating atherosclerosis (Brook et al. 2010; Rückerl et al. 2007).

The link between the inflammatory process in the lung and the systemic response is thought to be mediated by markers of inflammation released by alveolar macrophage and bronchial epithelial cells in response to exposure to ambient air pollution and capable of entering the systemic circulation and stimulating the production of acute-phase proteins (Demetriou et al. 2012; Hoffman et al. 2009). Inflammatory markers in peripheral blood might therefore reflect deregulation resulting from chronic exposure to ambient air pollution (Dubowsky et al. 2006, van Eeden et al. 2001).

A number of studies have reported on acute changes in blood markers of inflammation in response to day-to-day variability in air pollution. These studies have generally reported inconsistent results. Effects of short-term exposure to ambient air pollutants on the levels of fibrinogen have been reported, either with a negative association (in response to PM₁₀ or SO₂) (Panasevich et al. 2009; Seaton et al. 1999; Steenhof et al. 2013), or a positive association (in response to PM₁₀ or O₃) (Rückerl et al. 2007). Short-term exposure to particulate matter has been associated with an increase in IL-6 levels (van Eeden et al. 2001) or shown no relationship (Panasevich et al. 2009; Seaton et al. 1999; Zuurbier and Hoek 2011). A positive

association with TNF- α levels (in response to PM₁₀, NO₂) was reported by (Panasevich et al. 2009; Tsai et al. 2012), but Larsson et al. reported no association with diesel exhaust (Larsson et al. 2013).

We identified four studies that reported on the association between markers of long-term exposure to air pollution and chronic perturbations of blood inflammatory markers (Table S1) (Chuang et al. 2011; Forbes et al. 2009; Hoffman et al. 2009; Panasevich et al. 2009). All studies assessed a small set of blood markers of inflammation. Panasevich et al. (2009) assessed four markers (IL-6, TNF- α , CRP, fibrinogen) and reported significantly higher IL-6 levels and positive effect estimates of CRP levels after long term exposure to elevated residential levels of 30-year average traffic-related NO₂. Hoffmann et al. (2009) assessed two markers of inflammation (CRP, fibrinogen) and reported a positive association between annual particulate matter (PM_{2.5}) and CRP and fibrinogen (an increase of 16.7% in CRP and 2.4% in fibrinogen for a unit increase in PM_{2.5}). Forbes et al. (2009) also explored the association between estimates of long-term exposure to ambient air pollution and fibrinogen and CRP, but observed no associations. Chuang et al. (2011) assessed two markers of inflammation (IL-6 and neutrophils) and reported that an increase in annual average particulate air pollutant (PM₁₀ and PM_{2.5}) and NO₂ was marginally ($p < 0.1$) associated with elevated IL-6 and neutrophils.

We assessed the association between long-term exposure to nitrogen oxides (NO_x) and plasma concentration of a large panel of cytokines, chemokines, and growth factors in a sample of the general population. Our study contributes to the existing literature by combining a wide range of inflammatory markers (n=28) measured in a large number of individuals (n=587) with state of the art long-term air pollution exposure assessment. By comparing two prospective cohorts from Italy and Sweden, in which we applied the same exposure assessment strategy, we were

able to study the association over a wide-range of air pollution exposure and assess the between-cohort heterogeneity of our findings. In addition, in a sensitivity analysis, we restricted our analyses to the elderly and the overweight, to assess whether we observed stronger effects among individuals that have been reported to have a higher susceptibility to develop air pollution induced cardiovascular(Bentayeb et al. 2012) and other health effects (Dubowsky et al. 2006; Rckerl et al. 2007; Simoni et al. 2015).

2. Materials and methods

For the current study we combined data from two existing projects: inflammatory markers from Genomics Biomarkers of Environmental Health (EnviroGenoMarkers) (Chadeau-Hyam et al. 2014; Hebels et al. 2013) and NO_x data from the European Study of Cohorts for Air Pollution Effects (ESCAPE) (Beelen et al. 2013).

2.1. Study population

EnviroGenoMarkers was set up to investigate the association between a set of environmental agents (polychlorinated biphenyls, polycyclic aromatic hydrocarbons, cadmium, lead, phthalates, brominated flame retardants, ambient air pollutants and water treatment byproducts), intermediate OMICS markers (metabolomics, epigenomics, proteomics and transcriptomics), and various human diseases (Chadeau-Hyam et al. 2014). It has a nested case-control design, using biosamples of two prospective cohorts: the Italian contribution to the European Prospective Investigation into Cancer and Nutrition study (EPIC-Italy) (Palli et al. 2003), and the Northern Sweden Health and Disease Study (NSHDS) (Hallmans et al. 2003). EPIC-Italy was initiated in 1992 and completed the recruitment of 15,171 men and 32,578 women healthy middle-aged (35-70 years old) volunteers in 4 different areas of Italy: Varese, Turin, Ragusa, and Florence (Palli et al. 2003). The NSHDS cohort contains 3 sub-cohorts: the Västerbotten Intervention program (VIP), the Västerbotten Mammary Screening (MS) Program and the Northern Sweden MONICA project. It started in 1985 and the total cohort and biobank contains at present 85,000 unique healthy individuals (Hallmans et al. 2003). At the time of recruitment (around 2002), cohort members from both studies completed a standardized questionnaire focusing on dietary and life-style habits and donated blood.

The EnviroGenoMarkers data included in the current study were collected in two phases. In the first phase 100 Non-Hodgkin's lymphoma cases, 100 breast cancer cases, were identified through local Cancer Registries (loss to follow-up < 2%) and occurred on average 6 years (range 1 to 17 years) after recruitment/blood collection, and the same number of controls matched on sex, age, center and date of blood collection were included (Chadeau-Hyam et al. 2014). The lymphoma case-control data were subsequently supplemented with samples from additional cases (147 cases in NSHDS, 34 cases in EPIC-Italy) and equal number of similarly matched controls (phase 2) (Chadeau-Hyam et al. 2014; Kelly et al. 2013).

Our study population, a subset of the EnviroGenoMarkers data, includes 97 individuals (23 men, and 74 women) from two centers participating in the EPIC-Italy cohort (Turin and Varese) and 490 individuals (210 men, and 280 women) from the NSHDS cohort (Umeå). Our selection from the NSHDS cohort includes 195 future Non-Hodgkin's lymphoma cases (49 from phase 1 and 146 from phase 2), 50 future breast cancer cases, and 245 controls matched on sex, age, center and date of blood collection. Our selection from the EPIC-Italy cohort includes 38 future Non-Hodgkin's lymphoma cases (24 from phase 1 and 14 from phase 2), 12 future breast cancer cases, and 47 controls matched on sex, age, center and date of blood collection. The average time to diagnosis for breast cancer cases was 6 years (range 2 to 10 years) and for lymphoma cases was 6 years (range 2 to 16 years) after blood collection.

2.2. Cytokine measurements

Plasma levels of a panel of 28 inflammatory markers including interleukin (IL) 1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, interferon alpha (INF- α), interferon gamma (INF- γ), tumor necrosis factor alpha (TNF- α), eotaxin, IL1 receptor antagonist (IL1ra), interferon gamma-induced protein 10 (IP10), granulocyte-macrophage colony-stimulating factor (GMCSF), epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), fms-like tyrosine kinase receptor-3 (Flt3) ligand protein (Flt3 ligand), granulocyte colony-stimulating

factor (GCSF), melanoma growth stimulatory activity/growth-related oncogene (GRO), monocyte chemotactic protein-1 (MCP-1), monocyte chemotactic protein-3 (MCP-3), macrophage derived chemokine (MDC), macrophage inflammatory protein-1 alpha (MIP-1 α), macrophage inflammatory protein-1 beta (MIP-1 β), soluble CD40 ligand (sCD40L), soluble IL-2 receptor alpha (sIL2R α), transforming growth factor alpha (TGF- α), and vascular endothelial growth factor (VEGF) were measured in stored samples of all subjects using the milliplex hcytomag-60 19.Hum and hcytomag-60SPMX13 kits, according to the protocol described by the manufacturer (Saber Hosnijeh et al. 2012). Quality control samples were run in duplicate with the study samples in each batch (Saber Hosnijeh et al. 2010).

Samples from EPIC-Italy contained citrate as anticoagulant and had been stored in cryostraws in liquid nitrogen for 11–19 years. Their recorded collection-to-storage times were 55–347 min. Samples from NSHDS contained heparin or EDTA as anticoagulant and had been stored in plastic cryovials at –80 C for 4–19 years. Blood samples were generally collected in the morning and their collection-to-storage time was always < 1 hour (Hebels et al. 2013). A previous analysis showed that samples not cold-stored within 2 h after blood collection had significantly different expression profiles than fresh samples, and therefore only peripheral blood mononuclear cells samples that had been placed in cold storage within 2 h after blood collection were included in the current study (Chadeau-Hyam et al. 2014).

2.3. Exposure assessment

The European Study of Cohorts for Air Pollution Effects (ESCAPE) study was set up to investigate the effect of long-term exposure to ambient air pollution on human health in 15 European countries (Beelen et al. 2013; Cyrys et al. 2012). ESCAPE measurements, includes 36 cohorts which study areas cover Europe. Exposure levels were estimated using land use regression models that were developed using exposure measurements conducted between October 2008 and April 2011 (Beelen et al. 2013). Study area specific land use regression

models were used to assign estimates of long-term exposure to the cohort participants home-address. Land use regression models were developed combining exposure measurements conducted at a restricted number of sites (40 to 80) covering each study area with Geographic Information System (GIS) based predictors. Exposure to nitrogen oxides (NO_2 and NO_x) was assessed for all study areas participating in ESCAPE, while exposure to particulate matter ($\text{PM}_{2.5}$, $\text{PM}_{2.5}$ absorbance and PM_{10}) was assessed for a subset of study areas that contributed to the contrast in exposure in the ESCAPE dataset. Of the three study areas that were included in EnviroGenoMarkers (Umeå, Turin, and Varese) particulate matter estimates were only available for 13 subjects from Turin (Beelen et al. 2013). We therefore based our analyses on NO_x . In the cities included in the current analysis the squared correlations (R^2) between NO_x and NO_2 ranged between 0.94 and 0.97. The NO_2/NO_x ratio was 0.57, 0.54, 0.53 for Umea, Turin, Varese areas, respectively (Cyrus et al. 2012).

2.4. Data analysis

For four markers of inflammation (IL-12, IL1-RA, sIL2-RA, and Flt3 ligand) more than 50% of the samples were below the limit of detection (LOD). We excluded these markers from our analyses. Data were imputed when measurements were out of range of the calibration curve (either too low: $<\text{LOD}$, or too high) based on a maximum likelihood estimation procedure (Lubin et al. 2004). For imputation of samples $<\text{LOD}$ we imputed using the empirical LOD across all plates as the upper bound. For imputation of samples with a concentration exceeding the calibration curve we used a value of the twice the highest observed concentration that was not out of range as the upper bound. Among the analytes that were retained, the maximum percentage of imputed values was 47.7% (IL-13), while 70% of the retained markers had less than 30% imputed values.

For six individuals in the Varese population unrealistically low NO_x exposure estimates ($<0.16 \mu\text{g}/\text{m}^3$) were predicted by the ESCAPE model. We set these values at the 2.5th

percentile of the distribution of NO_x exposure estimates in the full Varese study population also including individuals for which markers of inflammation were not assessed (7.81 µg/m³). We assessed the sensitivity of this decision by replacing the 2.5th percentile by either the 1.5th or the 5th percentile of the distribution and observed that our results were robust.

We conducted linear mixed-effects modeling to investigate the association between long-term exposure to NO_x and markers of inflammation. We included random intercepts for microtiter plate in the model to capture nuisance variation generated in the assessment of the inflammatory markers (clustering of the inflammatory markers measurements by plate) (McHale et al. 2011) and included fixed-effects for NO_x and for a priori selected potential confounding factors, i.e., body mass index (BMI) (kg/m²), age (in three categories: (30,40], (40,50], (50,75], years), sex (female, male), smoking status (never, former, current), phase (1 and 2), future disease status (lymphoma case, breast cancer case, control) and sample storage time (years) consistent with previous analyses of the EnviroGenoMarkers data (Chadeau-Hyam et al. 2014; Kelly et al. 2013). As a sensitivity analysis, we explored the potential for confounding by the remaining factors available in our dataset including socio-economic status (education level (primary, technical, secondary, university)), and physical activity (moderately inactive, moderately active, active).

Due to differences in dietary habits, lifestyle, and air pollution exposure levels between the Italian and Swedish cohorts, we stratified our analyses by cohort (Table 1; Figure 1). As there was some overlap in the exposure distributions of the two cohorts, we also conducted analyses on the combined cohorts, while adjusting for country (Figure 1).

As sensitivity analysis we explored the association between NO_x and markers of inflammation in two potentially susceptible strata: among overweight individuals (BMI>25; n=323) and among individuals older than age 50; n=383. In addition, we performed analyses among never

smokers (n=353) to assess the effect of potential residual confounding by active smoking, and among controls only (n=292) to assess the effect of potential bias due to early manifestations of future disease.

We natural log transformed NO_x and markers of inflammation to limit the influence of high concentrations and normalize distributions. We computed unadjusted p-values and adjusted p-values for multiple testing (q values) by controlling the false discovery rate (FDR) at 5%. We used penalized splines (P-spline) in the generalized additive mixed-model (GAMM) framework to assess potential non-linearity of the relationships between inflammatory markers and exposure.

Correlation coefficients between all markers of inflammation were assessed using Pearson correlation. A two-sided q-value < 0.05 was considered statistically significant. Statistical analyses were performed using R version 3.0.2 (package lme4).

3. Results

We summarized the general characteristics of the study population by cohort in Table 1. The Swedish cohort had a lower proportion of women than the Italian cohort (57% versus 76%), a higher proportion of current smokers (21% versus 8%) and a lower proportion of never smokers (59% versus 66%). We observed a considerable difference in the distribution of NO_x concentrations between the two cohorts (Table 1; Figure 1). The median (5th, 95th) concentration of NO_x estimated for the Italian cohort (94.2 µg/m³ (7.8, 124.5)) was considerably higher than the median concentration estimated for the Swedish cohort (6.65 µg/m³ (4.8, 19.7)) ($p < 0.001$).

Univariate mixed-effect regression analyses yielded evidence for an association between long-term exposure to NO_x and several markers of inflammation. In Table 2 we report the significant effects and in Table S2 we report the results for the complete set of inflammatory markers. Our strongest finding is for IL-8, for which we observed a significant negative association in the Italian cohort as well as in the combined cohort (q-values of 0.001 and 0.02, respectively). For an inter quartile change in NO_x concentration in the combined cohort (10 µg/m³) IL-8 decreased 5.1 pg/mL (17% decrease). Although a negative association between NO_x and IL-8 was observed in the Swedish cohort as well, this association was not significant ($q = 0.655$). For several other markers we observed significant negative associations with long-term exposure to NO_x in the Italian cohort, but not in the Swedish or combined cohorts: IL-2 ($q = 0.012$), TNF- α ($q = 0.020$), IL-10 ($q = 0.017$). For IL-10 the non-significant association observed in the combined cohorts was in the same direction as it was in the Italian cohort ($q = 0.083$).

We observed little influence of the potential confounding factors on the effect of NO_x on the markers of inflammation (the effect size did not change after adjustment for the potential confounding factors).

Results from sensitivity analyses among never-smokers (Table 3 for the significant effects and Table S3 for the complete set of markers) and among controls only (see supplemental material, Table S7) were similar, though with larger standard errors, compared to the results from our main analyses, suggesting limited impact of potential residual confounding of smoking or future (lymphoma or breast cancer) case status. We assessed the impact on our results of additional correction for further covariates available in the EnviroGenoMarkers dataset, but observed no considerable changes in effect estimates (<5% for the markers of inflammation for which we observed a significant association with NO_x; results not shown). We did not observe any evidence for deviation from linearity for the markers significantly associated with NO_x at the log scale (results not shown).

The associations between NO_x and the inflammatory markers within different subgroups of the combined population are presented in Table 3 (cohort-specific results are presented in supplemental material, Tables S4, S5, and S6). In the stratified model, the size of the effect of NO_x on several inflammatory markers (especially IL-8) was often larger in overweight individuals than in non-overweight individuals. We observed smaller effect sizes of NO_x on the markers of inflammation among elderly individuals. However, we did not observe a significant interaction between NO_x and BMI or age in the unstratified models.

283 **Table 1. Descriptive characteristics of the study population**

Characteristics	N (%) or Mean/Median (SD ^a)	
	Swedish cohort (n=490)	Italian cohort (n=97)
Sex (N (%))		
Female	280(57%)	74(76%)
Male	210(43%)	23(24%)
Smoking Status (N (%))		
Current- smoker	104(21%)	8(8%)
Former- smoker	97(20%)	25(26%)
Never-smoker	289(59%)	64(66%)
Age (years)(N (%))		
<40	29(6%)	2(2%)
40-50	143(29%)	30(31%)
>50	318(65%)	65(67%)
BMI ^b (kg/m ²) (Mean $\pm$ SD [*])	26.1 $\pm$ 4.1	25.7(3.8)
NO _x ^c (µg/m ³) (Median (SD [*]))	6.65 (5.84)	94.2(42.4)

284 ^aSD, Standard deviation; ^bBMI, Body mass index; ^cNO_x, Nitrogen oxide

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Fig1. Box plot (left) and density plot (right) of log (NO_x) (μg/m³) exposure levels observed in the Swedish and Italian cohorts.

Table2. Significant effects of long-term exposure to NO_x on markers of inflammation adjusted for potential confounders

Inflammatory markers	Combined cohort (n=587)				Swedish cohort (n=490)				Italian cohort (n=97)			
	β^e	SE ^a	P-Value	Q-Value ^b	β	SE	P-Value	Q-Value	β	SE	P-Value	Q-Value
IL ^c -2	-0.058	0.118	0.623	0.703	-0.019	0.144	0.896	0.896	-0.421	0.143	0.003	0.012
IL-8	-0.179	0.068	0.008	0.020	-0.079	0.076	0.295	0.655	-0.520	0.133	0.000	0.001
IL-10	-0.256	0.116	0.028	0.083	-0.104	0.142	0.467	0.619	-0.690	0.218	0.002	0.017
TNF ^d - α	-0.028	0.057	0.620	0.643	0.036	0.073	0.625	0.689	-0.213	0.069	0.002	0.020

^aSE, standard error of β ; ^bQ-Value, False Discovery Rate correction for P-Value, ^cInterleukin (IL), and ^dtumor necrosis factor alpha (TNF- α), ^eeffect estimate per unit changes of the exposure

Results are based on linear mixed-effects models of log-transformed dependent and independent variables with fixed-effects: body mass index (BMI) (kg/m²), age (years), sex (female, male), smoking status (never, former, current), phase (1 and 2), future disease status (lymphoma case, breast cancer case, control) and sample storage time (years). And plate as random effects.

Table3. Significant effects of long-term exposure to NO_x on markers of inflammation within 3 sub-groups (overweight, elderly, and never-smokers)

Inflammatory markers	Overweight (n=323)				Elderly (n=383)				Never-smokers (n=353)			
	β^e	SE ^a	P-Value	Q-Value ^b	β	SE	P-Value	Q-Value	β	SE	P-Value	Q-Value
IL ^c -2	-0.073	0.144	0.613	0.843	-0.080	0.139	0.566	0.778	0.104	0.154	0.502	0.628
IL-8	-0.254	0.078	0.001	0.004	-0.039	0.078	0.619	0.847	-0.113	0.093	0.226	0.282
IL-10	-0.270	0.131	0.040	0.073	-0.301	0.135	0.026	0.071	-0.180	0.160	0.262	0.427
TNF ^d - α	0.032	0.067	0.629	0.766	0.012	0.069	0.866	0.951	0.017	0.068	0.803	0.882

^aSE, standard error of β ; ^bQ-Value, False Discovery Rate correction for P-Value, ^cInterleukin (IL), and ^dtumor necrosis factor alpha (TNF- α), ^eeffect estimate per unit changes of the exposure

Results are based on linear mixed-effects models of log-transformed dependent and independent variables. Sex (female, male), phase (1 and 2), future disease status (lymphoma case, breast cancer case, control) and sample storage time (years) were included as fixed effects and plate as random effect.

4. Discussion

This study provides some evidence for a perturbation of systemic inflammatory markers due to long-term exposure to NO_x in a cohort of healthy Italian and Swedish individuals. We observed significantly decreased levels of IL-8 in the combined cohort and for three other inflammatory markers in the Italian sub-cohort, but observed no evidence for any association in the Swedish sub-cohort.

Our finding of an inverse association between long-term exposure to air pollution and IL-2, IL-8, IL-10, and TNF- α is in contrast to results from most previous studies of long-term exposure to air pollution and markers of inflammation, which have generally reported positive associations (Hofmann et al. 2011; Panasevich et al. 2009). Similarly, studies focused on the biological mechanisms of these markers of inflammation have reported a primarily pro-inflammatory role. We summarize the current knowledge below. IL-2 performs critical functions for the elimination of diseased cells, including promotion of T and natural killer (NK) cells, cytolytic activities, and regulation of naive T cell differentiation into Th1 and Th2 subsets upon exposure to antigens (Liao et al. 2013). IL-8 is produced under inflammation stimulation and is attracting and activating neutrophilic granulocytes (Zarogoulidis et al. 2014). TNF- α is involved in the innate immune response (Clark 2007). IL-10 is an immunoregulatory cytokine mainly secreted by macrophages, but also by T helper 1 (Th1) and Th2 lymphocytes, dendritic cells, cytotoxic T cells, B lymphocytes, monocytes and mast cells. IL-10 inhibits the capacity of monocytes and macrophages to present antigen to T cells and therefore downregulates the expression of IL-1 β , IL-6, IL-8, IL-12 and TNF- α (Trifunović et al. 2015). Although we do observe downregulation of IL-8 and TNF- α in our study, this finding does not correspond to the observation that IL-10 is downregulated as well. Interestingly, a study by Forbes et al. (2009) also observed negative associations between long-term exposure to air pollution and markers of inflammation (fibrinogen and C-reactive

protein), triggering Forbes et al. (2009) to hypothesize that the health effects of chronic outdoor air pollution are not mediated by systemic inflammation. Some short-term air pollution studies have also reported inverse associations with selected markers of inflammation (e.g. (Seaton et al. 1999) (Fibrinogen), and (Rückerl et al. 2007), (IL-6)). Reasons for these inverse associations remain unclear. Furthermore, similar inverse patterns have been observed in relation to other exposures such as endotoxin (Lauw et al. 2000) and carbon monoxide (Morse et al. 2003) and can be seen as indicative of a de-regulation of the inflammatory system.

Our study was subject to some (potential) limitations. One potential limitation is related to the air pollution exposure metric we used in our analyses, as only NO_x exposure estimates were available. Although the correlation of spatial variation in NO_x and particulate matter is often high (and was high ($p = 0.83$) in the ESCAPE study from which we derived our exposure estimates (Eeftens et al. 2012)), generally stronger associations with chronic health effects have been observed for particulate matter (especially PM_{2.5}) than for NO_x (Beelen et al. 2014) and particulate matter has been suggested to be a better marker for ambient air pollution than NO_x (Hoek et al. 2013). Another limitation of our exposure metric, related to the design of ESCAPE, is the fact that current exposure levels were used to assign exposure for historical home addresses (in some cases more than 20 years retrospectively), which might have introduced a certain degree of measurement error. However, several studies have documented that the land use regression models can be applied successfully to estimate air pollution concentrations several years forwards or backwards in time. Although the absolute level of exposure to air pollution has generally decreased over time, spatial contrasts for NO₂ have been shown to remain stable over long periods of time (10 years and longer) (Cesaroni et al. 2012; Gulliver et al. 2013; Wang et al. 2013; Eeftens et al. 2011). Note, we performed a sensitivity analyses within the Swedish part of the study using back-extrapolated NO_x

exposure levels at time of the blood draw using the methodology described in (Beelen et al. 2014). Results of these analyses did not provide any different results. The markers of inflammation that we included in our analysis are subject to variability over time (e.g. diurnal and seasonal variation), likely contributing to increased standard errors (Forbes et al. 2009). Several studies have shown examples of considerably larger between-person variability in markers of inflammation than within-person variability, suggesting that single measurements of circulating inflammatory markers provide information about long-term perturbation of the inflammatory markers (Hofmann et al. 2011; Navarro et al. 2012).

Due to the nested case-control design of the EnviroGenoMarkers study our study population included future cancer cases (individuals that were diagnosed with lymphoma or breast cancer in the years following biological sampling) and matched controls. A potential implication of this design for our study is a perturbation of the inflammatory markers due to pre-diagnostic manifestation of the disease among cases. However our results did not change significantly when we restricted our analyses to controls only. In addition, the fact that our study population cannot be viewed as a random sample from the general population might have reduced the external validity of our findings (but should not have any impact on the internal validity).

We observed stronger associations in the Italian cohort than in the Swedish cohort. Although the exposure assessment strategy in both cohorts was the same, the cohorts differed with regards to certain aspects. The difference in the air pollution exposure level between the two cohorts likely contributed considerably to the level of statistical significance (but not the direction) of the observed association. Although most of the individuals in our analyses were included from the Swedish cohort, absolute exposure levels and the exposure contrast in that cohort were low (median $6.65\mu\text{g}/\text{m}^3$, SD 5.8), compared to the Italian cohort (median $94.21\mu\text{g}/\text{m}^3$, SD 43.0). Furthermore NO_x exposure levels observed in the Swedish cohort were the

lowest observed among the 36 European regions for which exposure to NO_x was estimated in the ESCAPE study, while exposure levels observed for the Italian cohort (especially the Turin cohort) were among the highest (Cyrys et al. 2012). If we restricted the analyses of the Swedish cohort to subjects exposed to NO_x levels higher than the lowest 5th percentile (7.8 µg/m³) of the Italian cohort, we did not observe a more homogeneous picture.

Differences between the cohorts also existed with regards to age, sex, BMI, and smoking behavior. As we controlled for these aspects in our analyses, a large impact on our results is unlikely. Further (not a priori selected) factors available in our dataset including, socioeconomic status, dietary patterns, and physical activity, did not significantly explain variability in markers of inflammation within the two cohorts independently and are therefore unlikely responsible for the observed differences between the cohorts. The cohorts differed with regards to the anticoagulant that was used in the blood samples (EDTA in Sweden, citrate in Italy). Although the use of different anticoagulants results in absolute differences in levels of inflammatory markers (See Table S8 for the variance of the concentration of each inflammation marker for the Swedish and Italian cohorts separately), correlations between measurements in split samples simultaneously treated with heparin, citrate, and EDTA have shown to be highly correlated (Hebels et al. 2013; Wong et al. 2008). The difference between the Swedish and the Italian cohort with respect to the anticoagulant that was used, for which we corrected in the statistical models on the combined dataset by adjusting for cohort, is therefore an unlikely explanation for the observed between-cohort differences in our results.

Our analysis among two groups of potentially susceptible populations (individuals with a BMI > 25 or individuals older than age 50) provided some indication (though not significant) for a larger effect among the overweight, but not among the individuals older than age 50. As individuals with conditions associated with chronic inflammation such as older or overweight individuals have been shown to have enhanced susceptibility for air pollution related health

effects (Dubowsky et al. 2006; Rückerl et al. 2007), we a-priori assumed a larger effect size in these subgroups compared to the overall population. For the overweight this was indeed the case. However, among the elderly we observed a smaller effect size compared to the effect size observed in the overall population. Considering the unexpected inverse relationship that we observed between exposure to air pollution and cytokine production, further biological interpretation of these patterns in the effect sizes is not evident.

5. Conclusion

Our results suggested some indication of an inverse association between long-term NO_x exposure and four systemic inflammatory markers: IL-2, IL-8, IL10, and TNF- α . These results might contribute to a future elucidation of the pathways through which long term exposure to air pollution induces adverse health effects.

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