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The impact of ambient air pollution on the human blood metabolome

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ABSTRACT

Background: Biological perturbations caused by air pollution might be reflected in the compounds present in blood originating from air pollutants and endogenous metabolites influenced by air pollution (defined here as part of the blood metabolome). We aimed to assess the perturbation of the blood metabolome in response to short term exposure to air pollution.

Methods: We exposed 31 healthy volunteers to ambient air pollution for 5 h. We measured exposure to particulate matter, particle number concentrations, absorbance, elemental/organic carbon, trace metals, secondary inorganic components, endotoxin content, gaseous pollutants, and particulate matter oxidative potential. We collected blood from the participants 2 h before and 2 and 18 h after exposure. We employed untargeted metabolite profiling to monitor 3873 metabolic features in 493 blood samples from these volunteers. We assessed lung function using spirometry and six acute phase proteins in peripheral blood. We assessed the association of the metabolic features with the measured air pollutants and with health markers that we previously observed to be associated with air pollution in this study.

Results: We observed 89 robust associations between air pollutants and metabolic features two hours after exposure and 118 robust associations 18 h after exposure. Some of the metabolic features that were associated with air pollutants were also associated with acute health effects, especially changes in forced expiratory volume in 1 s. We successfully identified tyrosine, guanosine, and hypoxanthine among the associated features. Bioinformatics approach Mummichog predicted enriched pathway activity in eight pathways, among which tyrosine metabolism.

Conclusions: This study demonstrates for the first time the application of untargeted metabolite profiling to assess the impact of air pollution on the blood metabolome.

1. Introduction

The blood metabolome has been defined as the collection of biologically active chemicals in human blood derived from endogenous processes and exogenous exposure to foods, drugs, and pollutants (Rappaport et al., 2014). Untargeted metabolomic techniques such as mass spectrometry (MS) and nuclear magnetic resonance can measure exogenous and endogenous compounds in blood (among other media) and provide a powerful tool to study the potential direct and indirect impact of environmental risk factors on the composition of the blood metabolome (Holmes et al., 2008).

A metabolic signature in the blood of exposure to ambient air pollution is plausible as some ambient air pollutants (ultrafine particles and gaseous air pollutions) have been shown to enter the bloodstream directly from the lungs (Ewetz, 1993; Nemmar et al., 2002). In addition, larger particles (particulate matter $(PM)_{2.5}$, PM_{10}), incapable of crossing the lung epithelium have been shown to induce inflammation in the lungs, triggering a systemic response that can be observed in the peripheral blood (Hogg and van Eeden, 2009). Assessment of the blood metabolome in relation to air pollution is therefore of interest as it might simultaneously detect specific metabolites related to air pollution and their biological response.

Abbreviations: FENO, Concentration of NO in exhaled breath; CRP, C-reactive protein; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; IL-6, interleukin 6; ICC, intra class correlation coefficient; *m/z*, mass-to-charge ratio; PNC, particle number concentration; PM, particulate matter; QTOF, quadrupole time-of-flight mass spectrometer; tPA/PAI-1, tissue plasminogen activator/plasminogen activator inhibitor-1 complex; UHPLC, ultra-high performance liquid chromatography; VWF, Von Willebrand factor; MS, mass spectrometry

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Two studies recently provided evidence for an association between (long- and short-term) exposure to air pollution (NO_2 and $PM_{2.5}$) and changes in the blood metabolome (Menni et al., 2015; Ward-Caviness et al., 2016). Both studies used a targeted approach in which a set of pre-specified metabolites (138 and 288) was assessed. Associations were observed with long-chain fatty acids, amino acids, a carbohydrate (glycerate), a salt (benzoate), and two cofactors and vitamins (a-tocopherol and threonate). Compared to targeted approaches, untargeted metabolomics has the potential to increase the coverage of the metabolome (increasing from several hundred markers to several thousands) and to reduce bias towards identifying well-studied metabolites. A recent study of cigarette smoking demonstrated the success of untargeted metabolomics by identifying 12 unexpected xenobiotic metabolites, in addition to a set of known nicotine metabolites, potentially opening up new avenues of studying the etiology of smoking-related diseases (Gu et al., 2016).

In this hypothesis generating study we used an untargeted high-resolution MS method to assess the profile of metabolic features in peripheral blood samples that were collected in the RAPTES study in which volunteers were exposed for five hours to different levels and mixtures of air pollution. The RAPTES study is highly standardized in terms of the exposure levels and potential confounding factors that were included. Furthermore, blood samples were taken before and after exposure allowing study participants to serve as their own control. Within RAPTES we previously observed associations between several air pollutants and changes in respiratory health parameters measured 2 and 18 h after exposure (Strak et al., 2013a). We also observed associations with markers of coagulation and vascular inflammation measured in peripheral blood that was collected at the same time points (Steenhof et al., 2014).

We assessed the association between changes in the serum concentrations of metabolic features, exposure to air pollutants, and markers of biological perturbations to acquire first insights into the perturbation of the blood metabolome in response to short term exposure to air pollution.

2. Methods

2.1. Study design

The study design has been described in detail before (Strak et al., 2012, 2013a, 2013b). Briefly, we exposed healthy human subjects to ambient air pollution for five hours at five locations in the Netherlands. On the day of exposure we standardized food intake and physical activity of the study participants. The study population consisted of healthy non-smoking volunteers (21 women and 10 men) with a median age of 22 years (range 19–26) and a median BMI of 22.3 (range 17–32). See Table A.1 for an overview of the characteristics of the study subjects. The locations were an underground train station, a continuous traffic location, a stop-and-go traffic location, a farm, and an urban background site (a large garden within a urban region). All locations were within 70 km of the Utrecht University campus where blood was collected in the morning (07:00–07:30), two hours before exposure (t_0) using standard venipuncture techniques. Blood was also collected two (t_9) and eighteen hours (t_{25}, the next morning) after exposure. In Table 1 we provide an overview of the total number of blood samples that were collected at t_0, t_9, and t_{25}, and indicate how many instances of repeated sampling (the same individual visiting a location twice) occurred at each location.

Serum samples were stored within 1 h of blood collection at −80 °C until analysis. We recruited healthy, young, nonsmoking Utrecht University students living on campus to minimize exposure to traffic-related air pollution when traveling to the data collection point. As the composition and the level of air pollution at the underground train station location has been shown to be very different from the other study locations in previous analyses (PM mass concentrations at the underground train station were up to 14 times higher than at the urban background, the sum of the concentrations of trace metals in fine and coarse PM was nearly 20 times above the outdoor levels, and elemental carbon was elevated in fine and coarse PM, in contrast to the outdoor sites where EC was predominantly found in fine PM) (Strak et al., 2011), we excluded this site from our main analysis, but assessed the impact of this decision in a sensitivity analysis.

Table 1
Number of blood samples[a] collected at the five study locations and included in the statistical analysis.

Timing of sampling	Urban background	Farm	Continuous traffic	Stop-and-go traffic	Underground
t_0[b,c]	29 (3)	28 (6)	31 (6)	37 (11)	45 (19)
t_9[b]	28 (3)	26 (6)	31 (6)	36 (10)	41 (17)
t_{25}[b]	28 (3)	24 (6)	30 (6)	36 (10)	43 (18)

[a] For some study participants blood was collected at the same location on multiple days. The number of repeated blood samples is indicated in brackets.
[b] t_0, blood was collected before exposure; t_9, blood was collected two hours after exposure; t_{25}, blood was collected eighteen hours after exposure.
[c] Blood samples collected at t_0 for which no matching blood sample at t_9 or t_{25} was available, were excluded from statistical analysis.

Participants were brought to one of the study locations once every 14 days in groups of eight and were exposed at the location for five hours, cycling 20 min each hour on a stationary bicycle (at a fixed minute ventilation rate of 20 L/min/m^2 body surface area) to increase and standardize inhalation. Each participant had to visit all five locations once, with the two remaining visits assigned randomly to a location. Visits were conducted from March until October 2009, and started at 09:00–09:30 in the morning. The participants completed an online screening questionnaire. Exclusion criteria included smoking or living in a household with a smoker; lifetime diagnosis of asthma or chronic obstructive pulmonary disease; or history of cardiovascular disease, diabetes mellitus, or pregnancy. Before the study, each participant was examined by a physician (blood pressure and pulmonary examination) and obtained medical clearance for participation. The study was approved by the ethics committee at University Medical Center Utrecht. Written informed consent was provided by all participants.

2.2. Exposure assessment

The methods used to characterize exposure to air pollutants have been described in detail before (Strak et al., 2011). Briefly, five hour air pollution measurements were collected at the five locations, each time study participants were present. We assessed the following markers of air pollution: particulate mass (PM) concentration for particles with a diameter < 2.5 μm ($PM_{2.5}$), < 10 μm (PM_{10}), and between 2.5 and 10 μm (PM_{coarse}), gaseous pollutants (nitrogen dioxide, NO_2; nitrogen oxides, NO_x; ozone, O_3), and particle number concentration (PNC, a proxy for ultrafine particles). Within the PM samples we assessed absorbance (a proxy for elemental carbon), trace metal composition, secondary organic and inorganic components, and endotoxin content. In Table A.1 we provide the mean and associated standard deviation of the measured levels of all air pollutants included in the current study. While the urban background location was generally the location where the lowest exposure levels were incurred, the ranking of the other locations based on exposure level varies from marker to marker (Strak et al., 2011). We conducted analyses using data from the 48 individual air pollutants.

2.3. Assessment of health markers

We assessed three respiratory health parameters (the concentration of NO in exhaled breath (FE_{NO}), Forced Vital Capacity (FVC), forced

expiratory volume in 1 s (FEV1)) and six markers of inflammation and coagulation measured in peripheral blood (interleukin 6 (IL-6), fibrinogen, tissue plasminogen activator/plasminogen activator inhibitor-1 complex (tPA/PAI-1), Von Willebrand factor (VWF), platelets, C-reactive protein (CRP)). These health markers were determined at t_0, t_9, and t_{25}. The methods that were used to assess the health markers were described previously (Strak et al., 2012, 2013a). In Table A.2 we provide the mean and range of the measured levels at t_0 of all health markers included in the current study.

2.4. Assessment of the human blood metabolome

We employed untargeted metabolite profiling using ultra-high performance liquid chromatography (UHPLC; Infinity 1290, Agilent Technologies Inc., Santa Clara, USA) coupled to a quadrupole time-of-flight mass spectrometer (QTOF; 6550, Agilent Technologies) equipped with an electrospray ion source operated in positive polarity. A full description of the serum sample preparation, UHPLC-QTOF analyses, and data processing is provided in Appendix B. Metabolome data was not available for eighteen sampling occasions (the combination of individual, location, and sampling day), either due to technical failure during the analysis or because a sample was not available for the current analysis. These sampling occasions were excluded from all statistical analyses.

We excluded metabolic features from statistical analysis if their peak height was lower than 5000 counts (i.e., the detection limit) in 60% or more of the samples. In the remaining samples, we imputed metabolic feature intensities with a peak height under 5000 using the imputeLCMD package in R, which has been developed for imputation of left-censored data using random draws from a truncated distribution with parameters estimated using quantile regression (Lazar et al., 2016).

2.5. Statistical analysis

We used a mixed model (Douglas et al., 2014; Team, 2012) to assess the association between the air pollutants and the peak area of the metabolic features measured in blood. Each model contained one air pollutant and one metabolic feature. We log-transformed all peak areas to normalize their distribution. Similar to previous analyses conducted within the RAPTES study we adjusted all analyses for age, BMI, sex, mean outside temperature, mean outside relative humidity and season. We included peak areas measured in blood collected at t_0 as predictor in the models and included a random intercept for subject and a random slope for the air pollutant (to allow between individual variation). We calculated p-values for the fixed effects of the air pollutant on the peak areas using a *t*-test. We assessed the influence on the noteworthy associations of leaving out all data from each sampling location (one at the time) and of adding data from the underground location. We further assessed the robustness of the associations to iteratively removing data from the locations for four measures of influence: Cook's D criterion (cut-off value < 1), dfbeta criterion (cut-off value < 1), 'association not changed in significance level' (i.e. moving from 'significant' to 'non-significant'; threshold $p < 0.05$), 'no change in the direction of the association' (Nieuwenhuis et al., 2012). We call associations that were robust to all measures of influence 'robust associations' and metabolic features involved in these associations 'robust metabolic features'.

We assessed the association between the change in metabolic feature concentration from t_0 to t_9, t_{25} and the change in health marker from t_0 to t_9,t_{25}. We included a random intercept for subject and a random slope for change in metabolic feature concentration. The metabolic feature – health marker analyses were adjusted for the same covariates as the exposure- metabolic feature analyses. We calculated p-values for the fixed effects of the air pollutant on the metabolite concentrations using a *t*-test.

To address the increased risk of false positive findings due to multiple comparisons we calculated q-values using the method developed by Storey and Tibshirani (2003). We consider an association with a *q*-value < 0.2 noteworthy.

We formally assessed causal mediation of effects of air pollutants on health markers by metabolic features using the mediation package (Tingley et al., 2014). We first fit the mediator model where the difference in metabolite levels between t9 and t0 or t25 and t0 was modeled as a function of the exposure and covariates. Next, we modeled the difference in health outcome levels between t9 and t0 or t25 and t0 as a function of the mediator (difference in metabolic feature level), exposure, and the same set of covariates used in the mediator model. We then used the mediate function (using the model fits of the mediator and outcome models as input) to estimate the average causal mediation effects (the effect of the air pollutant that is mediated by a metabolic feature) and average direct effects (the effect of the air pollutant on a health marker that is not mediated by a metabolic feature). P values for the mediation effects were estimated using 1000 Monte Carlo simulations, using the package default settings. We included exposure-health effect associations in mediation analysis if the p-value of that association was < 0.05, we considered a p value < 0.1 for the indirect effect noteworthy.

We created boxplots showing the distribution of robustly associated metabolic features at each sampling location to assess the contribution of the variation in exposure levels across sampling locations to variations in the change of the metabolic features. We estimated the trend across sampling locations with a simple linear regression using the exposure ranking of the sampling location as explanatory variable. We consider a *p* value < 0.1 for the trend estimate noteworthy.

All statistical analyses were conducted in R version 3.03 (R Core Team, 2014).

2.6. Annotation of metabolic features

For metabolic features identified in the statistical analysis to be robustly associated to an air pollutant and also to a health marker we confirmed the molecular weights and acquired fragmentation spectra whenever possible. We then searched the monoisotopic masses or mass-to-charge ratio (*m/z*) values (assuming $[M+H]^+$ or $[M+Na]^+$ ion) against three databases: HMDB (http://www.hmdb.ca/), METLIN (https://metlin.scripps.edu/), and MzCloud (https://www.mzcloud.org/), using a tolerance of 15 ppm for potential matches. We then evaluated the matches for plausibility based on retention time, structure, isotope patterns, and product ion spectra when available.

In parallel, we used the Mummichog bioinformatic approach to assess pathway activity among robust metabolic features identified in the statistical analysis (Li et al., 2013). Mummichog computes a list of tentative annotations based on the *m/z* value of the metabolic features. Mummichog considers 16 derivatives and adducts ($[M]^+$, $[M+H]^+$, $[M+2H]^{2+}$, $[M+3H]^{3+}$, $[M+Na]^+$, $[M+H+Na]^{2+}$, $[M+K]^+$, $[M\text{-}H_2O+H]^+$, $[M\text{-}H_4O_2+H]^+$, $[M\text{-}NH_3+H]^+$, $[M\text{-}CO+H]^+$, $[M\text{-}CO_2+H]^+$, $[M\text{-}HCOOH+H]^+$, $[M+HCOONa]^+$, $[M\text{-}HCOONa+H]^+$, $[M\text{-}C_3H_4O_2+H]^+$) to map *m/z* features to small molecular weight compounds. More than one compound can be mapped per *m/z* feature. Network activity of the mapped metabolites is then evaluated by assessing pathway enrichment in the human reference metabolic pathways included in MetaFishNet (Li et al., 2010) and assessing the activity in modules that can be built with them within the reference metabolic pathways. Annotations in Mummichog become more probable (high activity score) if annotated metabolites reflect biological activity (enrichment) on a local structure in the metabolic network. Annotations become less probable if matched metabolites are distributed more randomly across the metabolic network (Li et al., 2013). Predicted metabolites that play a role in significant modules or pathways together form the 'activity network'. We used 200 permutations and a mass accuracy of 8 ppm. Mummichog has been shown to correctly predict pathway when there was an overlap size (between the tentatively

Table 2
Number of significant[a] associations between molecular features and air pollutants.

Analysis	Number of associations at t_9[b]	Number of associations at t_{25}[b]	Overlap between associations identified at t_9 and at t_{25}[b]
All outdoor locations[c]	457	1548	18
Adding underground location[d]	0	0	
Jack-knifing analyses			
Excluding urban background location[d]	381	1248	8
Excluding stop-and-go traffic location[d]	403	1085	12
Excluding continuous traffic location[d]	262	741	14
Excluding farm location[d]	415	1368	17
Robust to all jack-kniving analyses	134	410	3
Influential groups analyses			
Fulfilling Cook's D criterion (< 1)	423	1104	12
Fulfilling dfbeta criterion (< 1)	350	854	11
Not changed in significance	157	437	3
Not changed in direction	421	1410	14
Robust to all influential groups criteria	**89**	**118**	**0**
Of these robust in jack-knifing analysis	*73*	*98*	*0*

[a] $q < 0.2$.
[b] t_9, blood was collected two hours after exposure; t_{25}, blood was collected eighteen hours after exposure.
[c] Main analysis to assess association between air pollutants and molecular features.
[d] Sensitivity analysis to assess the influence of adding or excluding a single study location on the observed associations.

identified metabolites and the metabolites in the pathway) of four metabolites or more, even though some of the individual metabolites were incorrectly identified (Li et al., 2013). We therefore only report pathway activity for those pathways that had an overlap size of four or more.

3. Results

We conducted high resolution MS analyses on 493 blood samples collected in the RAPTES study. 3873 metabolic features were successfully measured. In Table 1 we show the number of blood samples that was collected at the different sampling locations and were included in statistical analyses. A small number of blood samples (n = 16) was not included in the statistical analysis as the result of technical failure during the MS analyses or because a sample was not available for the current analysis. In Table 2 we show the total number of significant associations ($q < 0.2$) between metabolic features and air pollutants.

In our main analysis, which includes only the outdoor locations, we observed 457 associations between metabolic features and air pollutants at t_9 and 1548 associations at t_{25}. Of these, 18 associations between metabolic features and air pollutants were observed at both t_9 and at t_{25}. To assess the robustness of the associations we observed in our main analysis we conducted sensitivity analyses by adding or removing (jack-knifing analyses) sampling locations from the dataset. When we added data from the underground sampling location zero associations were observed at either t_9 or t_{25}. 134 associations at t_9 and 410 associations at t_{25} (3 of which overlapping) survived all jack-knifing analyses. We further assessed the robustness of the associations to iteratively removing data from the locations for four measures of influence: Cook's D criterion (cut-off value < 1), dfbeta criterion (cut-off value < 1), 'association not changed in significance level' (threshold $p < 0.05$), 'no change in the direction of the association'. 89 associations at t_9 and 118 associations at t_{25} met all 'measures of influence' criteria. These we call robust associations. Of these, 73 and 98 associations, respectively, also survived our jack-knifing analyses. Although none of the robust associations were observed both at t_9 and at t_{25}, four metabolic features were associated to an air pollutant at both t_9 and t_{25} (monoisotopic mass (MI) 151.0608, 175.0670, 232.0874, and 573.7178). Evaluation of trends in metabolic features levels across sampling locations confirmed the robust associations detected using linear mixed models (Appendix C).

In Tables 3 and 4 we list the metabolic features that were robustly associated to at least one air pollutant and were also associated to one or more health markers ($q < 0.2$). At t_9 many metabolic features were associated to multiple air pollutants and also to multiple health markers. Especially associations with FEV1 and fibrinogen were numerous. Roughly half of the metabolic features increased after exposure. At t_{25} metabolic features again were mostly associated to FEV1 and fibrinogen and appeared to be primarily associated to SO_4 determined in PM_{coarse}. Metabolic features were primarily upregulated after exposure. Correlations between the metabolic features that were robustly associated at t_{25} were rather high (median $r = 0.53$ at t_{25} versus 0.03 at t_9; density plot of Pearson correlation coefficients in Fig. D.1), inflating the number of significantly associated metabolic features, but also pointing towards a potential relationship via a shared pathway.

We included all exposure-metabolic feature-health associations listed in Tables 3 and 4 in a formal mediation analysis (complete results in Tables E.1 and E.2). We observed indications for mediation of the effect of several air pollutants (NO, NO_x, absorption (PM_{10}), and Cu (PM_{coarse})) on FEV1 for two metabolic features measured at t_9 (MI 178.415 and MI 272.1554). At t_{25} we observed an indication that MI 510.2516 mediated the effect of SO_4 ($PM_{2.5}$) on fibrinogen levels.

We report three compounds that were robustly associated to air pollution and for which we were able to confirm identity through MS/MS and standard analysis. The compounds that we identified were tyrosine (monoisotopic mass: 182.0813, retention time: 1.31 min, associated to air pollution at t_9), guanosine (monoisotopic mass: 152.0565, retention time: 1.73 min, a fragment was associated to air pollution at t_{25}), and hypoxanthine (monoisotopic mass: 137.0455, retention time 1.21 min, associated to air pollution at t_{25}). A description of the identification procedure and MS/MS spectra for the identified compounds is provided in Appendix G. No further metabolic features could be annotated.

Mummichog predicted differential enrichment of eight pathways for which the overlap size was four or larger. Most convincingly enriched pathways were 'tyrosine metabolism' (in agreement with our identification of tyrosine as one of the compounds robustly associated to air pollution), 'urea cycle/amino group metabolism', and 'N-Glycan degradation' (Table 5).

4. Discussion

We observed an association between short term exposure to air pollution and an acute perturbation of the blood metabolome. Some of the compounds measured in blood associated to air pollution were also associated to health markers that were previously associated with

Table 3
Small molecular weight compounds (n = 21) measured at t_9[a] that are associated to at least one air pollutant (q < 0.2) and to at least one health marker (q < 0.2).

MI (Da)[b]	RT[c] (min)	Associated exposure(s)[d]	Associated health marker (s) at t_9[e,f]	Associated health marker (s) at t_{25}[e,f]
178.0415[g]	1.84	***NO***	**FEV1**	**FEV1**
549.253	7.05	NO2	CRP	CRP
160.0168	1.29	NO2	fibrinogen/CRP	fibrinogen/CRP
178.0415[g]	1.84	***NO_X***	**FEV1**	**FEV1**
551.36	6.82	*Ba ($PM_{2.5}$)*	FEV1/platelets	FEV1
726.0044	6.91	*Ba ($PM_{2.5}$)*	FEV1/fibrinogen	FEV1/fibrinogen
480.9914	6.99	*Ba ($PM_{2.5}$)*	fibrinogen	fibrinogen
749.9941	6.9	*Ba ($PM_{2.5}$)*	FEV1/fibrinogen	FEV1/fibrinogen
573.7178	7	*Ba ($PM_{2.5}$)*	fibrinogen	FEV1/fibrinogen
184.0348	0.85	Ba ($PM_{2.5}$)		fibrinogen
772.0059	6.89	*Ba ($PM_{2.5}$)*	FEV1	FEV1
232.0557	3.51	*Pb ($PM_{2.5}$)*	FEV1	
133.0504	2.99	*Absorption ($PM_{2.5}$)*	FEV1	
320.1385	2.61	*Absorption ($PM_{2.5}$)*		CRP
272.1554	5.21	Absorption ($PM_{2.5}$)	**FEV1**/fibrinogen	FEV1
797.4201	7.05	Absorption ($PM_{2.5}$)	fibrinogen	CRP
461.8545	0.62	V ($PM_{2.5}$)		fibrinogen
135.0689	1.31	Endotoxin (PM_{10})	fibrinogen	fibrinogen
181.074	1.31	endotoxin (PM_{10})	fibrinogen	fibrinogen
300.093	5.4	*OP AA (PM_{10})*		CRP
272.1554	5.21	OP AA (PM_{10})	FEV1/fibrinogen	FEV1
772.0059	6.89	*OP ESR (PM_{10})*	FEV1	FEV1
272.1554	5.21	Absorption (PM_{10})	**FEV1**/fibrinogen	FEV1
797.4201	7.05	Absorption (PM_{10})	fibrinogen	CRP
178.0415[g]	1.84	*Cu (PM_{coarse})*	**FEV1**	**FEV1**
573.7178	7	*Cu (PM_{coarse})*	fibrinogen	FEV1/fibrinogen
184.0348	0.85	Cu (PM_{coarse})		fibrinogen
772.0059	6.89	*Cu (PM_{coarse})*	FEV1	FEV1
184.0348	0.85	EC (PM_{coarse})		fibrinogen
311.1048	1.2	*NO3 (PM_{coarse})*	fibrinogen	fibrinogen/CRP
184.0348	0.85	Sb (PM_{coarse})		fibrinogen
772.0059	6.89	*Sb (PM_{coarse})*	FEV1	FEV1
606.3815[g]	6.8	***Sb (PM_{coarse})***	FEV1	

[a] t_9, blood was collected two hours after exposure.
[b] Monoisotopic mass.
[c] Retention time in minutes.
[d] Trace elements, endotoxin, and absorption determined in particles with a diameter < 2.5 μm ($PM_{2.5}$), < 10 μm (PM_{10}), and between 2.5 and 10 μm (PM_{coarse}); OP AA (oxidative potential measured by the extent of ascorbate depletion), OP ESR (oxidative potential measured by electron spin resonance). Air pollutants in italic indicate an inverse association with the metabolic feature.
[e] t_9, determined two hours after exposure; t_{25}, determined eighteen hours after exposure.
[f] Concentration of NO in exhaled breath (FE_{NO}), Forced Vital Capacity (FVC), forced expiratory volume in 1 s (FEV1), interleukin 6 (IL-6), tissue plasminogen activator/plasminogen activator inhibitor-1 complex (tPA/PAI-1), Von Willebrand factor (VWF), c-reactive protein (CRP).
[g] For bold exposures and bold health markers an indication for mediation of the effect of the air pollutant on the health marker by the molecular feature was observed (See Supplemental Material V Table S2).

exposure to air pollution in this study, pointing towards a mediating role of the metabolic features.

Associations between specific PM components and cardio-respiratory health effects observed in the RAPTES study are discussed in detail in two articles by Strak et al. (2012, 2013b). Consistent with the literature no single or group of air pollutants could be identified as the primary culprit. In the current analysis, we primarily observed associations between metabolic features and PM components rather than PM mass concentration, which would point toward a stronger effect of particle composition to biological response than PM mass concentration itself. At t_{25} most features were associated to SO_4 measured in PM_{coarse}. SO_4 has been reported earlier to be associated with adverse health effects in epidemiological studies (Pope et al., 2007). Potential mechanisms through which biological effects could occur include increasing the

Table 4
Small molecular weight compounds (n = 62) measured at t_{25}[a] that are associated to at least one air pollutant (q < 0.2) and to at least one health marker (q < 0.2).

MI (Da)[b]	RT[c] (min)	Associated exposure(s)[d]	Associated health marker (s) at t_9[e,f]	Associated health marker(s) at t_{25}[e,f]
510.2516	3.57	NO_3 ($PM_{2.5}$)	fibrinogen/CRP	fibrinogen/CRP
509.7495	3.57	NO_3 ($PM_{2.5}$)	fibrinogen/ Platelets/CRP	fibrinogen/CRP
452.7383	2.96	NO3 ($PM_{2.5}$)	fibrinogen	fibrinogen/CRP
302.1696	4.18	*OP ESR ($PM_{2.5}$)*	FEV1/fibrinogen	FEV1/fibrinogen/ platelets
169.0848	0.62	*OP ESR ($PM_{2.5}$)*		fibrinogen
431.323	6.94	*OP ESR ($PM_{2.5}$)*	FEV1	fibrinogen
620.4016	6.98	*OP ESR ($PM_{2.5}$)*	FEV1	
510.2516	3.57	**SO_4 ($PM_{2.5}$)**	fibrinogen/CRP	**fibrinogen**/CRP
310.1154	1.82	SO_4 ($PM_{2.5}$)	Platelets	platelets
446.8133	6.65	*Zn ($PM_{2.5}$)*	FEV1	FEV1
780.5172	6.81	*Zn ($PM_{2.5}$)*	FEV1/fibrinogen	
136.0382	1.21	*Zn ($PM_{2.5}$)*	Platelets	
264.0155	1.01	Pb (PM_{coarse})	fibrinogen	
151.0492	1.73	Pb (PM_{coarse})	Platelets	platelets
474.3145	6.45	SO_4 (PM_{coarse})	FEV1	FEV1
512.8540[g]	6.62	SO_4 (PM_{coarse})	FEV1	
523.8418	6.63	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1
458.3098[g]	6.65	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1/fibrinogen
424.8007	6.66	SO_4 (PM_{coarse})	FEV1	FEV1
552.8643	6.72	SO_4 (PM_{coarse})	FEV1	FEV1
553.3675	6.72	SO_4 (PM_{coarse})	FEV1	
711.9881	6.75	SO_4 (PM_{coarse})	FEV1	FEV1
714.4672	6.75	SO_4 (PM_{coarse})	FEV1	FEV1
692.4554	6.76	SO_4 (PM_{coarse})	fibrinogen	fibrinogen
431.808	6.78	SO_4 (PM_{coarse})	FEV1	
418.31	6.79	SO_4 (PM_{coarse})	FEV1	
420.7857	6.79	SO_4 (PM_{coarse})	fibrinogen	FEV1/fibrinogen
601.4219	6.8	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1/fibrinogen
775.5642	6.81	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1
515.8453	6.84	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1/fibrinogen
696.9832	6.84	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1/fibrinogen
483.3439[g]	6.85	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1
493.8345	6.85	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1/fibrinogen
674.471	6.85	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1/fibrinogen
471.8211	6.87	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1
562.7231	6.87	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1/fibrinogen
548.3765	6.88	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1/fibrinogen
610.9052	6.88	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1/fibrinogen
447.3283[g]	6.89	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1/fibrinogen
772.5078	6.89	SO_4 (PM_{coarse})	fibrinogen	fibrinogen
427.7952	6.9	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1
706.4708[g]	6.91	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1/fibrinogen
684.4522[g]	6.92	SO_4 (PM_{coarse})	FEV1	FEV1
662.4424	6.94	SO_4 (PM_{coarse})		FEV1
661.9418	6.94	SO_4 (PM_{coarse})	FEV1/fibrinogen	fibrinogen
498.3647	6.96	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1/fibrinogen
639.9266	6.96	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1/fibrinogen
498.8651	6.96	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1/fibrinogen/ CRP
571.4084	6.99	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1/fibrinogen
574.3921	6.99	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1/fibrinogen
573.7178	7	SO_4 (PM_{coarse})	fibrinogen	FEV1/fibrinogen
549.3962	7	SO_4 (PM_{coarse})	FEV1/fibrinogen	FEV1
529.861	7.01	SO_4 (PM_{coarse})	fibrinogen	FEV1/fibrinogen
622.4425	7.06	SO_4 (PM_{coarse})	fibrinogen/ platelets	
624.9198	7.06	SO_4 (PM_{coarse})	fibrinogen	FEV1/fibrinogen
697.9672	7.08	SO_4 (PM_{coarse})	fibrinogen	fibrinogen
558.8823	7.1	SO_4 (PM_{coarse})	fibrinogen	
653.9403	7.11	SO_4 (PM_{coarse})	FEV1/fibrinogen	

(continued on next page)

Table 4 *(continued)*

MI (Da)[b]	RT[c] (min)	Associated exposure(s)[d]	Associated health marker (s) at t_9[e,f]	Associated health marker(s) at t_{25}[e,f]
631.9294	7.12	SO_4 (PM_{coarse})	fibrinogen	
663.4318	6.68	SO_4 (PM_{coarse})	Platelets	
514.3698	6.83	SO_4 (PM_{coarse})	FEV1	FEV1
770.4774	7	*SO_4 (PM_{coarse})*	FEV1/fibrinogen	FEV1/fibrinogen/ CRP
596.7477	6.92	SO_4 (PM_{coarse})	FEV1	FEV1

[a] t_{25}, blood was collected eighteen hours after exposure.
[b] Monoisotopic mass.
[c] Retention time in minutes.
[d] Trace elements, endotoxin, and absorption determined in particles with a diameter < 2.5 μm ($PM_{2.5}$), < 10 μm (PM_{10}), and between 2.5 and 10 μm (PM_{coarse}); OP AA (oxidative potential measured by the extent of ascorbate depletion), OP ESR (oxidative potential measured by electron spin resonance). Air pollutants in italic indicate an inverse association with the metabolic feature.
[e] t_9, determined two hours after exposure; t_{25}, determined eighteen hours after exposure.
[f] Concentration of NO in exhaled breath (FE_{NO}), Forced Vital Capacity (FVC), forced expiratory volume in 1 s (FEV1), interleukin 6 (IL-6), tissue plasminogen activator/ plasminogen activator inhibitor-1 complex (tPA/PAI-1), Von Willebrand factor (VWF), c-reactive protein (CRP).
[g] For bold exposures and bold health markers an indication for mediation of the effect of the air pollutant on the health marker by the molecular feature was observed (See Supplemental Material V Table S2).

acidity or hygroscopicity of the particulate matter (the latter would make components such as water-soluble metals, more bioavailable) (United States Environmental Protection Agency (USEPA), 2009). SO_4 might also be an indicator of unmeasured secondary aerosol components that may be biologically active. Interestingly, the SO_4 content of $PM_{2.5}$ in our study is, consistent with the literature, much higher than the SO_4 content of PM_{coarse}. (Supplemental Table A.1). Therefore, if we assume that SO_4 was the true culprit for the changes in the blood metabolome we observed, we would have expected the number of metabolic features that was associated with SO_4 in $PM_{2.5}$ to be higher.

This is the second study that reports an acute effect of exposure to air pollution on changes in the blood metabolome. Recently, Ward-Caviness et al. reported associations between short-term exposure to NO_2 and changes in long-chain fatty acids concentration in the German KORA cohorts (Ward-Caviness et al., 2016). In addition, two studies of exposures closely related to air pollution, environmental exposure to polycyclic aromatic hydrocarbons (Wang et al., 2015) (components of air pollution) and cigarette smoke (Hsu et al., 2013) (particulate exposure), provide support for the plausibility of our findings, although assessment of the actual overlap in reported metabolic features is not possible as many of the associated features could not be identified.

We observed significant associations between several metabolic features and a number of respiratory health and inflammation and coagulation parameters. This highlights the potential role of metabolomics for the identification of markers both associated to exposure and to intermediate health markers. The observed association between the blood metabolome and markers of inflammation is plausible due to the alteration of the metabolic profile of inflamed tissue and the influx of immune cells when inflammation is present (Fitzpatrick and Young, 2013). The association between metabolic markers and indices of lung function has been reported before (Flexeder et al., 2014). In a recent analysis in the KORA-F4 cohort, 30 metabolites were significantly associated with FVC (n = 29) or FEV1 (n = 23) (Flexeder et al., 2014). Though specific metabolites were not reported, the authors do mention an enrichment of metabolites involved in tyrosine metabolism, corresponding to what we have observed in our study. The authors of that analysis hypothesized about a role of pulmonary surfactant in the perturbation of the blood metabolites. For a number of health parameters (VWF, platelets, CRP, $FE_{NO,}$ FVC) the association with metabolic features (measured at t_9) was stronger at t_{25} than at t_9. A similar pattern was observed in RAPTES previously between air pollution and markers of inflammation and coagulation (Strak et al., 2013a).

Although our mediation analysis provided some indication for a potential mediating role of the blood metabolome in health effects induced by short term exposure to air pollution, the observed associations between health effects measured at t_9 and metabolic features measured at t_{25} would point towards an influence of these health effects on the blood metabolome rather than vice versa (reverse causation). The exact role of the blood metabolome in the development of health effects needs to be explored further.

A strength of our study is the experimental design in which we carefully measured exposure to many air pollutants on-site and standardized food intake and physical activity on the day of the experimental exposure. We also asked participants to avoid natural exposure to air pollution on the day before the experimental exposure to air pollution. We thereby limited the influence of other factors than air pollution on variations in the blood metabolome. We previously showed that these air pollution exposures induced various acute biological perturbations, some of which were measured in peripheral blood (Steenhof et al., 2014; Strak et al., 2013a). Another strength of our study design is the fact that we had one pre- and two post-exposure blood measurements for each individual in each exposure situation and that each exposure situation was visited twice by at least one individual. This allowed us to assess nuisance variance introduced by

Table 5
Pathway activity predicted using Mummichog bioinformatic approach.

Affected pathway[a]	Time point[b]	Count[c]	Percentage[d]	p-value[e]	adjusted p-value[f]
Tyrosine metabolism	t9	11	15.1%	0.035	0.001
Urea cycle/amino group metabolism	t9	7	15.9%	0.067	0.001
Glycerophospholipid metabolism	t9	5	12.8%	0.221	0.005
Tryptophan metabolism	t9	5	8.9%	0.509	0.026
N-Glycan Degradation	t9	4	66.7%	0.001	0.001
Pentose phosphate pathway	t9	4	12.1%	0.295	0.009
Aspartate and asparagine metabolism	t9	4	6.7%	0.756	0.102
Urea cycle/amino group metabolism	t25	5	11.4%	0.161	0.002
Arginine and Proline Metabolism	t25	4	12.9%	0.144	0.002
Aspartate and asparagine metabolism	t25	4	6.7%	0.572	0.028

[a] Only pathways for which counts ≥4 are shown.
[b] Time point at which the metabolic feature was robustly associated to an air pollutant. t9, blood was collected two hours after exposure; t25, blood was collected eighteen hours after exposure.
[c] Number of predicted metabolites active in pathway.
[d] Percentage of total number of metabolites in pathway.
[e] p-value for Fisher's exact test.
[f] Permutation based p-value based on the EASE score.

inter-individual variation in the blood metabolome. Because blood samples collected at t_0 and t_{25} were collected at the same wall-clock time, we were able to assess the contribution of ambient air pollution to changes in the blood metabolome 18 h after exposure, independent from natural diurnal variation. Changes in the blood metabolome observed 2 h after exposure (t_9) might have been affected by natural diurnal variation in metabolite levels. Because these measurements were conducted at the same time each study day, while air pollution levels varied from day to day, this phenomenon only had an impact on the precision with which we could assess the association with air pollution.

We observed no overlap between the associations observed at t_9 and those observed at t_{25}. It is plausible that certain changes in metabolite levels would be manifested only several hours after exposure (e.g. as the result of a systemic inflammatory response), and were therefore in this study detected at t_{25} (the next morning) and not at t_9 (two hours after exposure). However, metabolites with a short half-life would manifest only shortly after exposure and not the next morning. As we currently only have limited insight into which metabolites the associated features in this study represent, it is not possible to provide further in depth discussion on this topic.

We observed large intra-individual variation for many of the metabolic features when we calculated the Intra Class Correlation Coefficient (ICC) for variation across and within sampling days (extended description and results in Appendix F). Limited information is available from other studies about day to day variability in the blood metabolome (Breier et al., 2014; Carayol et al., 2015; Chaleckis et al., 2016; Floegel et al., 2011). Our median ICC values (0.4–0.5) were lower than what was reported in a recent study that assessed diurnal variation of 188 blood metabolites in 22 individuals (median ICC 0.66) (Breier et al., 2014). This difference is likely explained by differences in the nature of the metabolites measured in that study versus our study.

Many factors might have contributed to the modest ICC values that we observed for most metabolic features. Consumption of food and drugs have been shown to have an important influence on the blood metabolome (Rappaport et al., 2014). While we controlled food consumption and assessed drug use on the day of blood sampling we cannot rule out any influence of food consumption in the days before blood sampling. Uncontrolled variation in life style factors and air pollution exposure in the days before exposure might have had a similar influence on our results.

Most of the 48 pollutants measured in this study were highly correlated (Strak et al., 2011). In this analysis we were primarily interested in identifying perturbations in the blood metabolome due to exposure to air pollution, rather than identifying which of the specific air pollutants was the true cause of these perturbations. As a consequence a number of associations were likely false-positive. The exposure that was involved in most associations observed at t_{25} (SO_4 in PM_{coarse}) had a maximum Pearson correlation of 0.62 with any of the other exposures, thereby potentially reflecting a unique signal.

We were able to successfully annotate three metabolites (tyrosine, guanosine, and hypoxanthine). Tyrosine is a precursor to catecholamines (epinephrine, norepinephrine and dopamine) which have been shown to play a role in heart disease (Schömig, 1990). In addition, an inverse relationship between tyrosine supplementation and blood pressure has been reported in humans (Deijen et al., 1999). In our analysis tyrosine was associated to fibrinogen levels in blood. Fibrinogen increases in the presence of inflammation, a process which is induced by catecholamines among other agents (Martini et al., 2007). Fibrinogen was previously shown to be associated with several air pollutants in the RAPTES study (Strak et al., 2013a). Our result might point towards a potential role of tyrosine in the effects of air pollution on fibrinogen levels in blood. Although a role of physical activity or stress of the experiment cannot be excluded. Hypoxanthine and guanosine are two purine metabolites that have been reported to increase in response to physical activity (Gerber et al., 2014). A link between purine metabolism and exposure to air pollution is less evident. Hypoxanthine and guanosine were both associated with platelet count in our study. We were able to annotate guanosine based on the presence of a guanosine fragment (aglycone of guanosine) which was included in our set of compounds robustly associated to both air pollution and a health marker, while guanosine itself was not. However, nominal p-values for guanosine were all below 0.05.

Mummichog provides a possibility to assess impact on biological activity as measured by the metabolome while bypassing the need to confirm the identity of specific metabolites.

Mummichog analyses rely on the joint and tentative annotation (based on accurate mass matching) of several metabolites of the same metabolic pathways. Although not based on fully validated annotation of the metabolome, Mummichog results express the likelihood of the involvement of specific metabolic pathways in the response to air pollution exposure. Five of the pathways selected by mummichog are related to amino acid metabolism. Several of these amino acids have been linked to exposure to air pollution in in vivo or in vitro studies. These include tyrosine, tryptophan, aspartate and asparagine (Huang et al., 2015; Kendall, 2007). As described above, tyrosine metabolism was also found to be enriched in a set of metabolites associated with FVC or FEV1 (known to be affected by air pollution) in the KORA cohort (Flexeder et al., 2014). In addition, 'urea cycle/amino group metabolism' is involved in the disposal of ammonia, a common air pollutant. Furthermore, upregulation of glycerophospholipid (main component of biological membranes) metabolism has been reported in association to exposure to cigarette smoke in mice, another source of particulate exposure (Titz et al., 2016).

We provided insights into the perturbation of the blood metabolome in response to short term exposure to air pollution. Current developments of approaches for the annotation of metabolic features identified in untargeted assessment of the blood metabolome will allow future studies to delve deeper into the identity of specific metabolites that are affected by exposure to air pollution. Our results should be validated in other studies of both short and long-term effects of air pollution on human health.

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Ethical review

The study was approved by the ethics committee at University Medical Center Utrecht. Written informed consent was provided by all participants.

Appendix A–G. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envres.2017.03.042.

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