Prenatal exposure to air pollution affects autophagy, senescence and remodelling proteins in cord blood

Olga Gorlanova[1,2,*], Heide Oller[1,2], Uri Nahum[1,2,3], Noëmi Künstle[1,2], Loretta Müller[1,2,4], Andrea Marten[1,2], Céline Rüttimann[1,2], Danielle Vienneau[5,6], Kees de Hoogh[5,6], Primo Schär[7], David Schürmann[7], Carla Rebeca Da Silva Sena[1,2,8], Martin Röösli[5,6], Philipp Latzin[1,2], Pablo Sinues[1,9], Sven Schulzke[1,2], Ruth Steinberg[1,2], Jakob Usemann[1,2], Pawan Sharma[10] and Urs Frey[1,2], on behalf of the BILD study group

[1]University Children's Hospital (UKBB), University of Basel, Basel, Switzerland. [2]Division of Paediatric Respiratory Medicine and Allergology, Department of Paediatrics, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland. [3]Institute for Medical Engineering and Medical Informatics, University of Applied Sciences and Arts Northwestern Switzerland, Muttenz, Switzerland. [4]Lung Precision Medicine, Department for BioMedical Research, University of Bern, Bern, Switzerland. [5]Swiss Tropical and Public Health Institute, Allschwil, Switzerland. [6]University of Basel, Basel, Switzerland. [7]Department of Biomedicine, University of Basel, Basel, Switzerland. [8]Priority Research Centre GrowUpWell and Hunter Medical Research Institute, University of Newcastle, Newcastle, NSW, Australia. [9]Department of Biomedical Engineering, University of Basel, Allschwil, Switzerland. [10]Department of Pathology and Laboratory Medicine, Center for Inflammation and Lung Research, Lewis Katz School of Medicine, Temple University, Philadelphia, PA, USA.

Corresponding author: Urs Frey (urs.frey@ukbb.ch)

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Prenatal exposure to air pollution influences levels of autophagy-related and proinflammatory proteins in healthy term newborns: this study found four unique groups of healthy newborns, each showing different response patterns to air pollution in cord blood https://bit.ly/4iJHoAv

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Abstract

Background Air pollution increases inflammation and reactive oxygen species that can induce autophagy, thereby leading to airway inflammation and remodelling. However, it is unclear whether prenatal air pollution may impact proteins involved in autophagy. The objectives of the present study were to investigate the associations of prenatal air pollution with proteins indicative of autophagy, senescence and remodelling in infants.

Methods We included 387 healthy term newborns from the BILD cohort study and measured 11 proteins: interleukin (IL)-1β, IL-8, matrix metallopeptidase (MMP)-3, MMP-9, platelet-derived growth factor (PDGF)-AA, transforming growth factor-β (TGF-β), sirtuin-1, p62, LC3B, tumour necrosis factor-α (TNF-α), and Beclin-1 in cord blood serum and plasma. We assessed the association of whole pregnancy residential exposure to nitrogen dioxide (NO_2) and particulate matter (PM_{10}) with protein levels using multivariable Tobit regression models. We performed unsupervised hierarchical clustering based on protein concentrations with a network construction of identified clusters.

Results Our results indicate that NO_2 exposure during pregnancy can increase Beclin-1, a pivotal initiator of autophagy. Additionally, elevated NO_2 exposure was correlated with a reduction in IL-8 levels. Unsupervised hierarchical clustering of all measured proteins gave four distinct clusters with similar protein expression profiles. When analysing the clusters' clinical and exposure characteristics, significant differences were observed in NO_2 and PM_{10} exposure during pregnancy. Network analysis revealed distinct protein–protein correlation patterns among clusters.

Conclusions Our findings in healthy term newborns showed that prenatal air pollution exposure is associated with alterations in levels of autophagy-related proteins. For the first time, we identified four distinct clusters of newborns, suggesting that there are different air pollution response patterns in a healthy population.

Introduction

The prenatal period is particularly vulnerable to the adverse effects of air pollution exposure, with potential long-term consequences for lung development. Epidemiological evidence suggests that prenatal air pollution exposure increases the risk of adverse pregnancy outcomes, including prematurity and low birthweight [1]. Moreover, maternal exposure to air pollution is linked to respiratory morbidity [2] and impaired lung function in infancy [3, 4] and childhood [5]. These effects may persist into childhood, contributing to asthma development [6] and reduced lung function [7]. However, the host-defense mechanisms through which air pollution exerts deleterious effects in early life remain insufficiently understood.

Environmental pollutants have been identified as pivotal contributors to heightened inflammation and increased levels of reactive oxygen species (ROS), serving as direct indicators of oxidative stress [8]. ROS accumulation induces autophagy, a crucial regulatory mechanism for cellular homeostasis and ROS defense [9].

Autophagy is a fundamental mechanism of stress response, allowing cells to manage environmental oxidative stress, potentially linking lung growth with development, including *in utero* development [10, 11]. It is also involved in the regulation of pulmonary cell homeostasis in both healthy and disease states [12]. In disease states, dysfunctional autophagy can lead to cellular senescence or death [13], modulating the development of asthma [14]. Moreover, excessive autophagy contributes to airway remodelling [15]. Nevertheless, the relationship between autophagy, cellular senescence and tissue remodelling, particularly following exposure to air pollution, is not well studied.

Therefore, we hypothesised firstly that there is a functional relationship between autophagy, senescence and remodelling-related protein expression that might be affected by prenatal air pollution. Secondly, we postulated that within a healthy population there are subgroups of infants showing different autophagy and protein responses to air pollution. If it is confirmed that subgroups of healthy infants might show differences in susceptibility to air pollution, then, since this has not previously been shown, there is scope for impacts on risk group characterisation and even health policies.

We aimed to: 1) examine the association between exposure to nitrogen dioxide (NO_2) and particulate matter with an aerodynamic diameter of $\leqslant$10 µm (PM_{10}) during pregnancy and the expression of a defined set of proteins involved in autophagy, senescence and remodelling obtained from the cord blood of healthy term neonates; 2) cluster infants based on similarities in protein expression using an approach independent of clinical phenotype to identify subgroups with variations in response to air pollution; and 3) investigate the distribution of air pollution exposure during pregnancy and demographic parameters in these newly identified clusters.

Methods

Study population

The ongoing, prospective Bern-Basel Infant Lung Development (BILD) birth cohort study is conducted in Switzerland, as previously described [16]. Participants have been recruited antenatally in Bern since 1999 and Basel since 2011, and followed up *via* clinical visits and questionnaires. Inclusion criteria initially involved middle-European (self-reported) infants, with mothers having no serious health problems or drug abuse, and newborns without major birth defects or perinatal diseases. Parental written informed consent was obtained. The study protocol was approved by the Ethics Committee of Northwest and Central Switzerland (EKNZ, Basel, Switzerland) and the Bernese Cantonal Ethics Research Committee (KEK, Bern, Switzerland).

For the current study, we considered infants recruited until 2019 when the cord blood proteins were measured. Infants classified as preterm (<37 weeks gestational age at birth) were excluded from the analysis (n=100). Air pollution measurements were available for 525 infants. Cord blood proteins were measured in samples with sufficient volume of plasma (n=678) and serum (n=580). Also included were term infants with complete data on air pollution exposure, covariates and proteins. The final sample consisted of 387 infants after excluding samples where the time from blood collection to processing exceeded 3 days, samples from cord blood donation or those with unreadable labels due to freezing.

Outcomes: cord blood proteins

Umbilical cord blood was collected directly after birth in EDTA tubes (for plasma) or serum tubes (for serum), centrifuged and stored at −80°C for future analyses [17, 18].

We analysed 11 autophagy, cellular senescence and remodelling proteins involved in the development of pulmonary disease. Interleukin (IL)-1β, IL-8, matrix metallopeptidase (MMP)-3, MMP-9, platelet-derived growth factor (PDGF)-AA, transforming growth factor-β (TGF-β) and tumour necrosis factor-α (TNF-α) were detected in serum. Sirtuin 1 (SIRT1), p62 protein (also-called Sequestosome 1 (SQSTM1), hereafter referred to as p62), microtubule-associated protein 1 light chain 3 (LC3B/MAP1LC3) and Beclin-1/BECN1 were detected in plasma. The pattern for interpretation of protein concentration is shown in table 1.

Exposure assessment: air pollution

This study focused on long-term prenatal air pollution exposure, specifically using mean NO_2 and PM_{10} levels throughout the whole pregnancy and the third trimester. Details on prenatal air pollution exposure estimation, using time–space models, are provided in the supplementary material and were described previously [5, 19]. NO_2 was modelled using a long-term time series of bi-weekly and monthly passive samples from 146 locations along with spatial predictors (land use; roads; traffic; population; annual NO_2 from a dispersion model) and temporal predictors (meteorological conditions; NO_2 from a continuous monitoring station), and extensively validated [19]. Validation included 10-fold internal cross-validation and an external validation using 57 NO_2 passive measurements obtained at study participants' homes.

A more simplified time–space approach was used for PM_{10}. Daily mean PM_{10} levels from the monitoring station at Payerne, Switzerland, were used to adjust the concentration at the home addresses obtained from annual dispersion models from Pollumap (METEOTEST 2021), thus incorporating the spatial context [5]. Both models (NO_2 and PM_{10}) were then applied to calculate maternal exposures using all home addresses

TABLE 1 Proteins included in the analysis

Protein	Biological function
Autophagy proteins	
LC3B	Increases autophagosome formation by facilitating the elongation of autophagic membranes
Beclin-1/BECN1	Induces autophagy
Autophagy and senescence proteins	
p62	Serves as a cargo receptor for the selective degradation of ubiquitinated proteins; accumulation can indicate inefficient autophagy and contribute to senescence-related processes
SIRT1	• Overexpression of SIRT1 reverses senescence • Negatively regulates the expression of several senescence-associated secretory phenotype (SASP) factors, including IL-8 and IL-1β
Infammatory-associated SASP	
IL-8	• Pro-inflammatory cytokine • Promotes the senescence of fibroblastic mesenchymal progenitor cells (MPCs), allowing these senescent cells to evade immune-cell-mediated clearance. This contributes to the persistence of senescent cells, exacerbating inflammation and fibrosis
IL-1β	• Pro-inflammatory cytokine • Plays a role in the induction of senescence and drives chronic inflammation in the lungs, contributing to airway remodelling
TNF-α	• Induction of senescence and autophagy • Pro-inflammatory cytokine • Inhibition of SASP, including TNF-α, has been shown to prevent $PM_{2.5}$-induced airway remodelling
Remodelling-associated SASP	
PDGF-AA	• Promotes tissue remodelling and alveolar regeneration by activating matrix fibroblasts and supporting the differentiation of alveolar epithelial cells • Increased PDGF-AA signalling plays a crucial role in the repair of damaged lung tissue, particularly in aged lungs
MMP-9	Involved in the breakdown of the extracellular matrix and remodelling of tissue during inflammation and fibrosis
MMP-3	A metalloproteinase that contributes to tissue remodelling by degrading extracellular matrix components
TGF-β	A key regulator of tissue remodelling, involved in fibrosis and extracellular matrix production

LC3B/MAP1LC3: microtubule-associated protein 1 light chain 3; p62/SQSTM1: protein Sequestosome 1; SIRT1: Sirtuin 1; IL-8: interleukin-8; IL-1β: interleukin-1β; TNF-α: tumour necrosis factor-α; PDGF-AA: platelet-derived growth factor AA; MMP-9: matrix metallopeptidase-9; MMP-3: matrix metallopeptidase-3; TGF-β: transforming growth factor-β.

during pregnancy. Thus, adjustments for address changes during pregnancy were made by calculating the mean exposure weighted by the duration spent at each address.

Covariates

During a clinical examination of infants aged 1 month, study nurses conducted interviews and documented family characteristics and prenatal exposures, including birth order, parental education, family history of allergy and prenatal maternal smoking [16]. Using the birth records, information about gestational age (in weeks), birthweight, sex and mode of delivery was obtained. Small for gestational age (SGA) was defined when a birthweight was <10th percentile, assessed using the Fenton growth chart [20].

Statistical analysis

Regression analysis

First, the association between exposure to air pollutants during the whole pregnancy and selected proteins was assessed using the multivariable Tobit regression model to account for the left-censoring data at the level of detection (LOD) [21]. Zero values for all proteins were imputed with half the minimum non-zero value, and the data were log2-transformed to achieve normality prior to statistical analysis. For IL-1β, a logistic regression was applied since 67% of data was below LOD. IL-1β was categorised as 0 (below LOD) or 1 (above LOD). We used a single-pollutant regression model adjusted for sex, gestational age, season of birth (cosine function of the date of birth), prenatal maternal smoking (yes/no), mode of delivery, birth order (first and later birth), study centre, time from blood collection to processing and temporary fridge storage until processing. Covariates were selected on the basis of our previous studies [18, 22, 23]. In the sensitivity analyses, we also investigated the effect of short-term air pollution exposure (the third trimester before birth) on cord blood proteins. Effect estimates were presented as a coefficient (β) or odds ratio (OR) and 95% confidence intervals (CIs) per 10 $\mu g \cdot m^{-3}$ increase in each pollutant. Benjamini–Hochberg correction for multiple tests (n=11) was calculated with a cut-off of <0.05 to define significance.

Unsupervised hierarchical clustering

Unsupervised hierarchical clustering based on protein profiles without guidance from clinical and exposure data was applied to find whether the difference in proteins can separate infants according to the level of prenatal air pollution exposure. For hierarchical clustering, the log2 data were z-score-normalised. Clustering was done using Euclidean distance and Ward's linkage method with *hclust* algorithm and the heatmap with *pheatmap*. Clusters were defined by cutting top branches. Depending on the data distribution, a two-way ANOVA or a Kruskal–Wallis test was used to compare air pollution exposure, protein concentrations and clinical characteristics included in the regression analysis across the identified clusters. Residual analysis was performed to test for the assumptions of the ANOVA. Normality was assessed using Shapiro–Wilk's normality test and homogeneity of variances was assessed by Levene's test. *Post hoc* analysis was conducted using Tukey's Honest Significant Difference test or Dunn's test, following the ANOVA or Kruskal–Wallis test, as appropriate. For categorical variables, a Chi-squared test was used when all expected frequencies were ⩾5, while Fisher's exact test was applied when any expected frequency was <5 to assess differences across clusters.

Correlation network analysis

To identify modules of highly interconnected proteins in each cluster, we used *igraph* [24] with clustering with the Fruchterman–Reingold layout algorithm. Edges are weighted according to Spearman's correlation coefficient (r). The network integrity is preserved when edges are only shown if absolute correlation r⩾0.2. A higher correlation threshold would lead to a drastic drop in network connectivity in clusters with a low number of infants. Then we calculated network node centrality parameters for each node (protein): 1) the degree centrality that represents the number of edges connected to the node; 2) the closeness centrality that reflects the mean distance to other nodes; 3) the betweenness centrality – one of the important centrality measures that represents how often a node lies on the shortest path between others.

We also analysed the relationship between air pollution exposure and the network properties of the identified clusters using MATLAB (version 2024b). For each cluster, we constructed undirected graphs and calculated key network metrics, including density (the proportion of edges in the network compared to the maximum possible edges), mean degree (the mean number of edges per node), size of the largest connected component (LCC) and edge weight mean (the mean of the edge weights across the graph). The clustering coefficients were computed using the original weighted method, as well as the Onnela weighted clustering approach, which employs the geometric mean of normalised edge weights to measure the intensity of weighted triangles [25], and the Barrat weighted clustering method [26], which incorporates node strength and weighted contributions to triangle. These metrics were then integrated with mean NO_2 and PM_{10} levels calculated for each cluster to assess potential associations.

In a sensitivity analysis, we explored whether sex modifies the association between air pollution exposure during pregnancy and cord blood protein levels. First, we included an interaction term between sex and air pollutants in the multiple regression analysis. Next, we conducted a stratified analysis by sex to investigate potential differences. Finally, we performed sex-stratified hierarchical clustering to further evaluate these differences.

All calculations, except for the network analysis with air pollution, were conducted using R version 4.3.0 within Rstudio version 2022.07.2.

Results

A total of 387 infants with complete air pollution and protein expression data were analysed (supplementary figure S1). Demographics and exposure characteristics are described in table 2. The concentration of proteins along with LODs in the cord blood is given in supplementary table S1. Five proteins had concentrations above the LOD in 100% of the samples, while four proteins had fewer than 2% of their values below the LOD. The remaining proteins, p62 and IL-1β, had 31% and 65% of their values below the LOD, respectively. A heatmap with Spearman's correlation coefficients between proteins is shown in supplementary figure S2.

The correlation between air pollutants across different time windows (third trimester and the whole pregnancy) is reported in supplementary table S2. NO_2 and PM_{10} exhibited a strong correlation within the same time window, ranging from 0.74 to 0.77.

Association of air pollution with protein expression

The proteins significantly dysregulated by NO_2 include Beclin-1 and IL-8 (figure 1, supplementary table S3). A 10 $\mu g \cdot m^{-3}$ increase in NO_2 exposure during pregnancy was linked to a 0.76 decrease (95% CI −1.12– −0.40, p-value$_{adj}$ = <0.001) in IL-8 levels on a log2 scale. Conversely, a positive

TABLE 2 Demographic and exposure data of all infants and comparison of protein-based clusters of infants

	Overall	Cluster 1	Cluster 2	Cluster 3	Cluster 4	p-value
Infants, n	387	203	108	65	11	
Sex, boy	195 (50.4)	99 (48.8)	64 (59.3)	27 (41.5)	5 (45.5)	0.124
Gestational age weeks	39.79±1.13	39.75±1.10	39.79±1.19	39.87±1.16	39.91±0.79	0.875
Gestational weight g	3413±426	3401±429	3488±431	3334±397	3367±418	0.115
SGA, yes	32 (8.3)	22 (10.8)	5 (4.6)	3 (4.6)	2 (18.2)	0.079
Maternal asthma, yes	41 (10.6)	19 (9.4)	12 (11.1)	8 (12.3)	2 (18.2)	0.601
Birth order, later birth	206 (53.2)	122 (60.1)[#]	53 (49.1)	27 (41.5)	4 (36.4)	**0.023**
Mode of delivery, C-section	64 (16.5)	33 (16.3)	18 (16.7)	12 (18.5)	1 (9.1)	0.942
Maternal age years	32.46±4.23	32.37±4.38	32.05±4.24	33.06±3.81	34.64±2.69	0.150
Missing	8 (2.1)	3 (1.5)	5 (4.6)	0	0	
Prenatal maternal smoking, yes	23 (5.9)	15 (7.4)	6 (5.6)	2 (3.1)	0 (0)	0.634
PM_{10} during pregnancy $\mu g \cdot m^{-3}$	19.77±3.76	19.98±3.70	20.25±3.75[¶]	18.87±3.82	16.45±1.95[+]	**0.002**
NO_2 during pregnancy $\mu g \cdot m^{-3}$	17.40±6.27	17.79±6.37[#]	18.12±5.98[#]	15.26±5.82	16.00±7.54	**0.015**
PM_{10} in third trimester $\mu g \cdot m^{-3}$	17.21±7.23	17.49±7.36	18.22±7.22	15.01±6.55	15.28±6.53	**0.026**
NO_2 in third trimester $\mu g \cdot m^{-3}$	19.93±6.10	20.08±6.14	20.46±6.47[#]	19.15±5.48	16.66±4.13	0.161
Season at birth						**0.032**
Spring	100 (25.8)	55 (27.09)[¶]	28 (26.93)	15 (23.08)[¶]	2 (18.18)	
Summer	99 (25.6)	46 (22.66)	29 (26.85)	24 (36.92)	8 (72.73)	
Autumn	102 (26.4)	58 (28.57)	23 (21.3)	13 ((20)	0	
Winter	86 (22.2)	44 (21.67)	28 (25.93)	13 (20)	1 (9.09)	
Study centre						**<0.001**
Basel	37 (9.6)	9 (4.4)[¶,#]	6 (5.6)[¶]	11 (16.9)[¶]	11 (100)	
Bern	350 (90.4)	194 (95.6)	102 (94.4)	54 (83.1)	0 (0)	

Categorical variables are presented as n (%); continuous variables are presented as mean±SD. An ANOVA or Kruskal–Wallis test was used to compare continuous data across clusters, with *post hoc* analysis using Tukey's or Dunn's test as appropriate. For categorical variables, Chi-squared or Fisher's exact test assessed differences across clusters. Pairwise comparisons were performed with p-values adjusted using Benjamini–Hochberg correction. Boldface indicates p<0.05. Seasons are defined as follows: Winter (December, January, February), Spring (March, April, May), Summer (June, July, August), Autumn (September, October, November). SGA: small for gestational age (weight is <10th percentile for gestational age); PM_{10}: particulate matter 10 µm or less in diameter; NO_2: nitrogen dioxide. [#]: significantly different from cluster 3 at the p-value$_{adj}$ <0.05 level; [¶]: significantly different from cluster 4 at the p-value$_{adj}$ <0.05 level; [+]: significantly different from cluster 1 at the p-value$_{adj}$ <0.05 level.

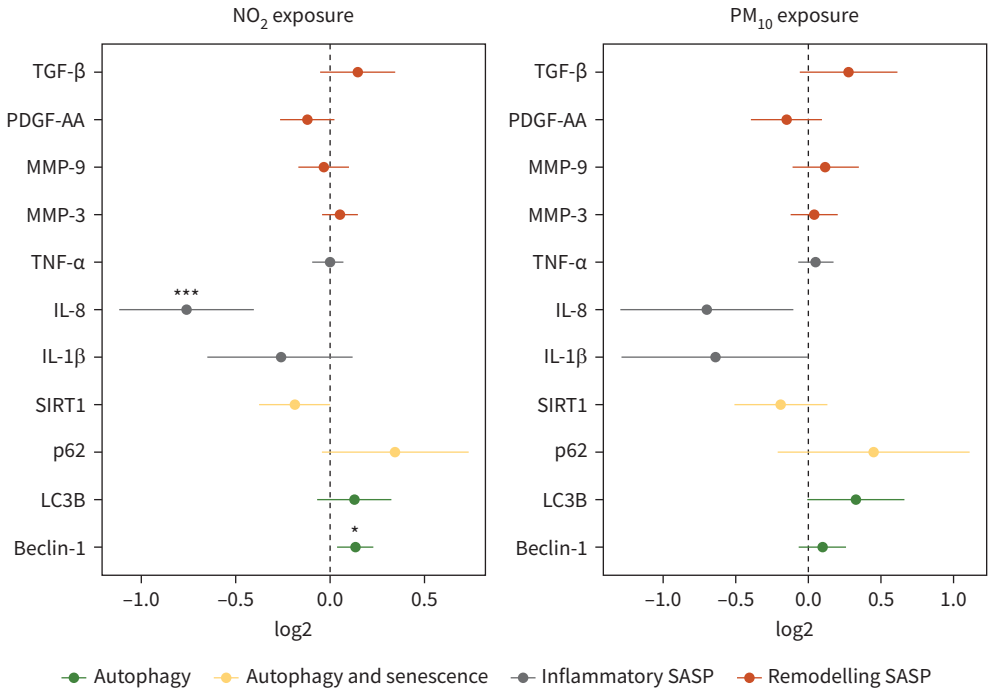

FIGURE 1 Adjusted associations of nitrogen dioxide (NO_2) and particulate matter (PM_{10}) during the whole pregnancy with proteins. Estimates were reported as a coefficient with corresponding 95% CI per 10 $\mu g \cdot m^{-3}$ increase in NO_2 and PM_{10}. Estimates were obtained using Tobit regression models adjusted for gestational age, birth order, sex, maternal smoking during pregnancy, mode of delivery, study centre, season of birth, time from blood collection to processing and temporary fridge storage until processing. For IL-1β estimates were obtained using a logistic regression model with the same adjustment. SASP: senescence-associated secretory phenotype; IL-1β: interleukin-1β; IL-8: interleukin-8; MMP-3: matrix metallopeptidase-3; MMP-9: matrix metallopeptidase-9; PDGF-AA: platelet-derived growth factor AA; TGF-β: transforming growth factor-β; TNF-α: tumour necrosis factor-α; SIRT1: Sirtuin 1; p62 (SQSTM1): protein Sequestosome 1. *: p<0.05; ***: p<0.001.

association was observed between NO_2 and Beclin-1 (0.13; 95% CI 0.04–0.23, $p\text{-value}_{adj} = 0.034$). After Benjamini–Hochberg correction, no significant association was found between PM_{10} exposure during pregnancy and cord blood proteins.

In the sensitivity analysis using air pollution exposure in the third trimester, we observed a similar pattern of associations for all proteins, but the effect was weaker than for the whole pregnancy (supplementary table S4).

Hierarchical clustering of proteins

Subsequent unsupervised hierarchical clustering of all 11 proteins resulted in an optimal outcome of four clusters. Cluster demographics and exposure characteristics are presented in table 2. The difference in clinical characteristics between clusters was observed only in terms of birth order. The clusters were distinguished by environmental factors such as air pollution and season of birth, which can impact pollutant levels, indicating that air pollution is a significant factor contributing to the overall difference in protein levels (figure 2 and table 2). Some clusters are dominated by participants from a single study area, which may be related to the significant variation in pollution levels by study centre (supplementary table S5).

Cluster 1 is the most common cluster compromising 203 infants (52% of the total study population) with similar clinical and exposure characteristics as observed in the whole population. This cluster is characterised by low p62 protein levels (figures 2, 3, and supplementary figure S3, supplementary table S6) and elevated levels of Beclin-1 and LC3B compared to the other clusters (supplementary figure S3). Furthermore, NO_2 levels were significantly higher compared to cluster 3, while PM_{10} levels were elevated relative to cluster 4 (table 2).

Cluster 2 comprises 108 infants. It shows lower levels of IL-1β when compared to clusters 1, 2 and 4.

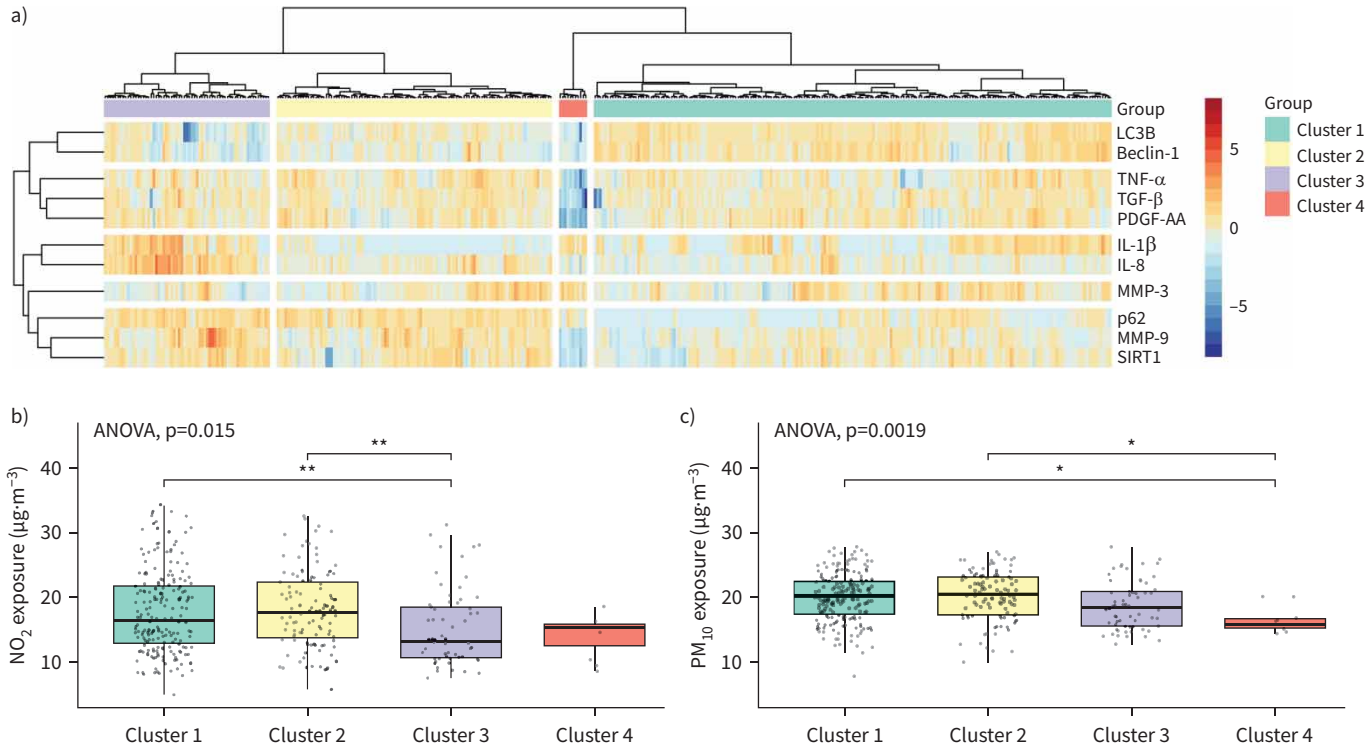

FIGURE 2 a) Heatmap of hierarchical clustering of all proteins and samples. Red denotes high expression of proteins and blue, low. b) and c) Nitrogen dioxide (NO_2) and particulate matter (PM_{10}) exposure during the whole pregnancy differed by infants' clusters according to protein level. An ANOVA test followed by Tukey's *post hoc* test was used to compare air pollution exposure. Only significant pairwise comparisons after adjustment for multiple testing are shown. IL-1β: interleukin-1β; IL-8: interleukin-8; MMP-3: matrix metallopeptidase-3; MMP-9: matrix metallopeptidase-9; PDGF-AA: platelet-derived growth factor AA; TGF-β: transforming growth factor-β; TNF-α: tumour necrosis factor-α; SIRT1: Sirtuin 1; p62 (SQSTM1): protein Sequestosome 1. *: p<0.05; **: p<0.01.

Cluster 3 comprises 65 infants. This cluster displayed general upregulation of pro-inflammatory proteins (IL-1β and IL-8) compared to other clusters. p62 and SIRT1 were also more highly expressed in this cluster (figures 2, 3, and supplementary figure S3, supplementary table S6).

Cluster 4 (n=11) consists only of infants without maternal smoking exposure during pregnancy, with the highest proportion of infants born in summer (72%), all in Basel (table 2). Cluster 4 infants show the lowest levels of all proteins except IL-1β and MMP-3, which are upregulated (figures 2, 3, and supplementary figure S3, supplementary table S6). This cluster has a lower level of PM_{10} exposure during pregnancy compared to cluster 1, but there is no difference observed in NO_2 exposure.

Protein correlation with network analysis

Figure 4 illustrates the network analysis between proteins by each cluster. Cluster networks were differentiated by connectivity structure. Networks in clusters 3 and 4 showed a higher number of edge connections among proteins and stronger relationships (high values for Spearman's correlation coefficients) than networks in clusters 1 and 2 (supplementary figures S4 and S5). Moreover, Beclin-1 had the highest node centrality parameters (degree, strength and/or betweenness) in both cluster 2 and cluster 4 (supplementary figure S6). MMP-3 was central for cluster 1. For cluster 3, one of the central proteins was SIRT1 with the highest degree and betweenness parameters. In all clusters, the strongest positive correlations were observed between LC3B and Beclin-1 (r varies from 0.39 to 0.69). All metrics (except LCC size) exhibit a potential negative association with air pollution (figure 5, supplementary figures S7 and S8). A decrease with higher air pollution suggests that environmental stress disrupts the coherence and strength of local clusters.

In the sensitivity analyses we examined whether the associations between prenatal air pollution exposure and cord blood proteins differed by sex. Interaction terms between sex and air pollutants (NO_2 and PM_{10}) were tested in the multiple regression models, and no significant interactions were detected after correction for multiple comparisons (supplementary tables S7 and S8). Additionally, sex-stratified hierarchical

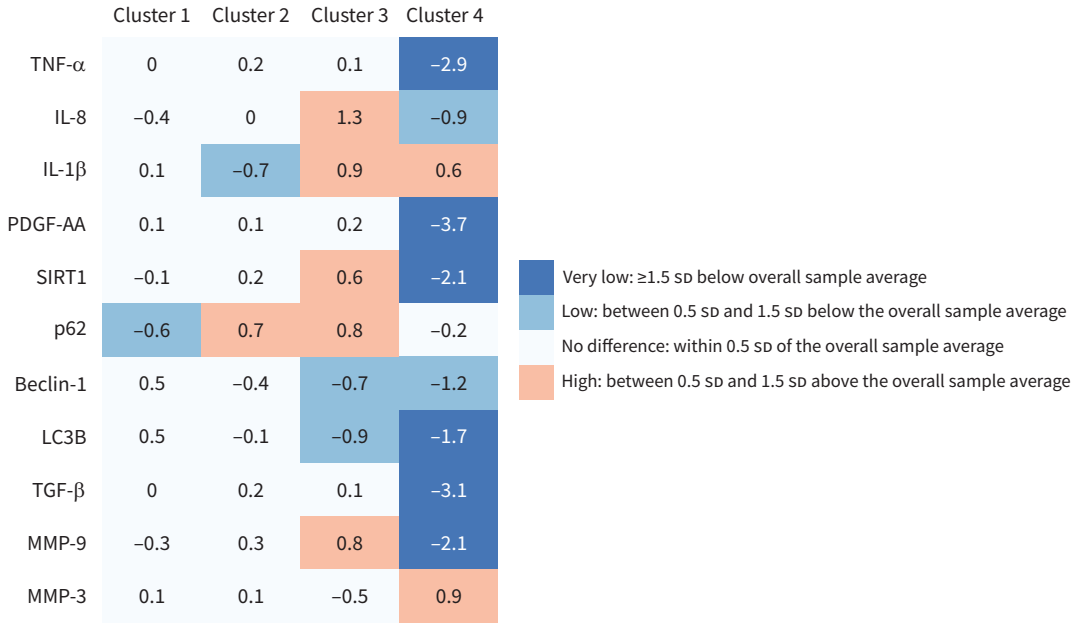

FIGURE 3 Relative degree of variability between clusters. Each cell represents the z-score of protein concentrations within a cluster, calculated relative to the overall sample average (set to 0) and scaled to a standard deviation (SD) of 1. The pairwise comparison for differences for all proteins by clusters can be found in supplementary figure S3. IL-1β: interleukin-1β; IL-8: interleukin-8; MMP-3: matrix metallopeptidase-3; MMP-9: matrix metallopeptidase-9; PDGF-AA: platelet-derived growth factor AA; TGF-β: transforming growth factor-β; TNF-α: tumour necrosis factor-α; SIRT1: Sirtuin 1; p62 (SQSTM1): protein Sequestosome 1.

clustering revealed broadly similar patterns between boys and girls, particularly in cluster 4, which displayed strong downregulation of autophagy-related proteins (LC3B, Beclin-1, SIRT1, p62) in both sexes (supplementary figures S9 and S10). Boys exhibited slightly more pronounced upregulation of pro-inflammatory markers (*e.g.*, IL-8, IL-1β), referred to as cluster 3, compared to girls (referred to as cluster 2). Overall, sex did not significantly modify the associations observed in our study.

Discussion

This is the first study showing that ambient air pollution during pregnancy is associated with differential expression of autophagy-related proteins in healthy newborns. Our results indicate that NO_2 exposure during pregnancy can increase Beclin-1 levels, a pivotal initiator of autophagy. Moreover, elevated NO_2 exposure was correlated with a reduction in IL-8 levels. Unsupervised hierarchical clustering of all proteins measured allowed us to group newborns into four distinct clusters with similar protein expression profiles. When analysing the clinical and exposure characteristics of these groups, the significant differences observed were NO_2 and PM_{10} exposure during pregnancy.

These findings align with substantial evidence from both mouse and cell studies suggesting that air pollution, particularly $PM_{2.5}$, induces autophagy, which is detected by elevated levels of Beclin-1 and LC3B in lung tissue [27–29]. In our study in healthy human newborns, we observed a significant increase in Beclin-1 that correlated with an increase in NO_2 exposure during pregnancy. We also observed that LC3B levels tended to be increased in relation to NO_2 exposure. Since NO_2 is the source of nitrate aerosol (an essential component of $PM_{2.5}$), this observation is in-line with findings from *in vitro* studies where there was an upregulation of LC3B following exposure to $PM_{2.5}$ [29, 30]. When calculating the mean over the whole pregnancy period, a high correlation between these two pollutants has also been observed [31].

Our results suggest a potential association between NO_2 exposure and compromised antioxidative capacity through decreased SIRT1 levels, although this association was not significant after adjustment for multiple testing. SIRT1 protects against inflammation and oxidative stress and its levels are reduced in the cells or lung tissue of mice exposed to smoke [32] or $PM_{2.5}$ [33]. Additionally, an epidemiological study has reported that long-term exposure to $PM_{2.5}$ is associated with decreased SIRT1 levels in the elderly population [34].

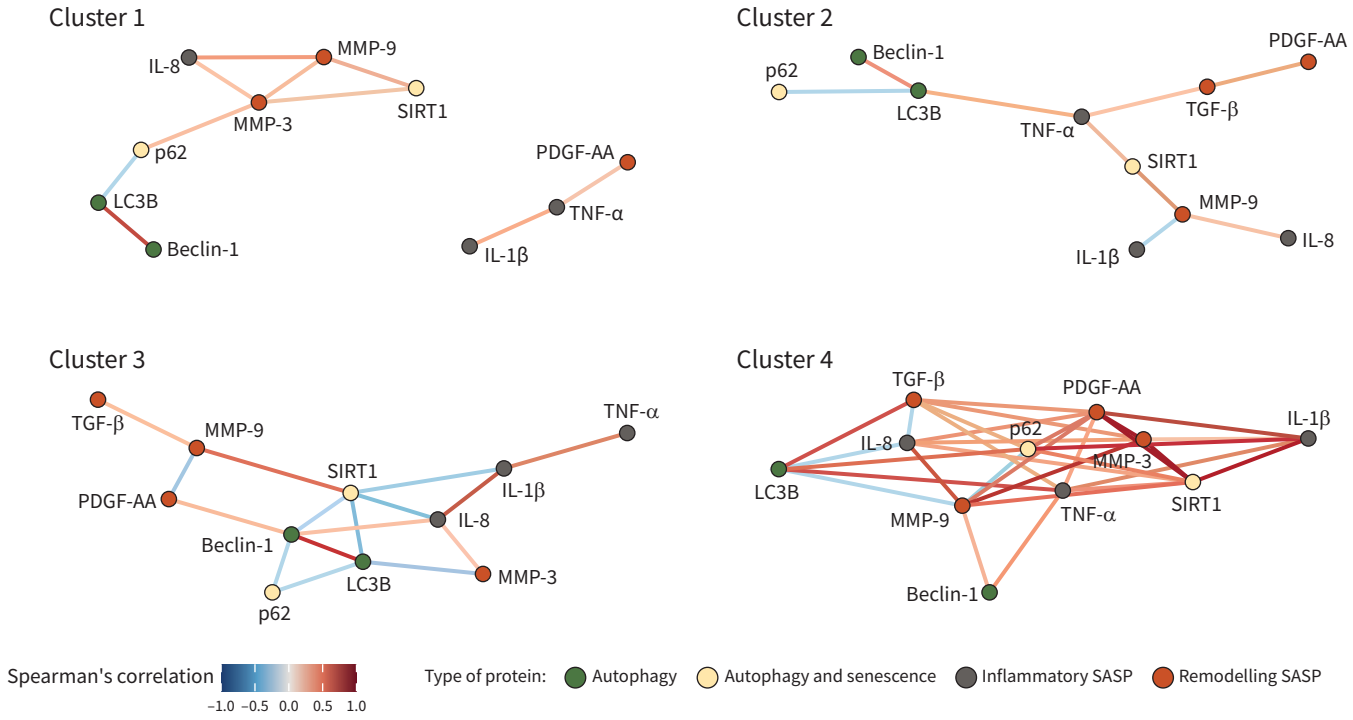

FIGURE 4 Correlation network in each cluster. Connections (edges) are established using Spearman's correlation coefficient. Correlations are displayed when the absolute correlation coefficient is >0.2. Each protein is represented by a node, with node colours indicating protein types, and edge colours reflecting the strength of correlation between proteins. SASP: senescence-associated secretory phenotype; IL-1β: interleukin-1β; IL-8: interleukin-8; MMP-3: matrix metallopeptidase-3; MMP-9: matrix metallopeptidase-9; PDGF-AA: platelet-derived growth factor AA; TGF-β: transforming growth factor-β; TNF-α: tumour necrosis factor-α; SIRT1: Sirtuin 1; p62 (SQSTM1): protein Sequestosome 1.

We found negative associations of NO_2 and PM_{10} exposure during pregnancy with IL-1β and IL-8. Notably, the results concerning the relationships between air pollution and cytokines exhibit inconsistencies across studies involving diverse population groups, including newborns, pregnant women and adults. While some studies have suggested that prenatal $PM_{2.5}$ and NO_2 exposure induced the levels of IL-1β [17, 35], and IL-8 [36, 37] in infants or pregnant women [38], others have also reported a negative relationship between air pollution exposure and these cytokines [36, 37], consistent with our findings. Additionally, research in adults conducted by Mostafavi *et al*. [39] indicated that long-term exposure to NO_2 was linked to a reduction in IL-8 levels, which contrasts with results from other studies reporting a pro-inflammatory effect of air pollution [40]. The differences observed in these studies could be attributed to the heterogeneity of pollutants contributing to air pollution, which may exert different biological responses and effects. Indeed, downregulation or inhibition of IL-8 secretion *in vitro* depends on particle composition, sites (industrial or rural) and time of exposure [41]. The discrepancy with our previous study [17] may stem from differences in time period, assay methods and air pollution exposure calculation models. Additionally, we demonstrate for the first time in a healthy population that distinct groups of individuals exhibit different protein response patterns to air pollution.

A pivotal finding in our study is the categorisation of newborns into four distinct clusters based on their autophagy-related protein expression. Notably, these clusters are distinguished mainly by air pollution exposure during pregnancy and not by perinatal and demographic factors. This suggests the existence of individual response patterns to air pollution in healthy newborns. Cluster 3 was identified as pro-inflammatory, characterised by high levels of IL-1β and IL-8, accompanied by increased expression of p62, SIRT1 and MMP-9. Increased levels of p62 correlates with impaired autophagy [42], potentially leading to the accumulation of autophagosomes and the promotion of pro-inflammatory cytokine production, thereby contributing to airway inflammation [42]. Moreover, IL-1β expression may precede an increase in MMP-9, promoting remodelling responses in the lung [43]. Impaired autophagy could also activate MMP-9 *via* nuclear factor kappa B-dependent signalling [44]. Given the lower air pollution exposure in this cluster when compared to cluster 1, we think that this particular group of infants is more susceptible to the adverse effects of air pollution. This underscores the complex interplay between

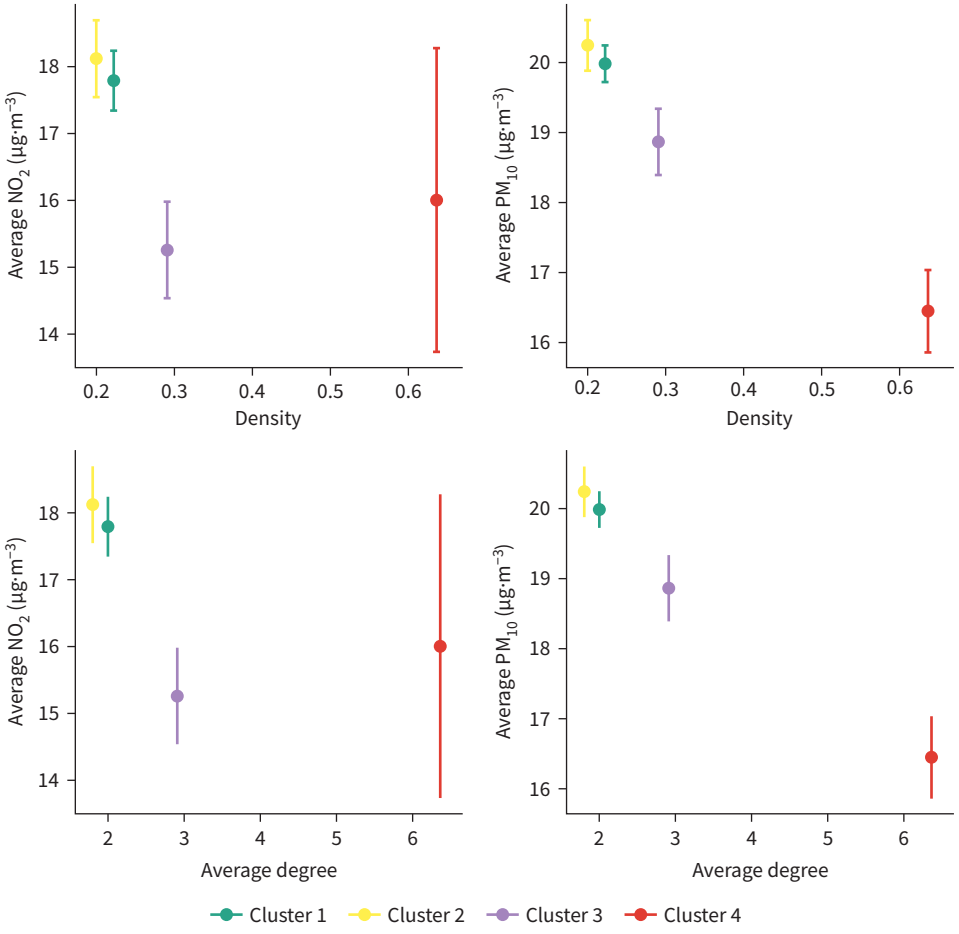

FIGURE 5 Association of network metrics (density and average degree) with air pollution exposure during pregnancy: average nitrogen dioxide (NO_2) and particulate matter (PM_{10}) levels, with points representing means and bars showing standard errors for air pollution exposure.

autophagy, cellular senescence and tissue remodelling, and their role in determining an infant's vulnerability to the effects of air pollution.

Our study demonstrates several strengths. We conducted a comprehensive analysis of autophagy-related proteins that play a crucial role in the cellular response to oxidative stress. Notably, the analysis of the autophagy response represents a novel and valuable addition to the field of infant air pollution studies. Furthermore, we observed robust associations of low to moderate levels of air pollution exposure during pregnancy and expression of stress response proteins within a homogeneous healthy population of newborns. Additionally, to the best of our knowledge, this is the first description of a "healthy" unselected large birth cohort which provides "normative values" of these protein markers in cord blood.

Several limitations should be noted when interpreting our findings. First, NO_2 and PM_{10} were estimated by modelling, rather than by continuous personal or in-home exposure monitoring. Exposure assessment in our studies varied between the two different pollutants, and we did not evaluate exposure to fine and ultra-fine particles, known to induce a more pronounced oxidative stress response. Furthermore, regression and cluster analysis did not provide information about signalling pathways. Simultaneous protein production by multiple cells adds complexity to our interpretation.

Although our previous study demonstrated differences in key autophagy markers between preterm and term infants [18], and another study highlighted the increased susceptibility of preterm infants to the adverse effects of air pollution on lung function [4], preterm infants were not included in the current study due to the limited sample size with complete data on both air pollution exposure and protein levels. Future studies

should aim to address these gaps by including larger preterm cohorts and investigating potential pathways mediating air pollution effects on protein expression.

In conclusion, in our study we first showed that air pollution exposure during pregnancy is associated with changes in autophagy-related proteins in the cord blood. We identified four subgroups among healthy newborns, each characterised by unique profiles of autophagy, pro-inflammatory and remodelling-associated proteins. Future longitudinal studies should explore whether these infant subgroups are more prone to asthma or experience greater lung function changes in response to air pollution exposure. Expanding autophagy-related protein panels and exploring diverse pollutants may uncover additional sub-clusters and underlying biological responses to air pollution.

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Data availability: The data and code supporting the findings of this study are available from the corresponding author upon request, subject to availability.

Ethics statement: The study protocol was approved by the Ethics Committee of Northwest and Central Switzerland (EKNZ, Basel, Switzerland) and the Bernese Cantonal Ethics Research Committee (KEK, Bern, Switzerland).

Author contributions: Conception and design: O. Gorlanova, H. Oller and U. Frey. Data collection and analysis: O. Gorlanova, A. Marten, L. Müller, C. Rüttimann, N. Künstle, R. Steinberg, P. Latzin, J. Usemann, D. Vienneau, K. de Hoogh and M. Röösli calculated the air pollution exposure. Data interpretation: O. Gorlanova, H. Oller and U. Frey. Manuscript drafting: O. Gorlanova and U. Frey. Manuscript revision and final approval: O. Gorlanova, H. Oller, A. Marten, L. Müller, U. Nahum, C. Rüttimann, N. Künstle, R. Steinberg, P. Latzin, D. Vienneau, K. de Hoogh, M. Röösli, C.R. da Silva Sena, P. Schär, S. Schulzke, P. Sinues, P. Sharma, D. Schürmann and U. Frey.

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