Cardiovascular Disease-Related Serum Proteins in Workers Occupationally Exposed to Polycyclic Aromatic Hydrocarbons

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The authors certify that all research involving human subjects was done under full compliance with all government policies and the Helsinki Declaration.
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ABSTRACT

Chimney sweeps have higher incidence and mortality of cardiovascular disease (CVD), likely related to their exposure to polycyclic aromatic hydrocarbons (PAH). In order to identify underlying mechanisms of PAH-related CVD, we here investigated whether PAH exposure was associated with levels of putative CVD-related proteins in serum among currently working chimney sweeps. We enrolled 116 chimney sweeps and 125 unexposed controls, all nonsmoking male workers from Sweden. We measured monohydroxylated PAH metabolites in urine by liquid chromatography coupled to tandem mass spectrometry and a panel of 85 proteins in serum using proximity extension assay. Linear regression analysis adjusted for age and body mass index showed that 25 proteins were differentially expressed between chimney sweeps and the controls ($p < .05$, adjusted for false discovery rate). Of the 25 proteins, follistatin (FS), prointerleukin-16 (IL-16), and heat shock protein beta-1 (HSP 27) showed positive associations with the monohydroxylated metabolites of PAH in a dose-response manner ($p < .05$). Pathway and gene ontology analyses demonstrated that the differentially expressed proteins were mainly involved in inflammatory response and immunological functions, such as leukocyte migration, cell movement of leukocytes, and adhesion of immune cells. In conclusion, we found a number of putative CVD-related proteins differentially expressed, between PAH-exposed and unexposed individuals, and mainly involved in inflammation and immune function. Our data warrant protective measures to reduce PAH exposure and longitudinal investigations of the protein profile in chimney sweeps and other occupational groups exposed to PAH.

Key words: chimney sweeps; work-related; PAH metabolites; circulating; biomarkers; inflammation.

Cardiovascular disease (CVD) is the leading cause of death worldwide, accounting for about 18 million deaths annually (31% of all global mortalities) (WHO, 2017). Although a number of risk factors are well-established, such as older age, smoking, hypertension, diabetes, low physical activity, and obesity; less is known about the contribution of occupational factors to the

burden of CVD (WHO, 2017). The Swedish agency for health technology assessment and assessment of social services (SBU) has recently concluded that occupational exposures such as silica dust, engine exhaust, and welding fumes are associated with CVD (SBU, 2017). Another occupational exposure linked to increased cardiovascular events is soot sweeping, ie, removal of soot from chimneys (Gustavsson et al., 2013; Hansen, 1983). Epidemiological studies have shown increased mortality of ischemic heart disease as well as increased incidence of myocardial infarction among chimney sweeps (Gustavsson et al., 1987, 2013; Hansen, 1983; Jansson et al., 2012). Polycyclic aromatic hydrocarbons (PAH), a group of lipophilic chemicals composed of at least 2 benzene rings, constitute a main part of soot (ATSDR, 1995; IARC, 2012). PAH are omnipresent as they can originate in nature (petroleum oil and natural gas) and be produced by incomplete combustion of organic material such as diesel fuel, wood, and cigarettes (IARC, 2010, 2012). Adverse cardiovascular outcomes have also been reported in other occupational groups exposed to PAH. Burstyn et al. investigated more than 12 000 male asphalt workers and found that exposure to PAH (benzo[a]pyrene; BaP) was associated with ischemic heart disease and showed a dose-response relationship (Burstyn et al., 2005). Similar association between BaP exposure and ischemic heart disease was also found among aluminum smelters ($n = 7026$) (Friesen et al., 2010). A few studies have suggested systemic inflammation and oxidative stress to be involved in the etiology of PAH-related CVD (Curfs et al., 2005; Jeng et al., 2011). Moreover, decreased heart rate variability, which is linked to adverse cardiovascular events, was associated with exposure to PAH among coke oven workers ($n = 1333$) (Li et al., 2012). In our previous work on Swedish chimney sweeps, we found associations between chimney sweeping and increased levels of homocysteine and cholesterol in serum, which might indicate potential interactions between PAH and cholesterol metabolism and/or the 1-carbon metabolism (Alhamdow et al., 2017). Still, knowledge about mechanisms of PAH-related CVD is limited and warrants further investigation.

We aimed in this study to explore the serum profile of putative CVD-related proteins among currently working chimney sweeps and related underlying mechanisms of PAH-induced CVD.

MATERIALS AND METHODS

Study participants. Participants in this study were nonsmoking male chimney sweeps (exposure group; $n = 116$) and unexposed individuals (control group; $n = 125$) selected from the parent study (Alhamdow et al., 2017), which included 151 chimney sweeps and 152 controls. The chimney sweeps were employed at different chimney sweeping companies, whereas the controls were workers at warehouses and municipalities with no reported occupational exposure to PAH. The controls matched the chimney sweeps in gender, age, smoking status, and geographical area of residence (southern Sweden). The enrollment process took place during 2010–2015 (more controls were recruited in the beginning of the recruitment period than chimney sweeps). More details about both study groups are published elsewhere (Alhamdow et al., 2017). Because tobacco smoking is a well-known source of exposure to PAH and a risk factor for CVD, current smokers or individuals with missing smoking information in the parent study were excluded (28 chimney sweeps and 25 controls) (Talhout et al., 2011; WHO, 2017). Participants with no blood sample (3 chimney sweeps) or with invalid proteomics data (4 chimney sweeps and 2 controls) were further excluded. As a result, the final number of

participants in this study was $n = 116$ chimney sweeps and $n = 125$ controls (Supplementary Figure 1). Written informed consent was obtained from all participants. The study was approved by the Regional Ethics Committee at Lund University (Lund, Sweden) and performed in accordance with the 1964 Helsinki Declaration and its later amendments.

Recruitment and questionnaire. Recruitment was initiated by contacting managers of the companies. The managers who agreed to participate were instructed to invite their employees for participation. Thus, we were able to calculate the response rate for companies, not individuals. The company response rate was 87% for chimney sweeps and 58% for controls. A trained occupational nurse arranged a company visit for each participant and performed a structured interview, measurement of weight, height, and blood pressure (only chimney sweeps), and sampling of blood and urine.

All participants filled in a questionnaire including questions about sociodemographic factors, lifestyle, disease history, and occupational history. The questionnaire queried level of education (from elementary school to university or higher), chronic diseases (eg, hypertension, myocardial infarction, stroke, and diabetes), family history of disease, prescribed and nonprescribed medication, consumptions of fish, vegetables, and fruits, physical activity during the past 12 months (from sedentary to regular exercising), smoking habit (current smoker, former smoker, and nonsmoker), passive smoking, use of snus, residential area, employment history including working years and job titles, and exposure to dust, diesel exhaust or smoke (such as from welding and soldering) during leisure time. The questionnaire of chimney sweeps further included questions regarding chimney sweeping. For instance, chimney sweeps were asked to estimate, on a scale from 0 to 100, the percentage of time spent on different work tasks from 1963 until the past 12 months before recruitment. Similar questions were also introduced to collect information about the use of protective equipment (eg, gloves, masks, and overall suits) during different sweeping tasks and the type of fuel (wood, petroleum oil, and wood pellets) used in residential houses and industrial facilities where chimney sweeps did soot sweeping.

Sampling of blood and urine. Postshift venous blood and urine samples were collected from participants on the same day during the company visit. There was no preferred day of sampling for controls; however, Wednesdays or Thursdays were chosen for chimney sweeps, because they were expected to be exposed to PAH from work. BD vacutainer SST tubes (Becton, Dickinson and Company, Plymouth, UK) were used for blood sampling. Blood samples were left in room temperature for 10 min to allow coagulation and then centrifuged at $1800 \times g$ for 15 min. Serum was separated, aliquoted, and transported on dry ice to the laboratory of Division of Occupational and Environmental Medicine at Lund University, where the samples were stored at $-80°C$. Urine samples were collected, transported at room temperature to the laboratory and stored at $-20°C$.

Analysis of PAH metabolites in urine. Monohydroxylated PAH metabolites of pyrene (4 benzene rings), phenanthrene (3 rings), BaP (5 rings), and benzo[a]anthracene (BaA; 4 rings) were measured in urine. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS; QTRAP 5500, AB Sciex, Foster City, California) was used for the analyses. In general, there were several isomers of each monohydroxy PAH; however, the quantification was performed using a pure standard of 1 isomer for

each PAH, ie, 1-hydroxypyrene (1-OH-PYR) for quantification of pyrene, 2-hydroxyphenanthrene (2-OH-PH) for phenanthrene, 3-hydroxybenzo[a]pyrene (3-OH-BaP) for BaP, and 3-hydroxybenzo[a]anthracene (3-OH-BaA) for BaA. Details about the LC-MS/MS analysis were published elsewhere (Alhamdow et al., 2017). Briefly, urine samples were treated with β-glucuronidase (Escherichia coli K12) and internal standards for 1-OH-PYR, 2-OH-PH, 3-OH-BaP, and 3-OH-BaA were added. The samples were then injected onto an LC system (2-dimensional) with 2 columns. All samples were run in duplicates and average values were used for statistical analysis. Concentrations of PAH metabolites were adjusted for urinary creatinine.

Measurement of protein concentrations in serum. Serum samples of chimney sweeps and controls were thawed on ice. Aliquots of 40 μl were transferred and randomized in 96-well plates (ThermoFisher Scientific, Waltham, Massachusetts). The plates were then covered by MicroAmp Clear Adhesive Film (ThermoFisher Scientific) and shipped on dry ice to Olink Proteomics (Olink Proteomics, Uppsala, Sweden). A pooled serum sample randomly aliquoted from several chimney sweep and control samples was shipped along with the sample plates for calculations of inter- and intra-assay coefficients of variation (%CV). All samples had been thawed once before aliquoting and plating. The samples were analyzed in 2 batches. Fifty-nine samples (29 chimney sweeps and 30 controls) were included in batch I and 204 samples (100 chimney sweeps and 104 controls) in batch II. Fourteen samples from batch I were rerun in batch II in order to adjust for batch effect. Failed samples were excluded from analysis, and thus we had 116 chimney sweeps and 125 controls for the final statistical analysis (Supplementary Figure 2).

A commercially available proteomics panel including 92 low-abundance serum proteins was used in this study; so-called Cardiovascular II (Olink). The panel encompasses proteins linked to CVD and inflammation, in addition to new exploratory proteins related to CVD. The proteins in this panel are, according to their biological process, involved in angiogenesis ($n = 16$), blood vessel morphogenesis ($n = 16$), catabolic process ($n = 20$), cell adhesion ($n = 29$), coagulation ($n = 10$), heart development ($n = 5$), immune response ($n = 38$), inflammatory response ($n = 28$), MAPK cascade ($n = 25$), platelet activation ($n = 9$), proteolysis ($n = 16$), regulation of blood pressure ($n = 8$), response to hypoxia ($n = 8$), response to peptide hormone ($n = 11$), wound healing ($n = 14$), and other gene ontology (GO) terms ($n = 10$) (olink.com; last accessed January 21, 2019). To note, a given protein can participate in several biological processes. For example, heat shock protein beta-1 (HSP 27; also called HSPB1) is involved in, among other biological processes, blood vessel morphogenesis, angiogenesis, and catabolic process.

The analysis was based on Proximity Extension Assay (PEA) where 92 different proteins were quantified simultaneously in 96 samples. Details of the method are described elsewhere (Alhamdow et al., 2019; Assarsson et al., 2014). Briefly, 2 protein-specific antibodies coupled with complementary oligonucleotides are used for each protein. Once the antibodies bind their target, the 2 oligonucleotides are in close proximity allowing hybridization and sequence extension by DNA polymerase. The resulting DNA sequence, which is protein-specific, is then amplified and quantified by real-time polymerase chain reaction. Protein concentrations were reported as log$_2$-scaled arbitrary units so-called Normalized Protein eXpression (NPX). Thus, concentrations of a given protein can be compared across different individuals (samples); however, concentrations of different proteins may not be compared with each other in a given individual.

Quality of both assay performance and analysis of individual samples was ensured by using different internal controls, ie, incubation controls 1 and 2 (for protein-antibody reaction), an extension control (for monitoring DNA extension), and a detection control (for monitoring the readout step). In addition, the following external controls were included; (1) a triplicate interplate control; an artificial sample including the same 92 proteins, (2) a triplicate of a negative control, and (3) a duplicate of a pooled serum sample (randomly selected from the study samples). The extension control and the interplate control were used for data normalization across both samples and plates. The signal of the negative control was used for calculations of limit of detection (LOD) of proteins. Proteins that had call rate (percentage of samples with valid proteomics data higher than LOD) < 90% were excluded from analysis. Proteomics measurements below LOD (of a specific protein) were replaced by LOD values. Inter- and intra-assay coefficients of variance (%CV) were computed using data from the pooled serum sample and proteins that had %CV > 20 were excluded from analysis. The readouts from the incubation and detection controls were used for pass/fail determination for each individual sample. Further details are provided on (olink.com; last accessed January 21, 2019).

Pathway and GO analyses. We selected the top 25 differentially expressed proteins (DEP) (based on the linear regression analysis; false discovery rate, FDR < 0.05) to explore potential diseases and biological functions and upstream regulators using Ingenuity Pathway Analysis software (Ingenuity IPA-42012434, Qiagen, Hilden, Germany). Log$_2$-fold change (comparing chimney sweeps with controls) was calculated and used for pathway analysis. Further, GO analysis was carried out for the same 25 DEP using the online tool WebGestalt (www.webgestalt.org; last accessed January 8, 2019) according to the following parameters; *Homo sapiens* as the organism, overrepresentation enrichment analysis as the method, geneontology as the functional database, uniprot_swissprot as the gene ID type, and genome_protein-coding as the reference set for enrichment analysis. The 3 GO domains molecular function, biological process, and cellular component were explored and the top 5 enriched GO in each domain were presented.

Statistical analyses. Median and interquartile range were calculated for continuous variables, eg, age (years), body mass index (BMI, kg/height in meters squared), PAH metabolites, and serum protein concentrations. Frequencies were calculated for categorical variables ie, sampling season (spring, summer, fall, and winter), smoking status (nonsmoker, former/party smoker), use of snus (yes, no), passive smoking (yes, no), exposure to smoke from hobbies (yes, no), intake of vegetables (more or less than 5 times a week), intake of fruits (more or less than 5 times a week), intake of fish (more or less than once a week), physical activity (high, low), residential area (big city, small city), education (university or higher, lower than university level), personal history of CVD (yes, no), family history of CVD (yes, no), diabetes (yes, no), prescribed medicines (yes, no), nonprescribed medicines (yes, no), myocardial infarction (yes, no), angina pectoris (yes, no), hypertension (yes, no), stroke (yes, no), thrombosis (yes, no), and kidney disease (yes, no). Differences between study groups (chimney sweeps vs controls) were evaluated by Mann-Whitney *U* test for continuous variables and by Fisher's exact test for categorical variables. The effect of potential confounders and covariates on dependent variables (serum

proteins) was examined by principal component regression analysis followed by Kolmogorov-Smirnov test (KS test) (Xu et al., 2016). Associations between study groups (chimney sweeps vs controls) and serum protein concentrations were analyzed by general linear models; model I (unadjusted) and model II (adjusted for age and BMI). Although the adjustment for age and BMI did not influence the effect estimates, we performed this adjustment in order to compare with other studies. Adjustment for multiple comparisons was applied using FDR method (FDR < 0.05) (Benjamini and Hochberg, 1995). Principal component analysis (PCA) was also performed, including the 25 DEP, to evaluate the proteomics signature across chimney sweeps and the controls. Further, the 25 DEP were regressed against PAH metabolites (log$_2$-transformed) in chimney sweeps using a general linear regression model adjusted for age, BMI, and day of sampling. This analysis was not adjusted for FDR because we had a direct hypothesis that the 25 DEP would be associated with the PAH metabolites. Effect estimates for all linear regression models were presented as unstandardized B value and 95% confidence interval (95% CI). Dose-response relationship was further evaluated for proteins that showed associations with PAH metabolites by linear regression models. In these models, the trends in serum protein concentrations across tertiles of PAH metabolites (low/medium/high; treated as continuous variables) were examined among chimney sweeps (adjusting for age, BMI, and day of sampling) and controls (adjusting for age and BMI). In our previous study (Alhamdow et al., 2017), we found higher serum concentrations of homocysteine, cholesterol, and high-density lipoprotein (HDL) in chimney sweeps compared with controls. Therefore, we examined the correlations between these serum markers and the 25 DEP among chimney sweeps and controls separately. We further examined whether the DEP mediated the higher levels of serum markers in chimney sweeps. For this purpose, we fit a linear regression model for the serum markers (homocysteine, cholesterol, or HDL) versus study groups (chimney sweeps/controls) adjusting for age and BMI. We then added 1 protein at a time to the model and calculated the change in the estimate B (for being a chimney sweep as a predictor vs serum markers as outcomes) as a percentage of mediation. An attenuation of > 10% in B was considered as significant mediation (Anderson et al., 2016).

The effect of various covariates on serum protein concentrations was evaluated by Spearman's correlations (continuous covariates) and Kruskal-Wallis test (categorical covariates) combined with FDR correction. For covariates that did not differ between chimney sweeps and controls (age, BMI, season of sampling, and intake of fish, fruits, and vegetables), the evaluation was restricted to the control group because the exposure to PAH among chimney sweeps might blur the correlations between the covariates and serum proteins. However, for covariates that significantly differed between chimney sweeps and controls (ie, sample storage time, use of snus, physical activity, and residential area), extra caution was enforced by performing the evaluation in chimney sweeps and controls separately.

Statistical significance was set at $p < .05$ (two-tailed). Statistical analyses were carried out using SPSS 23 (IBM SPSS Statistics, New York) and R (version 3.5.1).

RESULTS

Characteristics of the Study Participants
This study comprised 116 chimney sweeps and 125 unexposed controls for whom complete questionnaire information, blood and urine samples, and proteomics data were available (Supplementary Figures 1 and 2). Basic characteristics of the study participants are described in Table 1. The chimney sweeps did not significantly differ from controls in age and BMI ($p > .05$). As reported previously, chimney sweeps had higher serum concentrations of homocysteine, cholesterol, and HDL than controls ($p < .05$). Further, chimney sweeps had higher urinary concentrations of all 4 PAH metabolites than the controls ($p < .001$) (Alhamdow et al., 2017). It is worth mentioning that the PAH metabolites were intercorrelated among the chimney sweeps (average Spearman's correlation coefficient $r_S = .8$, $p < .001$). In addition, chimney sweeps who were sampled on Thursdays appeared to have higher concentrations of PAH metabolites in urine compared with those sampled on Wednesdays, but the differences were not significant ($p > .05$; adjusted for age and BMI). Chimney sweeps also had higher use of snus, higher physical activity, and lived in smaller cities compared with controls ($p < .05$).

Protein Inclusion Criteria and Influencing Factors
Information about the serum proteins analyzed and respective analytical parameters are summarized in Supplementary Tables 1 and 2. We excluded proteins when > 10% of the samples were below LOD (SLAMF7, BNP, PARP-1, and ITGB1BP2) as well as proteins with intra- and/or inter-assay CV > 20% (GH, FGF-21, and HAOX1). In total, we had 85 proteins for the final analysis. Principal component regression analysis combined with KS test showed that none of the potential confounders/covariates influenced the associations of study groups versus serum protein concentrations (ie, differential expression analysis comparing serum proteins between chimney sweeps and controls; KS test $p > .05$ for all model comparisons) (Supplementary Figure 3 and Table 3).

After investigating the effect of multiple covariates on serum protein concentrations among the controls, we found that age was correlated with 13 proteins and BMI with 22 proteins (FDR < 0.05) (Supplementary Table 4). Season of sampling, intake of vegetables, intake of fruits, and intake of fish did not influence serum protein concentrations (Supplementary Table 4). Similarly, none of the covariates sample storage time, use of snus, and residential area was influential on protein concentrations when evaluated among chimney sweeps and controls separately (Supplementary Table 5). Physical activity appeared to influence 4 proteins (PRSS8, CCL3, LEP, and SCF) in controls but not in chimney sweeps (Supplementary Table 5). To note, BMI showed strong positive correlations with leptin (LEP) among the controls ($r_S = .6$, $p = 7 \times 10^{-14}$) and chimney sweeps ($r_S = .7$, $p = 1 \times 10^{-16}$).

CVD-related Proteins in Serum Were Differentially Expressed Between Chimney Sweeps and Controls
Linear regression analysis adjusted for age, BMI, and FDR resulted in 25 DEP between chimney sweeps and controls (Table 2 and Supplementary Table 6). Examples of the 25 DEP are protein-glutamine gamma-glutamyltransferase 2 (TGM2), glyoxalase I (GLO1), NF-kappa-B essential modulator (NEMO), follistatin (FS), prointerleukin-16 (IL-16), and heat shock protein beta-1 (HSP 27). All the DEP showed higher levels in chimney sweeps compared with controls, except spondin-2 (SPON2). For instance, chimney sweeps had higher TGM2 concentrations (0.9 NPX) in serum compared with controls (Table 2). PCA plots including the 25 DEP showed partial separation between study groups: principal component 1 (PC1) and PC2 explained 39% and 12% of the dataset variability, respectively (Supplementary Figure 4).

Linear regression analysis (adjusted for age, BMI, and day of sampling), examining the associations between the 25 DEP and

Table 1. Characteristics of Chimney Sweeps and Controls

	Chimney Sweeps $n = 116$	Controls $n = 125$	p^a
Continuous variables	Median (IQR)[b]	Median (IQR)[b]	
Age (years)	41 (20)	43 (14)	0.879
Body mass index (BMI; kg/m^2)	26.7 (4.8)	26.9 (5.0)	0.371
1-hydroxypyrene (μg/g crea.)	0.38 (0.65)	0.05 (0.04)	< 0.001
2-hydroxyphenanthrene (μg/g crea.)	0.53 (0.67)	0.12 (0.09)	< 0.001
3-hydroxybenzo[a]pyrene (ng/g crea.)	3.04 (3.70)	0.93 (1.89)	< 0.001
3-hydroxybenzo[a]anthracene (ng/g crea.)	4.26 (4.68)	1.54 (1.05)	< 0.001
Homocysteine (μmol/l)	15 (6)	12 (5)	< 0.001
Cholesterol (mmol/l)	5.41 (1.8)	4.96 (1.94)	0.017
High-density lipoprotein (HDL; mmol/l)	1.32 (0.63)	1.16 (0.45)	0.001
Categorical variables[c]	n (%)[d]	n (%)[d]	
Sampling season (spring/summer/fall/winter)	33 (28) / 15 (13) / 58 (50) / 10 (9)	40 (32) / 26 (21) / 51 (41) / 8 (6)	0.263
Former or party smoker	47 (41)	42 (34)	0.164
Nonsmoker	69 (59)	83 (66)	
Use of snus	42 (36)	24 (19)	**0.002**
Passive smoking	21 (18)	16 (13)	0.161
Exposure to smoke from hobbies	8 (7)	5 (4)	0.397
Intake of vegetables (≥ 5 times/week)[e]	79 (68)	81 (65)	0.375
Intake of fruits (≥ 5 times/week)[f]	62 (53)	76 (61)	0.136
Intake of fish ($\geq$ once/week)[g]	56 (48)	59 (47)	0.485
Physical activity (high)[h]	65 (56)	53 (42)	**0.023**
Residential area (big city)[i]	36 (31)	55 (44)	**0.029**
Education (university or higher)	20 (17)	17 (14)	0.281
Personal history of cardiovascular disease[j]	33 (28)	25 (20)	0.174
Prescribed medicine[k]	34 (29)	26 (21)	0.138
Nonprescribed medicine[l]	20 (17)	28 (22)	0.337
Diabetes[m]	1 (1)	4 (3)	0.209
Myocardial infarction[m]	4 (3)	0 (0)	0.052
Angina pectoris[m]	5 (4)	6 (5)	0.546
Hypertension[m]	24 (21)	16 (13)	0.074
Stroke[m]	1 (1)	1 (1)	0.732
Thrombosis[m]	5 (4)	2 (2)	0.194
Kidney disease[m]	6 (5)	6 (5)	0.563
Family history of cardiovascular disease[n]	51 (44)	44 (35)	0.182

Data are median (interquartile range) for continuous variables or frequency (%) for categorical variables.

[a]P-value of Mann-Whitney *U* Test for continuous variables and Fisher's Exact Test for categorical variables.

[b]Three missing cases with analyses of 1-hydroxypyrene and 2-hydroxyphenanthrene, up to 19 missing cases with 3-hydroxybenzo[a]pyrene, and up to 7 missing cases with 3-hydroxybenzo[a]anthracene.

[c]Variables were categorized into (yes/no), unless otherwise stated.

[d]One missing case for some of the categorical variables. The percentage was calculated based on the whole number of participants (including missing cases).

[e]Intake of all kinds of vegetables, legumes, and root vegetables.

[f]Intake of all kinds of fruits and berries.

[g]Intake of all kinds of fish.

[h]Once a week or more of at least 30 min of regular physical activity.

[i]Big city compared with small city.

[j]Hypertension, myocardial infarction, angina pectoris, stroke, thrombosis in the arm or leg, arrhythmia or myocarditis.

[k]Predominantly included medication for cardiovascular disease, inflammation, lowering blood lipids, gastroesophageal reflux, and asthma.

[l]Vitamins, analgesics, and omega 3 fatty acids.

[m]Participants were asked "do you have and/or have you had the respective disease?".

[n]Participants were asked "Have any of your parents or siblings had myocardial infarction, stroke, or hypertension before 65 years of age?".

PAH metabolite concentrations in the chimney sweeps, revealed significant positive associations between FS and the metabolites 1-OH-PYR, 2-OH-PH, and 3-OH-BaP; IL-16 and 1-OH-PYR, 2-OH-PH, and 3-OH-BaA; and HSP 27 and all 4 PAH metabolites ($p < .05$) (Supplementary Table 7). For example, for every doubling in the urinary concentration of 3-OH-BaP, there was a 5% increase in the serum concentration of HSP 27 (Supplementary Table 7). Trend analysis, among chimney sweeps, showed dose-response relationships between FS and 1-OH-PYR, 2-OH-PH, and 3-OH-BaP ($p < .05$; adjusted for age, BMI, and day of sampling; Figure 1); IL-16 and 1-OH-PYR, 3-OH-BaP, and 3-OH-BaA; and HSP 27 and 1-OH-PYR, 3-OH-BaP and 3-OH-BaA. Trends for FS, IL-16, and HSP 27 among the controls were

less pronounced and significance was reached for FS with 2-OH-PH and 3-OH-BaA, and for HSP 27 with 3-OH-BaA ($p < .05$; adjusted for age and BMI; Figure 1).

Based on data derived from the comparative toxicogenomics database (ctdbase.org; last accessed January 15, 2019), several of the DEP showed, in different experimental models, altered protein and/or gene expression in association with PAH exposure (Supplementary Table 8).

Pathway and GO Enrichment Analyses

Pathway analysis of the 25 DEP showed that several proinflammatory cytokines, eg, TNF (tumor necrosis factor), IL-1β (interleukin-1 beta), and CSF2 (colony stimulating factor 2)

Table 2. Differences in Serum Protein Concentrations Between Chimney Sweeps and Controls, Evaluated by Linear Regression Analysis

Protein	Model 1 (Unadjusted)		Model 2 (Adjusted for Age and BMI)	
	p	B (95% CI)	p	B (95% CI)
TGM2	$1.5E^{-24}$	0.90 (0.75, 1.05)	$1.4E^{-24}$	0.91 (0.75, 1.06)
GLO1	$6.6E^{-19}$	0.59 (0.47, 0.71)	$6.0E^{-19}$	0.60 (0.47, 0.72)
FS	$8.4E^{-7}$	0.37 (0.22, 0.51)	$1.1E^{-7}$	0.38 (0.25, 0.52)
NEMO	$9.8E^{-7}$	0.40 (0.24, 0.56)	$2.7E^{-6}$	0.38 (0.23, 0.54)
IL-16	$1.4E^{-5}$	0.27 (0.15, 0.39)	$1.1E^{-5}$	0.28 (0.16, 0.40)
HSP 27	0.0001	0.23 (0.12, 0.35)	$6.8E^{-5}$	0.24 (0.12, 0.36)
CXCL1	0.0004	0.23 (0.11, 0.36)	0.0003	0.24 (0.11, 0.36)
PIgR	0.0004	0.06 (0.03, 0.10)	0.0005	0.06 (0.03, 0.10)
BOC	0.0007	0.13 (0.06, 0.21)	0.0002	0.13 (0.06, 0.20)
TIE2	0.0007	0.12 (0.05, 0.18)	0.0008	0.11 (0.05, 0.18)
STK4	0.0009	0.23 (0.10, 0.37)	0.002	0.21 (0.08, 0.35)
IL-27	0.001	0.11 (0.05, 0.18)	0.002	0.11 (0.04, 0.17)
LOX-1	0.001	0.23 (0.09, 0.37)	0.002	0.22 (0.08, 0.36)
PRSS27	0.003	0.15 (0.05, 0.24)	0.004	0.14 (0.05, 0.24)
SOD2	0.003	0.09 (0.03, 0.15)	0.002	0.09 (0.03, 0.15)
IL-17D	0.003	0.09 (0.03, 0.16)	0.004	0.09 (0.03, 0.15)
ANG-1	0.003	0.10 (0.03, 0.17)	0.004	0.10 (0.03, 0.17)
MARCO	0.003	0.09 (0.03, 0.15)	0.003	0.09 (0.03, 0.15)
AGRP	0.005	0.17 (0.05, 0.28)	0.004	0.16 (0.05, 0.27)
TNFRSF11A	0.009	0.14 (0.04, 0.24)	0.004	0.15 (0.05, 0.25)
TM	0.011	0.11 (0.03, 0.20)	0.011	0.11 (0.03, 0.20)
TF	0.013	0.11 (0.02, 0.19)	0.020	0.10 (0.02, 0.19)
SORT1	0.013	0.08 (0.02, 0.14)	0.008	0.09 (0.02, 0.15)
SPON2	0.014	−0.05 (−0.09, −0.01)	0.033	−0.04 (−0.08, −0.004)
MERTK	0.014	0.12 (0.02, 0.21)	0.015	0.11 (0.02, 0.21)

Unstandardized beta (B) and 95% confidence interval (95% CI) are reported for effect estimation. All 25 proteins presented were statistically significantly different after adjustment for false discovery rate; FDR < 0.05. BMI, body mass index.

Table 3. Top Diseases and Functions Predicted by Ingenuity Pathway Analysis (IPA) Examining the 25 Differentially Expressed Proteins

Diseases or Functions Annotation	p	Molecules
Inflammatory response	1.3E-13	ANG-1, CXCL1, HSP 27, NEMO, IL-16, IL-17D, IL-27, MARCO, LOX-1, PIgR
Cell movement	1.7E-12	ANG-1, CXCL1, TF, FS, HSP 27, IL-16, NEMO, MARCO, MERTK, LOX-1
Migration of cells	3.6E-12	ANG-1, CXCL1, TF, HSP 27, NEMO, IL-16, MARCO, MERTK, LOX-1, PIgR
Leukocyte migration	2.0E-11	ANG-1, CXCL1, TF, HSP 27, NEMO, IL-16, MARCO, LOX-1, PIGR, SOD2
Quantity of cells	1.8E-10	ANG-1, BOC, CXCL1, TF, FS, HSP 27, NEMO, IL-27, MERTK, PIgR
Attachment of cells	4.0E-10	ANG-1, TF, MERTK, LOX-1, SOD2, TIE2, TGM2
Necrosis	8.2E-10	ANG-1, CXCL1, TF, FS, GLO1, HSP 27, NEMO, IL-17D, IL-27, MERTK
Function of leukocytes	1.2E-09	TF, HSP 27, IL-16, IL-27, MARCO, MERTK, PIgR, STK4, TGM2, TM
Cell movement of leukocytes	1.4E-09	ANG-1, CXCL1, TF, HSP 27, NEMO, IL-16, MARCO, PIgR, SPON2, STK4
Adhesion of immune cells	1.5E-09	ANG-1, CXCL1, TF, MARCO, LOX-1, SPON2, STK4, TGM2, TM

were predicted to be activated as upstream regulators (Figure 2). Similarly, NFkB complex (nuclear factor NF-kappa-B; involved in regulating the immune system, stress response, and inflammation) and LPS (lipopolysaccharide; an endotoxin involved in inflammation and regulation of immune system) were also predicted to be activated (Figure 2). Top diseases and functions associated with the 25 DEP (including FS, IL-16, and HSP 27) were inflammatory response, cell movement and attachment, and various functions related to leukocytes and immune cells (Table 3). GO analysis suggested signal transducer activity and cytokine activity as top molecular functions. As well, leukocyte migration, positive regulation of response to external stimulus, and inflammatory response were the top biological processes (Supplementary Table 9). The analysis also showed that extracellular space and plasma membrane region were the top cellular components.

Correlations Between the 25 DEP and Homocysteine, Cholesterol, and HDL

Eight DEP were correlated with either homocysteine or cholesterol among chimney sweeps, whereas 2 DEP correlated with HDL among the controls ($p < .01$; Table 4). Less significant correlations were observed among chimney sweeps for IL-16 with cholesterol and HSP 27 with HDL ($p < .05$). In addition, kidney injury molecule-1 (KIM-1) was positively correlated with cholesterol in both chimney sweeps ($r_S = .24$; $p = .01$) and controls ($r_S = .29$; $p = .001$), however, this protein was not differentially expressed. Mediation regression analysis showed that 6 DEP, including FS and IL-16, were mediators (> 10% attenuation in B) for the association between study groups and homocysteine, and 1 protein (TGM2) for the associations between study groups and cholesterol or HDL (Supplementary Table 10). The percentage of mediation was 15% on average.
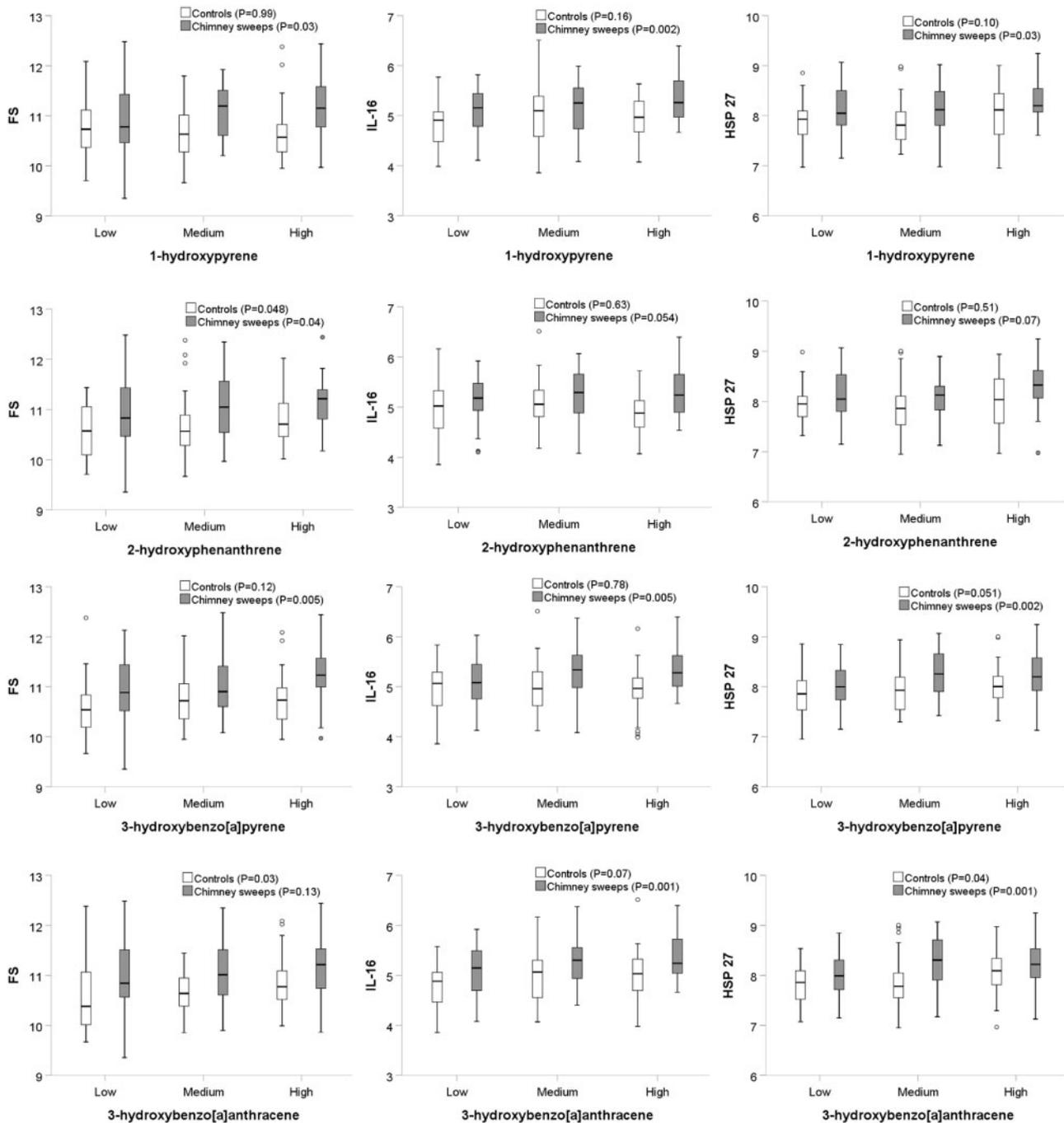
Figure 1. Differences in concentrations of the serum proteins follistatin (FS), prointerleukin-16 (IL-16), and heat shock protein beta-1 (HSP 27) across tertiles (low/medium/high) of the polycyclic aromatic hydrocarbons (PAH) metabolite concentrations in urine among chimney sweeps and controls. Serum protein concentrations were measured as normalized protein expression (NPX) values. P-value of trend test was reported. Trend test was performed using linear regression model where the variables of PAH metabolites tertiles were treated as continuous variables. The models for chimney sweeps were adjusted for age, body mass index (BMI), and day of sampling, whereas the models for the controls were adjusted for age and BMI.

DISCUSSION

In this cross-sectional study, we found a number of putative CVD-related serum proteins differentially expressed between PAH-exposed workers and unexposed controls. Of these proteins, FS, IL-16, and HSP 27 were, in a dose-response manner, associated with urinary concentrations of monohydroxylated metabolites of PAH. Pathway analysis identified inflammation and immune response as key functions for the DEP. Our study

highlights that PAH exposure might dysregulate proteins involved in inflammation and immune response; biological functions relevant for CVD development.

Little research has so far been accomplished in exploring underlying mechanisms of PAH-induced CVD. There are a few, but inconsistent, associations reported between PAH exposure and inflammatory markers (eg, C-reactive protein (CRP) and white blood cell count), and these are mainly based on environmentally exposed individuals, including the U.S. general population
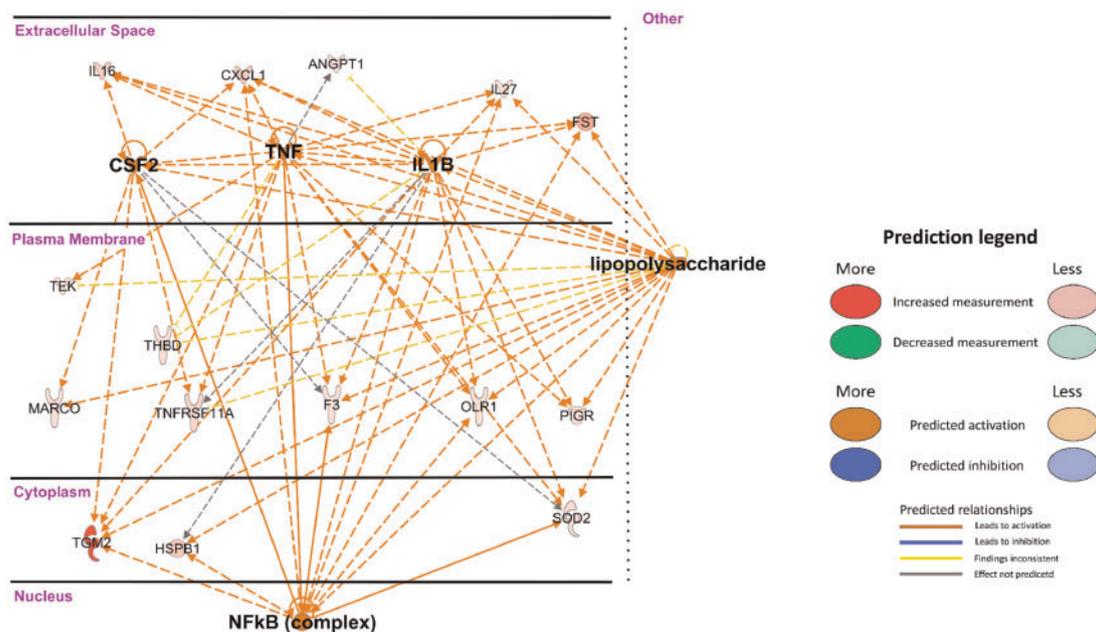
Figure 2. Top upstream regulators (shown in bold) that were predicted to be activated (activation z-score > 2.0) and connected to a mechanistic network. Data were derived from the top 25 differentially expressed proteins (false discovery rate; FDR < 0.05). Dashed lines indicate indirect interaction, whereas the solid ones indicate direct interaction. Some proteins displayed in this figure have different names eg, HSPB1 is HSP 27, F3 (TF), TEK (TIE2), THBD (TM), OLR1 (LOX-1), FST (FS), and ANGPT1 (ANG-1).

Table 4. Spearman's Rank Correlation Coefficients for Correlations Between the 25 Differentially Expressed Proteins and Homocysteine, Cholesterol, and High-density Lipoprotein (HDL), Stratified by Study Group

	Chimney Sweeps			Controls		
Protein	Homocysteine	Cholesterol	HDL	Homocysteine	Cholesterol	HDL
TGM2	−0.02	0.00	−0.04	−0.06	0.01	0.10
GLO1	0.11	−0.03	−0.08	−0.05	0.03	0.00
FS	0.07	0.05	0.13	0.08	0.183*	**−0.250****
NEMO	0.03	**−0.241****	0.09	0.02	−0.07	0.03
IL-16	0.08	−0.196*	−0.04	0.01	−0.01	−0.14
HSP 27	0.07	−0.08	−0.225*	−0.01	0.01	0.04
CXCL1	0.07	−0.02	−0.09	−0.06	−0.04	0.05
PIgR	**0.390*****	0.03	−0.05	0.14	0.13	−0.03
BOC	−0.11	−0.213*	0.09	−0.15	−0.08	−0.12
TIE2	0.02	−0.18	0.03	0.04	0.06	−0.04
STK4	0.06	**−0.284****	0.06	0.08	−0.05	0.13
IL-27	−0.01	**−0.327*****	−0.05	0.06	−0.06	0.01
LOX-1	−0.05	**−0.281****	0.12	0.03	−0.05	0.00
PRSS27	0.13	−0.16	−0.05	0.229*	0.14	−0.04
SOD2	0.02	0.01	0.02	0.02	0.05	−0.07
IL-17D	**0.341*****	−0.03	0.04	0.02	−0.03	−0.06
ANG-1	0.15	0.06	−0.11	0.04	0.04	0.03
MARCO	−0.01	−0.14	−0.05	0.12	0.13	−0.215*
AGRP	0.14	−0.16	−0.05	0.08	−0.212*	−0.01
TNFRSF11A	0.17	−0.10	−0.194*	−0.03	−0.02	−0.18
TM	0.04	**−0.253****	−0.04	0.05	0.06	−0.177*
TF	0.16	−0.16	0.11	−0.02	−0.02	−0.08
SORT1	0.190*	−0.07	−0.11	0.04	−0.05	0.06
SPON2	0.189*	−0.04	−0.11	0.03	0.07	−0.17
MERTK	**0.252****	−0.07	−0.04	0.03	0.09	**−0.231****

*$p < .05$ (two-tailed).
**$p < .01$ (two-tailed).
***$p < .001$ (two-tailed).

(Alshaarawy et al., 2013; Clark et al., 2012; Everett et al., 2010). These inconsistencies could, at least in part, be due to different analytical methodologies used for PAH metabolites analysis during different recruitment cycles or unmeasured confounders such as infections (Alshaarawy et al., 2013). A recent study including 3640 participants from China found higher odds of having dyslipidemia among individuals in the highest tertiles of the monohydroxylated metabolites of naphthalene, fluorene, and phenanthrene as compared to those in the lowest tertiles (Ma et al., 2019).

In our previous study (the parent study), we found higher concentrations of serum homocysteine, cholesterol, and HDL in chimney sweeps relative to the controls (Alhamdow et al., 2017). In the present study (nonsmoking participants from the parent study), 8 DEP were correlated with homocysteine, cholesterol, and HDL in chimney sweeps, whereas 2 DEP showed correlations among the controls. This indicates that the DEP may play a role in the interaction between PAH exposure and homocysteine, cholesterol, and HDL. Notably, several of the DEP were inversely correlated with cholesterol, which may seem inconsistent with the higher serum concentrations of cholesterol in chimney sweeps compared to controls (Alhamdow et al., 2017). We speculate that these inverse associations between DEP and cholesterol may reflect a response mechanism in chimney sweeps to reduce the higher concentrations of cholesterol by activating several pathways in which, the DEP are involved. Mediation regression analysis showed weak mediation by a few DEP, including FS, IL-16, and TGM2, for the associations between study groups and homocysteine, cholesterol, and HDL. Still, the pathway analysis did not reveal that the 1-carbon metabolism or lipid metabolism were related to the DEP, so the question is whether the associations between the DEP and serum homocysteine and cholesterol are down-stream effects of the inflammatory response. Elevated level of homocysteine has been suggested as a risk factor for CVD (McCully, 2015). That is because homocysteine may induce cardiovascular endothelial damage and cause vasoconstriction by reducing the levels of nitric oxide (vasodilator). Another suggested mechanism is through oxidative stress resulted from homocysteine metabolism imbalance (Marti-Carvajal et al., 2017). It should be noted that KIM-1 (kidney injury molecule-1) was positively associated with cholesterol, LDL, and triglycerides in both chimney sweeps and controls ($p < .05$), which is in line with a previous study (Figarska et al., 2018). However, this protein was not differentially expressed.

Reactive oxygen species (ROS) play an important role in pathways involved in vascular endothelial function (Touyz and Briones, 2011). Oxidative stress, an excessive production of ROS accompanied by impairment in antioxidative system, can induce endothelial dysfunction and inflammation leading to atherosclerosis and hypertension; primary risk factors for CVD (Cervantes Gracia et al., 2017; Elahi et al., 2009; Kondo et al., 2009; Touyz and Briones, 2011; Willerson and Ridker, 2004). PAH can induce oxidative stress, and thus promote endothelial dysfunction (Cervantes Gracia et al., 2017; Eom et al., 2013; Kuang et al., 2013; Moorthy et al., 2015). Interestingly, several of the DEP have been linked to oxidative stress. For instance, upregulation of TGM2 (transglutaminase 2) was associated with oxidative stress (Caccamo et al., 2012; Tatsukawa et al., 2016). Expression of GLO1, NEMO, FS, and HSP 27 were shown to be protective against oxidative stress (Jo-Watanabe et al., 2014; Lin et al., 2016; Luedde et al., 2007; Wyttenbach et al., 2002). For instance, a study in mice lung tissues and cultured human lung cells showed that exposure to oxidative stress-causing toxicants such as silica nanoparticles induced expression of FS protein, which in turn,

suppressed ROS production demonstrating an antioxidant activity (Lin et al., 2016; Nel et al., 2006). In this context, the increased expression of GLO1, NEMO, FS, and HSP 27 in chimney sweeps may represent a compensatory antioxidant response against the oxidative stress caused by PAH exposure.

Pathway analysis of the 25 DEP predicted dysregulation in biological processes related to immune function and inflammation such as leukocyte migration, positive regulation of response to external stimulus, and inflammatory response. Because the chimney sweeps in our study were clearly exposed to PAH (up to 7 times higher monohydroxylated PAH metabolites compared with unexposed controls) (Alhamdow et al., 2017), we speculate that the dysregulation in immune function and inflammatory response could be related to PAH exposure.

IL-16 is a proinflammatory cytokine that can promote expression of other proinflammatory proteins and function as a chemoattractant for a wide spectrum of immune cells (Mathy et al., 2000). Several studies using bronchoalveolar lavage have found higher concentrations of extracellular IL-16 in tobacco smokers as compared with nonsmokers (Andersson et al., 2004, 2011; Laan et al., 1999). In this context, higher serum levels of IL-16 in chimney sweeps could indicate an activation of the immune system; possibly, in response to PAH exposure as IL-16 was associated with PAH metabolites.

FS belongs to a family of extracellular proteins containing the follistatin domain (Phillips and de Kretser, 1998) and functions as an antagonist for activin, which can act as a proinflammatory factor (Sidis et al., 2006). In vivo studies showed an increase in circulating FS following inflammatory stimuli suggesting an anti-inflammatory role for FS (Hedger et al., 2011; Jones et al., 2004). Another study demonstrated an advantageous role of FS in cardiomyocyte injury (Chen et al., 2014). HSP 27 is an antiapoptotic protein that protects cells from different internal and external stressors and maintains cellular integrity by regulating protein folding (Rane et al., 2003; Samali et al., 2001; Wang et al., 2014). There is a growing body of evidence suggesting an important role of HSP 27 in lowering systemic inflammation and thereby reducing atherosclerotic events (Rayner et al., 2008). We found higher levels of FS and HSP 27 in chimney sweeps compared with controls, as well as higher levels of these 2 proteins with higher concentrations of PAH metabolites. This might indicate that the increase in FS and HSP 27 reflects a high demand for reducing inflammation in the chimney sweeps due to their exposure to PAH.

A main advantage of this study is the individual data for PAH exposure as well as the fact that we could take important confounders into account by analyzing nonsmoking workers that were matched for age, gender, and BMI. All serum samples were processed homogeneously and randomized prior to analysis of the 85 proteins, which reduces the chance of technical errors. Further, we had information about the general dietary habits concerning intake of vegetables, fish, and fruits, for which, no effect was observed on serum protein levels. This is in line with earlier studies that have shown limited impact of diet on levels of serum proteins when using the same technology as here (Dencker et al., 2017a,b). On the other hand, some limitations should be acknowledged. The company response rate was higher for chimney sweeps (87%) than controls (58%), which might have introduced a selection bias. However, it is unlikely that the controls' health status was the reason for the low response rate. The work nature of the controls, eg, food storage and warehouses, requires continuous workflow, and hence some managers decided not to participate as their employees

could not leave their work tasks for taking part in a research study as ours. Most of the DEP did not show associations with PAH metabolites and there is a possibility that the observed differences in protein expression between chimney sweeps and controls were due to unmeasured exposures, such as particles and degreasing chemicals. However, the lack of associations between the DEP and PAH metabolites could also be due to differences in their half-lives. Monohydroxylated PAH metabolites can be used as proxy for short-term exposure to PAH because their half-lives range between 4 and 36 h (Buckley and Lioy, 1992; Jongeneelen et al., 1990). The half-lives of PAH-DNA adducts range between days to months mirroring long-term exposure, however, it was not possible to measure PAH-DNA adducts in our study (Castano-Vinyals et al., 2004). Serum proteins, on the other hand, have much variable half-lives ranging from several minutes to a few weeks (Bachmair et al., 1986; Doherty et al., 2009). We do not know the half-lives for FS, IL-16, and HSP 27 in serum, but it is tempting to speculate that the associations found in our study between these proteins and PAH metabolites reflect similarities in the half-lives. In contrast, the absence of associations between the other DEP and PAH metabolites would suggest broader differences in half-lives between the DEP and PAH metabolites. Recruitments of the chimney sweeps and controls were not performed at the same time (more controls recruited in the beginning than sweeps), which might have influenced the levels of serum proteins due to differences in storage time. The impact of sample storage time (around 30 years at −80°C) on plasma protein levels was investigated by Enroth et al. using the same proteomic analysis technique (PEA, Olink) and including 108 proteins. After adjustment for multiple comparisons, the authors found negligible effect of storage time on plasma protein levels as only 1 protein, ie, mucin 16 (out of 108) was significantly influenced (Enroth et al., 2016). Another study has evaluated a storage time of up to 45 years (−70°C) in relation to serum/plasma proteins and found a minor effect of storage time; 5 proteins were influenced out of 92 (Björkesten et al., 2017). We evaluated the effect of sample storage time (up to 16 months at −80°C) in chimney sweeps and controls and found that none of the proteins was influenced. Taken together, storage time is an unlikely confounder in our study. We did not observe any effect of age or BMI on serum protein levels in the differential expression analysis because the controls matched chimney sweeps in age and BMI. However, age and BMI affected 13 and 22 proteins, respectively (FDR < 0.05) when evaluated among the controls, which is in agreement with previous studies (Enroth et al., 2014; Larsson et al., 2015a,b). Other covariates such as use of snus, physical activity, residential area, season of sampling, and intake of vegetables, fruits, and fish did not have significant influence on serum protein levels.

CONCLUSION

Our study showed a number of putative CVD-related proteins differentially expressed between PAH-exposed workers and unexposed controls. These changes in protein expression seem to be, at least in part, linked to PAH exposure. These results warrant protective measures such as use of protective gloves, masks, and long-sleeved workwear to reduce PAH exposure among workers. In addition, longitudinal investigations of the proteomic profile in chimney sweeps as well as in other occupational groups exposed to PAH are encouraged.

SUPPLEMENTARY DATA

DECLARATION OF CONFLICTING INTERESTS

ACKNOWLEDGMENTS

FUNDING

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