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ORIGINAL RESEARCH

The Association Between Inflammatory and Oxidative Stress Biomarkers and Plasma Metabolites in a Longitudinal Study of Healthy Male Welders

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Introduction: Human metabolism and inflammation are closely related modulators of homeostasis and immunity. Metabolic profiling is a useful tool to understand the association between metabolism and inflammation at a systemic level.

Objective: To investigate the longitudinal associations between the concentration of plasma metabolites and biomarkers related to inflammation and oxidative stress.

Methods: We conducted a repeated cross-sectional analysis consisting of 8 short-term panels that included 88 healthy adult male welders in Massachusetts, USA. In each panel, we collected 1–6 repeated measurements of blood and urine. We used a human vascular injury panel assay and custom cytokine/chemokine assay to quantify inflammatory biomarker plasma levels, liquid chromatography-mass spectrometry to quantify the concentrations of 665 plasma metabolites, and a competitive enzyme-linked immunoassay to quantify urinary 8-OHdG and 8-isoprostane levels. We used linear mixed effects models to estimate the longitudinal association between each inflammatory and oxidative stress biomarker and each metabolite.

Results: At a 5% FDR threshold, we detected ≥1metabolite association for 8 unique inflammatory and oxidative stress biomarkers: urinary 8-isoprostane, plasma C-reactive protein (CRP), serum amyloid A (SAA), intercellular adhesion molecule 1, circulating vascular cell adhesion molecule-1, interleukin 8 (IL-8), interleukin 10 (IL-10) and vascular endothelial growth factor. Specifically, 3 metabolites in the androgenic steroids pathway were negatively associated with SAA; 3 dihydrosphingomyelins metabolites were positively associated with 1 or more of CRP, SAA, IL-8 and IL-10; 4 metabolites in acyl choline metabolism pathways were negatively associated with IL-8; 7 lysophospholipid metabolites were negatively associated with 1 or more of CRP, SAA and IL-8; 4 sphingomyelins were positively associated with CRP and/or SAA; and 10 metabolites in the xanthine pathway were positively associated with urinary 8-isoprostane.

Conclusion: We found that metabolites in phospholipid groups had strong associations with multiple inflammatory biomarkers, especially CRP, SAA and IL-8. The mechanism of these associations warrants further investigation.

Keywords: metabolism, inflammation, phospholipids, metals, particulates, metabolomics, occupational health

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Introduction

Inflammation is a central component of the innate immune response. Acute inflammation is a protective mechanism triggered by irritants, infection, and injury, and initiated by the release of pro-inflammatory cytokines.[1] Following inflammation,

immune cells release signaling biochemicals, such as the major acute phase proteins C-reactive protein (CRP) and Serum Amyloid A (SAA), which mediate a series of biochemical events in response to cell and tissue damage. Blood flow increases, and leukocytes are recruited to eliminate foreign substances, clear necrotic cells, and initiate tissue repair.[2]

Inflammation plays an important role in many common diseases. Conditions such as obesity cause persistent immune activation, causing chronic inflammation in which these biochemical cascades reoccur over a prolonged period of time.[3] Chronic inflammation also plays a role in the etiology of many diseases, including cardiovascular diseases (CVD), diabetes mellitus (DM), and arthritis.[3–6]

The biochemical pathways of inflammation and metabolism are interconnected in the development of CVD and DM. Numerous studies have investigated the association between inflammatory biomarkers, especially CRP, and the risk of CVD and metabolic syndromes.[5,7–12] Lipid metabolism is especially important in maintaining the health of the cardiovascular system. A diet intervention study found that inflammation was an effect modifier for reduced-fat /low-cholesterol diet intervention to control CVD.[13,14] After the diet intervention, participants with increased CRP level showed less total and low-density lipoproteins (LDL) cholesterol reduction and a greater increase in triglycerides, compared to participants with lower CRP level. This study suggested a mechanism by which inflammation might increase CVD risk by affecting lipid metabolism. Particularly, inflammation and lipid signaling interact with each other and play important roles in the etiology of insulin resistance, for example, saturated fatty acids (SFA) promote chronic tissue inflammation which can cause insulin resistance.[15]

Understanding the relationship between inflammation and metabolism is important to improve knowledge of the risk of CVD and metabolic syndrome. Each of the above epidemiological studies examined a few particular pathways. However, more metabolic pathways may also interact with inflammation simultaneously. Given this complexity, metabolomics techniques could be useful tools to study this dynamic. To our knowledge, no previous studies have examined the longitudinal associations between inflammation and metabolomics.

In this study, we analyzed the longitudinal association between a panel of inflammatory and oxidative stress biomarkers and the plasma metabolome. We aimed to expand our understanding of the relationship between inflammation and metabolism from a broader view at the molecular level.

Method

Study Population

The participants are members of the International Brotherhood of Boilermakers, Local 29, whose job duties are welding and cutting mild steel, and repairing commercial boilers. The union provides welding technique training programs in summers and winters, in which we invited instructors and apprentices to participate this study. The detailed sample collection timeline is described in Table 1.

During the winters and summers of 2010–2012, we conducted 8 short panel studies in the welding school. In 7 of the 8 study periods, we collected 3–4 repeated measurements of both plasma and urine from each participant. Some participants joined multiple times in different panels.

All study procedures were approved by the Institutional Review Board of Harvard T.H. Chan School of Public Health.

Measurements of Biomarkers

The measurement method of vascular injury biomarkers and pro-inflammatory cytokines was published previously.[16] Briefly, EDTA-treated plasma was obtained from participants by venipuncture of the median cubital vein of the antecubital fossa using BD vacutainer, and centrifuging at 1200 RPM for 12 minutes. The aliquots were stored in cryogenic tubes at −80°C before the assay. Plasma concentrations of CRP, SAA, soluble intercellular adhesion molecule 1 (sICAM-1), circulating vascular cell adhesion molecule-1 (sVCAM-1) were determined by multiplex electrochemiluminescence using the MULTI-SPOT® 96-well Human Vascular Injury Panel II assay (Meso Scale Discovery, Rockville MD). Plasma concentrations of interleukin-1β (IL-1β), IL-2, IL-6, IL-8, IL-10, tumor necrosis factor alpha (TNFα), and vascular endothelial growth factor (VEGF) were determined using the 7-plex MULTI-ARRAY® 96-well custom cytokine/chemokine assay. The average within-plate coefficients of variation (the ratio of the standard deviation to the mean) ranged from 7.6% to 10.9% for different assays. The average between-plate CVs ranged from 12.3% to 17.4% for different assays.

Table 1 The Timeline and the Number of Blood Samples Collected in Each Study Panel

Sampling Period[a]	Panel	N	N	Non-Welding Day		Welding Day		Non-Welding Day	
		Participants	Samples	The Previous Morning	The Previous Afternoon	Morning (Pre-Shift)	Afternoon (Post-Shift)	The Next Morning	The Morning a Few Days Later
Jan. 2010 - Feb. 2010	1	31	94		33 samples	31 samples	30 samples		
Jun. 2010 - Jul. 2010	2	25	80		24 samples	24 samples	23 samples	9 samples	
Jun. 2010 - Jul. 2010	3	18	56	4 samples	19 samples	17 samples	16 samples		
Sept. 2010- Oct. 2010	4	23	37			22 samples	15 samples		
Jan. 2011 - Feb. 2011	5	23	92	15 samples	12 samples	22 samples	22 samples	4 samples	17 samples
Jun. 2011	6	26	84	17 samples	15 samples	26 samples	26 samples		
Feb. 2012	7	9	9			9 samples			
Jun. 2012	8	23	77	21 samples	19 samples	19 samples	18 samples		

Notes: [a]A total of 88 welders participated in the study. A total of 529 observations have both inflammatory biomarkers and metabolite levels available. The inflammatory biomarkers were analyzed from blood and urine samples, but this form only shows numbers of blood samples. Overall, each participant provided 1–18 repeated measurements, with a median of 4 and an average of 6. Within each study panel, each participant provided 1–6 samples, with a median of 3 and mean of 3 samples.

U-8-OHdG and u-8-isoprostane levels were both determined using competitive enzyme-linked immunoassay method performed by Genox Corporation (Baltimore, MD), as published previously.[17]

Profiling of Metabolites

Metabolite profiling procedures were described previously.[18] EDTA-plasma aliquots were taken from the same samples used for inflammatory biomarker measurements. Morning blood samples were taken after fasting, prior to a provided breakfast, and afternoon blood samples were drawn after the participants performed 5 to 6 hours of welding without lunch.

Proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. Samples were stored in liquid nitrogen before analysis. At the time of sample processing, each sample was aliquoted into 4 fractions for different analytical techniques and each method differentiates and quantifies some metabolites.

Sample extracts were dried then reconstituted in proper solvents for each of the four methods. We prepared each reconstitution solvent based on standards at fixed concentrations to ensure injection and chromatographic consistency.

All methods utilized Waters ACQUITY ultra-performance liquid chromatography (UPLC) method and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source, as well as Orbitrap mass analyzer operated at 35,000 mass resolution. One aliquot was analyzed under acidic positive ion conditions using electrospray ionization (ESI) method, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol that contain 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion mode ESI, but chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted using the same method above and was operated at an overall higher organic content. The third aliquot was analyzed under basic negative ion optimized conditions using ESI, eluted from a separate dedicated C18 column by methanol and water with 6.5mM Ammonium

Bicarbonate at pH 8. The last aliquot was analyzed via negative ESI following elution from a HILIC column (Waters UPLC BEH Amide 2.1×150 mm, 1.7 μm) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate, pH 10.8. We used MS and data-dependent MSn scans with dynamic exclusion for quantification. The scan range for the above methods covered 70–1000 m/z.

A total of 665 biochemical metabolites were analyzed from each plasma sample, including amino acid, carbohydrates, cofactor/vitamins, lipids, nucleotides, ally characterized molecules, peptides, and xenobiotic metabolites. Further information of these metabolites is given in supplementary materials (Supplementary material – a list of metabolites).

Before the analysis of metabolites, internal standards were added to each sample for quality control purposes. Instrument variability was 4%, determined by calculating the median relative standard deviation (RSD) for the internal standards that were added to each sample before injection into the mass spectrometers. Additionally, a reference sample pooled from a few plasma samples served as technical replicates in the analysis (RSD = 8%). As the procedure spanned multiple days, the instrument's inter-day variation was normalized per batch per compound. Missing values due to low concentration were imputed by the lowest detectable concentration of each compound. Concentrations were the median-scaled for each sample. Measurements for each compound were log-transformed and mean-centered across samples statistical analysis.

Covariates

The covariates of the analysis include self-reported age, race, current smoking status (current smoker or not); body mass index (BMI) measured by research staff; the time of sample collection (AM/PM), and whether it is on welding day or not, and study panel (nominal). If participants attended more than one panel, the measurements were taken in each panel. There was no missing value on these covariates.

Statistical Analysis

We used linear mixed-effect models (LMMs) to evaluate the association between each biomarker and each metabolite to account for unbalanced repeated measurements. Because many biomarker concentration measurements had skewed and heavily tailed distributions, we applied rank-normal transformations to each biomarker prior to analysis to improve robustness.

For each biomarker and each metabolite, we fit an LMM to model rank-normal transformed biomarker measurement (the response variable) as a function of metabolite concentration and control covariates (listed in the previous paragraph). We compared 2 model specifications for random effects: (1) including a participant-specific random intercept, and (2) including both a participant-specific and participant-and-study-panel-specific random intercept. Here study panel refers to a period of 1–2 days wherein each participant provided 1–6 samples (median 3). For example, one participant joined two study panels in winter 2010 and summer 2012 and provided 3 samples and 4 samples, respectively. Under the first model, there is a single participant-specific intercept across 7 samples, whereas the second model further includes participant-specific intercepts for the first 3 (winter 2010) and second 4 (summer 2012) samples. All models were conducted on a complete case basis.

Formally, we assumed the model

$$y_{ijk} = m_{ijk}\beta + x_{ijk}^{\top}\alpha + b_i + b_{ij} + \in_{ijk}$$

where y_{ijk} denotes the rank-normal transformed biomarker measurement for participant i at study panel j and sample number k, m_{ijk} denotes metabolite measurement, and $x_{ijk}^{\top}$ denotes the vector covariates (age, BMI, race, smoking status, weld day/non-weld day, AM/PM, and study panel). The random intercepts are independently distributed $b_i \sim N(0, \tau^2)$ and $b_{ij} \sim N(0, \gamma^2)$, and the residuals $\in_{ijk} \tilde{N}(0, \sigma^2)$, where $N(,)$ denotes the normal distribution. In model 1, we estimate τ^2 and fix $\gamma^2 = 0$, while in model 2, we estimate both τ^2 and γ^2.

The linear mixed-effect model was conducted using the lme4 R package.[19]

Next, we used the likelihood ratio test to compare the full model (estimating both τ^2 and γ^2) with the nested model with only a per-participant random effect (fixing. In nested model comparisons, the full model fit significantly better than the single-intercept model ($p<0.05$) for 9/13 biomarkers; we therefore used the full model in subsequent analysis. The model comparison was performed using the varTestnlme R package.[20]

Then, we used likelihood ratio tests to evaluate whether we should include panel, AM/PM, and welding day/non-welding day in the covariate list. We finalized the full model and applied it for each biomarker and each metabolite with the restricted maximum

likelihood method, and used the false discovery rate (FDR) method for multiple comparisons.[21]

The metabolites with at least one association at the significance level of <0.1 were selected for visualization by feature-expression heat map using the corrplot and pheatmap R package.[22,23] In this plot, the metabolites and biomarkers were reordered using the hierarchical clustering method.[24]

Furthermore, we selected participants who provided more than 10 biological samples. Additional analysis of longitudinal change of metabolites/biomarkers was performed using mixed models. The model specification and results are provided in Supplementary Table 2 and 3. Scatter plots of inflammatory biomarker and metabolite profiles over days of follow-up are shown in Supplementary Figure 1–31.

Results

The participants' characteristics are shown in Table 2. The majority of the participants were Caucasians. The average BMI was 29.5%, and 78% of participants had BMI >25. Only 6% of the participants participants reported ever diagnosed with asthma and diabetes, respectively. According to published data, the average breathing-zone $PM_{2.5}$ level over a welding shift was 421 μg/m^3, with a standard deviation (SD) of 360 μg/m^3, the average breathing-zone $PM_{2.5}$ level in the classroom area was 120 μg/m^3, with an SD of 60 μg/m^3. The methods of breathing-zone $PM_{2.5}$ measurements were described elsewhere.[18] We had a total of 529 plasma samples from 88 participants. Each participant provided 1–18 repeated measurements, with a median of 4 and an average of 6 samples. During each study panel, each participant provided 1–4 samples, with a median of 3 and an average of 3 samples.

The profile of inflammatory and oxidative stress biomarkers are shown in Supplementary Table 1. U-8-isoprostane and U-8-OHdG were measured only once on panel 1, thus had greater number of missing observations. Other missing values can be considered missing at random. The biomarkers that we analyzed were correlated. The longitudinal association between each two inflammatory biomarkers is shown in Figure 1. U-8-isoprostane and U-8-OHdG are associated. The level of CRA, SAA, sICAM-1, sICAM-1 are highly associated with each other, and they are all associated with IL-6.

Among 665 metabolites, seventeen metabolites were associated with U-8-isoprostane; twenty-one metabolites were associated with CRP; twenty-two metabolites were associated with SAA; two metabolites were associated with

Table 2 Participants' Characteristics

Characteristics	Mean or N	SD or %
Age (years)	42.9	13.2
BMI (kg/m^2)	29.5	6.5
Smoking status		
Non-current-smoker	54	62%
Current smoker	33	38%
Gender		
Male	88	100%
Race		
Caucasian	76	87%
African American	6	7%
Asian	3	4%
Other	2	2%
Diabetes (ever)		
No	80	94%
Yes	6	6%
Asthma (ever)		
No	79	94%
Yes	5	6%

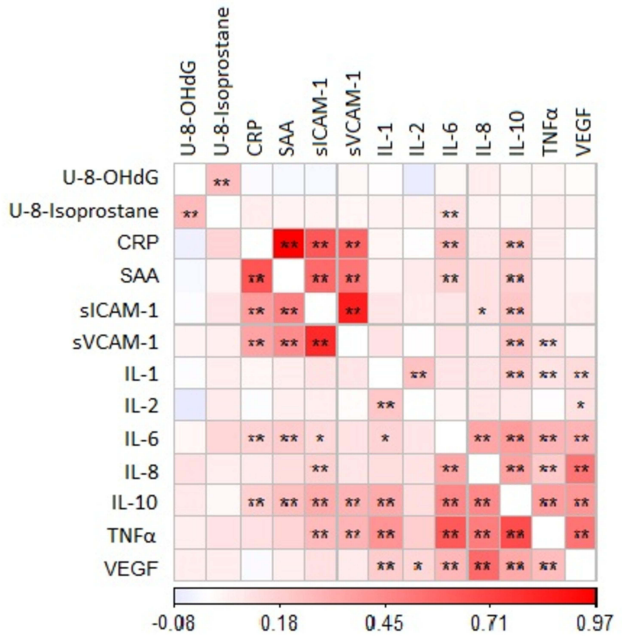

Figure 1 The association between inflammatory biomarkers. This is an association matrix between each two inflammatory biomarkers. The association was analyzed using the mixed-effect model taking repeated measurements into consideration. All biomarkers were rank-normal transformed. The color and color intensity shows the direction (red: positive, blue: negative) and effect size, respectively. The star marks represent the false discovery rate (FDR) adjusted statistical significance (*FDR P-value < 0.1; **FDR p-value < 0.05).

sICAM-1; one metabolite was associated with sVCAM-1; eighteen metabolites were associated with IL-8; eleven metabolites were associated with IL-10 and seven metabolites were associated with VEGF (Tables 3 and 4). The below associations we report are all at the significance level of <0.05 after FDR adjustment, unless specified.

Seven lipid metabolites were positively associated with U-8-isoprostane ($0.22 < \beta < 0.32$), including N-palmitoyl-sphinganine (d18:0/16:0) (dihydroceramide metabolism), two dicarboxylate metabolites, two monohydroxy fatty acid metabolites, 1-oleoyl-GPE (18:1) (lysophospholipid), and 1-stearoyl-2-oleoyl-GPE (18:0/18:1) (phosphatidylethanolamine, or PE). Ten xanthine metabolites were positively associated with U-8-isoprostane ($0.12 < \beta < 0.19$).

CRP and SAA had similar association profiles, as shown in Figure 2. The color code represents the direction and the effect size, while the number of stars shows the significance level of <0.05 (**) and <0.1 (*), respectively. CRP and SAA were negatively associated with 7 ($-0.57 < \beta < -0.25$) and 5 ($-0.71 < \beta < -0.34$) lysophospholipid metabolites, respectively. CRP and SAA were positively associated with 2 ($\beta = 0.22$ and 0.32) and 3 ($0.31 < \beta < 0.44$) dihydrosphingomyelins metabolites, respectively, as well as 4 ($0.37 < \beta < 0.64$) and 2 ($\beta = 0.45$ and 0.60) sphingomyelins, respectively. CRP was negatively associated with 3 phosphatidylcholine (PC) ($-0.23 < \beta < -0.43$) and 1 phosphatidylinositol (PI) ($\beta = -0.23$) metabolites. SAA was negatively associated with 3 ($-0.29 < \beta < -0.23$) androgenic steroid metabolites.

Plasma sICAM-1 was positively associated with alpha-hydroxyisocaproate and methylmalonate ($\beta = 0.28$ and 0.26), and plasma sVCAM-1 was positively associated with pyroglutamine ($\beta = 0.33$).

IL-8 and VEGF has several associated metabolites in common, including arachidonoylcholine ($\beta = -0.12$ vs $\beta = -0.02$), palmitoylcholine ($\beta = -0.14$ vs $\beta < 0$, $p < 0.1$), AMP ($\beta = 0.28$ vs $\beta = 0.37$), 3-phosphoglycerate ($\beta = 0.15$ vs $\beta = 0.12$), and thioproline ($\beta = 0.52$ vs $\beta > 0$, $p < 0.1$). IL-8 was also associated with dihydrosphingomyelins ($\beta = 0.43$ and 0.64), lysophospholipids ($\beta = -0.11$ and -0.18), and fatty acids ($-0.22 < \beta < -0.09$).

Plasma VEGF was also positively associated with metabolites in purine metabolism. IL-10 was found associated with amino acids, peptide, and several lipid metabolites.

Furthermore, from the additional analysis on participants who provided more than 10 biological samples, we found 18 metabolites that changed over time ($p<0.0001$, Supplementary Table 2, Supplementary Figure 1–18). Five inflammatory biomarkers of these participants changed over time ($p<0.05$, Supplementary Table 3, Supplementary Figure 19–31).

Discussion

In this study, we found plasma metabolites that are longitudinally associated with urinary oxidative stress biomarker U-8-isoprostane and plasma inflammatory biomarkers including CRP, SAA, sICAM-1, sVCAM-1, IL-8, IL-10 and VEGF. Phospholipids, including dihydrosphingomyelins, sphingomyelins, lysophospholipids, PC and PI had stronger associations with inflammatory and oxidative biomarkers. In each of above pathways, we found multiple metabolites that were associated with one or more inflammatory and/or oxidative stress biomarkers.

We conducted this study in active workers, who tend to be healthier and more resilient than community populations. No other exclusions were applied in the workers who were willing to participate in the study.

Few studies have evaluated the inflammatory and metabolic profile of active workers. In the same cohort study, our group previously examined the metabolic change and the association between inflammatory biomarker and telomere length.[16,18] An animal study analyzed the spatial distribution and abundance changes of lipid species in Sprague Dawley rat liver maintained on a high-fat diet combined with welding fume inhalation. This study showed that the combined effects of welding fume inhalation and a high-fat diet significantly altered the hepatic lipidome and researchers found higher abundance of ceramide-1-phosphate in the regular diet welding fume-exposed group which has been shown to regulate the eicosanoid pathway involved in pro-inflammatory response.[25] Thus, our study is the first study focused on the longitudinal association between metabolic profile and inflammatory biomarkers in a working population. The knowledge generated from this study can be generalized to broader active working population. Previous studies elucidated several mechanisms by which lipid species act positively and negatively in regulating inflammatory responses.[15] For example, eicosanoids are signaling molecules derived by the oxidation of arachidonic acid or other polyunsaturated fatty acids (PUFAs) similar to arachidonic acid, and they are bioactive lipid mediators that promote inflammation via innate immunity.[26] SFA are pro-inflammatory lipid compounds that mediate the activation

Table 3 The Metabolite-Inflammatory Biomarker Associations at a Significance Level of p-value <0.05 (False Discover Rate Adjusted)*

Biomarker	Metabolite	Super Pathway	Sub Pathway	Effect Size	FDR p-value
U-8-isoprostane, pg/mg creatinine	N-palmitoyl-sphinganine (d18:0/16:0)	Lipid	Dihydroceramides	0.25	0.04
	Azelate (C9-DC)	Lipid	Fatty Acid, Dicarboxylate	0.22	<0.01
	Docosadioate (C22-DC)	Lipid	Fatty Acid, Dicarboxylate	0.22	0.02
	2-hydroxydecanoate	Lipid	Fatty Acid, Monohydroxy	0.26	<0.01
	2-hydroxyoctanoate	Lipid	Fatty Acid, Monohydroxy	0.32	0.01
	1-oleoyl-GPE (18:1)	Lipid	Lysophospholipid	0.32	0.01
	1-stearoyl-2-oleoyl-GPE (18:0/18:1)	Lipid	Phosphatidylethanolamine (PE)	0.23	0.03
	1,7-dimethylurate	Xenobiotics	Xanthine Metabolism	0.16	<0.01
	1-methylurate	Xenobiotics	Xanthine Metabolism	0.12	0.02
	1-methylxanthine	Xenobiotics	Xanthine Metabolism	0.12	0.01
	3-methylxanthine	Xenobiotics	Xanthine Metabolism	0.16	<0.01
	5-acetylamino-6-amino-3-methyluracil	Xenobiotics	Xanthine Metabolism	0.19	<0.01
	5-acetylamino-6-formylamino-3-methyluracil	Xenobiotics	Xanthine Metabolism	0.12	0.01
	7-methylxanthine	Xenobiotics	Xanthine Metabolism	0.15	<0.01
	Paraxanthine	Xenobiotics	Xanthine Metabolism	0.14	0.02
	Theobromine	Xenobiotics	Xanthine Metabolism	0.14	<0.01
	Theophylline	Xenobiotics	Xanthine Metabolism	0.18	<0.01
Plasma CRP, ng/mL	Retinol (Vitamin A)	Cofactors and Vitamins	Vitamin A Metabolism	−0.59	<0.01
	Androstenediol (3beta,17beta) monosulfate (2)	Lipid	Androgenic Steroids	−0.21	<0.01
	Sphingomyelin (d18:0/18:0, d19:0/17:0)*	Lipid	Dihydrosphingomyelins	0.32	<0.01
	Sphingomyelin (d18:0/20:0, d16:0/22:0)*	Lipid	Dihydrosphingomyelins	0.22	0.01
	Methylmalonate (MMA)	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	0.19	<0.01
	1-linolenoyl-GPC (18:3)*	Lipid	Lysophospholipid	−0.25	<0.01
	1-oleoyl-GPC (18:1)	Lipid	Lysophospholipid	−0.39	<0.01
	1-palmitoleoyl-GPC* (16:1)*	Lipid	Lysophospholipid	−0.34	<0.01
	1-palmitoyl-GPC (16:0)	Lipid	Lysophospholipid	−0.57	<0.01
	1-palmitoyl-GPE (16:0)	Lipid	Lysophospholipid	−0.31	<0.01
	1-stearoyl-GPC (18:0)	Lipid	Lysophospholipid	−0.43	<0.01
	1-stearoyl-GPE (18:0)	Lipid	Lysophospholipid	−0.33	<0.01
	1-linoleoyl-2-arachidonoyl-GPC (18:2/20:4n6)*	Lipid	Phosphatidylcholine (PC)	−0.43	0.01
	1-palmitoyl-2-palmitoleoyl-GPC (16:0/16:1)*	Lipid	Phosphatidylcholine (PC)	−0.23	0.01
	1-stearoyl-2-oleoyl-GPC (18:0/18:1)	Lipid	Phosphatidylcholine (PC)	−0.41	<0.01
	1-palmitoyl-2-arachidonoyl-GPI (16:0/20:4)*	Lipid	Phosphatidylinositol (PI)	−0.23	<0.01
	1-(1-enyl-palmitoyl)-2-palmitoyl-GPC (P-16:0/16:0)*	Lipid	Plasmalogen	−0.57	0.01
	Sphingomyelin (d18:1/18:1, d18:2/18:0)	Lipid	Sphingomyelins	0.64	0.01
	Sphingomyelin (d18:1/20:1, d18:2/20:0)*	Lipid	Sphingomyelins	0.54	<0.01
	Sphingomyelin (d18:1/25:0, d19:0/24:1, d20:1/23:0, d19:1/24:0)*	Lipid	Sphingomyelins	0.37	<0.01
	Stearoyl sphingomyelin (d18:1/18:0)	Lipid	Sphingomyelins	0.58	0.00

(*Continued*)

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Table 3 (Continued).

Biomarker	Metabolite	Super Pathway	Sub Pathway	Effect Size	FDR p-value
Plasma SAA, ng/mL	Hydantoin-5-propionate	Amino Acid	Histidine Metabolism	0.11	0.02
	Arabitol/xylitol	Carbohydrate	Pentose Metabolism	−0.24	0.03
	Retinol (Vitamin A)	Cofactors and Vitamins	Vitamin A Metabolism	−0.66	<0.01
	5alpha-androstan-3beta,17beta-diol disulfate	Lipid	Androgenic Steroids	−0.23	0.04
	Androstenediol (3beta,17beta) disulfate (1)	Lipid	Androgenic Steroids	−0.28	<0.01
	Androstenediol (3beta,17beta) monosulfate (2)	Lipid	Androgenic Steroids	−0.29	<0.01
	Myristoyl dihydrosphingomyelin (d18:0/14:0)*	Lipid	Dihydrosphingomyelins	0.44	0.01
	Sphingomyelin (d18:0/18:0, d19:0/17:0)*	Lipid	Dihydrosphingomyelins	0.44	<0.01
	Sphingomyelin (d18:0/20:0, d16:0/22:0)*	Lipid	Dihydrosphingomyelins	0.31	<0.01
	Arachidonoylcarnitine (C20:4)	Lipid	Fatty Acid Metabolism (Acyl Carnitine, Polyunsaturated)	−0.21	0.01
	Methylmalonate (MMA)	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	0.33	<0.01
	1-oleoyl-GPC (18:1)	Lipid	Lysophospholipid	−0.39	0.04
	1-palmitoleoyl-GPC* (16:1)*	Lipid	Lysophospholipid	−0.39	<0.01
	1-palmitoyl-GPC (16:0)	Lipid	Lysophospholipid	−0.71	<0.01
	1-stearoyl-GPC (18:0)	Lipid	Lysophospholipid	−0.50	0.01
	1-stearoyl-GPE (18:0)	Lipid	Lysophospholipid	−0.34	0.05
	1-palmitoyl-2-arachidonoyl-GPI (16:0/20:4)*	Lipid	Phosphatidylinositol (PI)	−0.34	<0.01
	1-(1-enyl-palmitoyl)-2-palmitoleoyl-GPC (P-16:0/16:1)*	Lipid	Plasmalogen	−0.58	<0.01
	Sphingomyelin (d18:1/25:0, d19:0/24:1, d20:1/23:0, d19:1/24:0)*	Lipid	Sphingomyelins	0.45	<0.01
	Stearoyl sphingomyelin (d18:1/18:0)	Lipid	Sphingomyelins	0.60	<0.01
	Gabapentin	Xenobiotics	Drug - Neurological	0.41	<0.01
	3-hydroxycotinine glucuronide	Xenobiotics	Tobacco Metabolite	−0.20	<0.01
Plasma sICAM-1, ng/mL	Alpha-hydroxyisocaproate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.28	0.01
	Methylmalonate (MMA)	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	0.26	<0.01
Plasma sVCAM-1, ng/mL	Pyroglutamine*	Amino Acid	Glutamate Metabolism	0.33	<0.01

Plasma IL-8, pg/mL	Sarcosine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.18	<0.01
	3-phosphoglycerate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	0.15	<0.01
	Quinolinate	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	−0.14	0.05
	Myristoyl dihydrosphingomyelin (d18:0/14:0)*	Lipid	Dihydrosphingomyelins	0.43	0.01
	Palmitoyl dihydrosphingomyelin (d18:0/16:0)*	Lipid	Dihydrosphingomyelins	0.64	0.03
	Arachidonoylcarnitine (C20:4)	Lipid	Fatty Acid Metabolism (Acyl Carnitine, Polyunsaturated)	−0.22	0.01
	Arachidonoylcholine	Lipid	Fatty Acid Metabolism (Acyl Choline)	−0.12	<0.01
	Linoleoylcholine*	Lipid	Fatty Acid Metabolism (Acyl Choline)	−0.12	<0.01
	Oleoylcholine	Lipid	Fatty Acid Metabolism (Acyl Choline)	−0.09	<0.01
	Palmitoylcholine	Lipid	Fatty Acid Metabolism (Acyl Choline)	−0.14	<0.01
	1-palmitoyl-GPI* (16:0)	Lipid	Lysophospholipid	−0.11	0.03
	1-stearoyl-GPI (18:0)	Lipid	Lysophospholipid	−0.18	<0.01
	AMP	Nucleotide	Purine Metabolism, Adenine containing	0.28	<0.01
	Dihydroorotate	Nucleotide	Pyrimidine Metabolism, Orotate containing	−0.27	<0.01
	N-methylpipecolate	Xenobiotics	Bacterial/Fungal	0.15	0.02
	EDTA	Xenobiotics	Chemical	0.76	<0.01
	Iminodiacetate (IDA)	Xenobiotics	Chemical	0.15	0.02
	Thioproline	Xenobiotics	Chemical	0.52	<0.01
Plasma IL-10, pg/mL	Hydantoin-5-propionate	Amino Acid	Histidine Metabolism	0.10	0.03
	2-hydroxy-3-methylvalerate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.22	0.01
	Isobutyrylcarnitine (C4)	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.18	<0.01
	Methionine sulfoxide	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.22	0.03
	2-oxoarginine*	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.15	0.02
	Urea	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.39	<0.01
	Androstenediol (3beta,17beta) monosulfate (2)	Lipid	Androgenic Steroids	−0.20	0.04
	Sphingomyelin (d18:0/18:0, d19:0/17:0)*	Lipid	Dihydrosphingomyelins	0.31	0.01
	Methylmalonate (MMA)	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	0.24	<0.01
	Propionylcarnitine (C3)	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	0.21	0.01
	Gamma-glutamylvaline	Peptide	Gamma-glutamyl Amino Acid	0.26	0.01
Plasma VEGF, pg/mL	3-phosphoglycerate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	0.12	0.03
	Bilirubin (E,Z or Z,E)*	Cofactors and Vitamins	Hemoglobin and Porphyrin Metabolism	−0.22	0.02
	Arachidonoylcholine	Lipid	Fatty Acid Metabolism (Acyl Choline)	−0.07	0.02
	Sphingosine	Lipid	Sphingosines	0.15	0.02
	Hypoxanthine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.13	<0.01
	Inosine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.19	0.05
	AMP	Nucleotide	Purine Metabolism, Adenine containing	0.37	<0.01

Notes: *The effect size was derived from linear mixed-effect model adjusted for age, race, current smoking status, body mass effect, time of sample collection and whether it is on a welding day or not, as well as adjusted for random intercept effect of each participant and each study panel. The p-value was corrected using false discover rate (FDR) method.

Abbreviations: IL-8, interleukin 8; IL-10, interleukin 10; VEGF, vascular endothelial growth factor; CRP, C-reactive protein; SAA, serum amyloid A; ICAM-1, intercellular adhesion molecule 1; sVCAM-1, circulating vascular cell adhesion molecule-1; 8-isoprostane, also known as 15F2t-IsoP or 8-iso-PGF2a.

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Table 4 The Number of Metabolite-Inflammatory Biomarker Associations by Super Pathway and Sub Pathway

Super Pathway	Sub Pathway	Biomarker (Number of Associated Metabolites)
Amino Acid	Glutamate Metabolism	sVCAM-1 (1)
	GlycineSerine and Threonine Metabolism	IL-8 (1)
	Histidine Metabolism	SAA (1), IL-10 (1)
	LeucineIsoleucine and Valine Metabolism	sICAM-1 (1), IL-10 (2)
	MethionineCysteineSAM and Taurine Metabolism	IL-10 (1)
	Urea cycle; Arginine and Proline Metabolism	IL-10 (2)
Carbohydrate	GlycolysisGluconeogenesisand Pyruvate Metabolism	IL-8 (1), VEGF (1)
	Pentose Metabolism	SAA (1)
Cofactors and Vitamins	Hemoglobin and Porphyrin Metabolism	VEGF (1)
	Nicotinate and Nicotinamide Metabolism	IL-8 (1)
	Vitamin A Metabolism	CRP (1), SAA (1)
Lipid	Androgenic Steroids	CRP (1), SAA (3), IL-10 (1)
	Dihydroceramides	U-8-isoprostane (1)
	Dihydrosphingomyelins	CRP (2), SAA (3), IL-8 (2), IL-10 (1)
	Fatty Acid Metabolism (Acyl CarnitinePolyunsaturated)	SAA (1), IL-8 (1)
	Fatty Acid Metabolism (Acyl Choline)	IL-8 (4), VEGF (1)
	Fatty Acid Metabolism (also BCAA Metabolism)	CRP (1), SAA (1), sICAM-1 (1), IL-10 (2)
	Fatty AcidDicarboxylate	U-8-isoprostane (2)
	Fatty AcidMonohydroxy	U-8-isoprostane (2)
	Lysophospholipid	U-8-isoprostane (1), CRP (7), SAA (5), IL-8 (2)
	Phosphatidylcholine (PC)	CRP (3)
	Phosphatidylethanolamine (PE)	U-8-isoprostane (1)
	Phosphatidylinositol (PI)	CRP (1), SAA (1)
	Plasmalogen	CRP (1), SAA (1)
	Sphingomyelins	CRP (4), SAA (2)
	Sphingosines	VEGF (1)
Nucleotide	Purine Metabolism(Hypo)Xanthine/Inosine containing	VEGF (2)
	Purine MetabolismAdenine containing	IL-8 (1), VEGF (1)
	Pyrimidine MetabolismOrotate containing	IL-8 (1)
Peptide	Gamma-glutamyl Amino Acid	IL-10 (1)
Xenobiotics	Bacterial/Fungal	IL-8 (1)
	Chemical	IL-8 (3)
	Drug – Neurological	SAA (1)
	Tobacco Metabolite	SAA (1)
	Xanthine Metabolism	U-8-isoprostane (10)

of toll-like receptors and stimulation of IKKβ/NFκB and JNK1/AP1, and then promote inflammation and the release of cytokines.[15] FSA palmitate was found to induce the activation of the NLRP3-ASC inflammasome, promoting caspase-1, IL-1β, and IL-18 production, which also mediates insulin resistance.[27] LDLs promote cholesterol accumulation and inflammatory response in the artery wall, which drives the process of atherosclerosis.[28]

High-density lipoproteins (HDL) and certain unsaturated fatty-acid can be anti-inflammatory, thus beneficial to arterial health.[15,29] For example, omega-3 fatty acids, such as DHA and EPA, have an anti-inflammatory effect by inhibiting the production of arachidonic acid and other pro-inflammatory eicosanoids, although the molecular mechanism remains unclear. Omega-3 fatty acid was also found to stimulate GPR120 and the production of resolvins and protectins, which is anti-inflammatory.[30]

On the other hand, studies have shown how inflammation alters lipid metabolism at the whole-body level and in different tissues. For example, inflammation triggered

infection by bacteria or viruses may change lipid and lipoprotein metabolism. TNF-α was found to suppress lipoprotein lipase synthesis, which could contribute to hypertriglyceridemia.[31] IL-6 selectively alters skeletal muscle lipid metabolism.[32] Lipopolysaccharides (LPS), TNF-α, and IL-1β are involved in lipolysis in primary adipocytes and adipocyte cell lines.[33]

There are few overlaps between the known mechanisms above and the detailed metabolite-biomarker association in our findings. For example, we have not found significant associations between any examined metabolites and IL-1β, which played important role in above mechanisms. It is possible that our repeated cross-sectional analysis only reflects systemic association, while temporal relationship between the metabolites and inflammatory markers cannot be determined. In this longitudinal setting, we identified multiple metabolites that were associated with U-8-isoprostane, CRP, SAA, sICAM-1, sVCAM-1, IL-8, IL-10, and VEGF. We further examined the literature for insight into possible mechanisms of these associations.

The isoprostane family is prostaglandin-like compounds formed in vivo from the free radical-catalyzed peroxidation of essential fatty acids (primarily arachidonic acid).[34] An imbalance between the production and elimination of reactive oxygen species, including free oxygen radicals, are closely associated with inflammation. Creatinine-adjusted urinary 8-isoprostane is the most commonly used biomarker for oxidative stress in human studies.[35] U-8-isoprostane was found positively associated with xanthine metabolites that are related to caffeine digestion. One study showed that the superoxide-producing enzyme xanthine oxidase may elevate the release of isoprostane in human isolated pulmonary artery smooth muscle cells.[36]

In addition, U-8-isoprostane was associated with several lipids; however, the mechanism is not well understood. Azelate (C9-DC) was found negatively associated with circulating IL-6 in older adults in a cross sectional study,[37] but not oxidative stress biomarkers.

CRP is an acute phase pro-inflammatory protein made by the liver following the stimulation of IL-6. CRP levels in the blood increase when there is a condition causing inflammation, such as infection, rheumatic, and other inflammatory diseases. It is an important biomarker of acute conditions, as well as of the severity of disease in chronic conditions. Healthy people's CRP level should be under 3000 ng/dL, and the average CRP level of our measurements was ~4000 ng/dL (median ~2000 ng/dl), indicating that our study results can be generalized to health working men or man with acute or chronic inflammation conditions.

CRP and SAA were both associated with several sphingomyelins (positive) and lysophospholipids (negative) metabolites, while the mechanism was not yet clear. One previous study postulates that lysophospholipids constitute the ligand for CRP in the ischemic myocardium that CRP can bind to phosphatidylcholine vesicles containing lysophosphatidylcholine.[38] Another study suggested that the sphingomyelin-ceramide pathway participates in the induction of CRP and SAA by IL-6 and IL-1β in cell cultural experiments, most likely by transducing the effects of IL-1β.[39]

VEGF is a signaling protein that regulates endothelial cell function and promotes angiogenesis. We found VEGF and IL-8 were both positively associated with the plasma level of 5′ adenosine monophosphate (AMP). Few studies discussed the relationship between AMP and VEGF but many studies demonstrated how AMP-activated protein kinase (AMPK) and VEGF stimulates each other in the process of angiogenesis.[40–43] For example, cell culture studies showed that AMP-activated protein kinase stimulates VEGF expression and angiogenesis in skeletal muscle.[43] AMP-activated protein kinase mediates VEFG-stimulated endothelial NO production.[44] In addition, VEGF stimulates AMPK by a CaMKK-dependent mechanism and stimulation of AMPK activity is required for proliferation in response to either VEFG -A or -B and migration in response to VEGF-A.[45]

IL-8 is a chemokine produced by macrophages and other cell types. Known as a neutrophil chemotactic factor, it has two major functions. First, it induces chemotaxis in target cells, primarily neutrophils but also other granulocytes, causing them to migrate toward the site of infection, and stimulates phagocytosis once they have arrived. Second, it promotes angiogenesis,[46] which echoed our finding that it is associated with AMP along with VEGF. IL-8 was found to up-regulates VEGF mRNA and protein levels in endothelial cells by acting on its cognate receptor, CXCR2, and it results in the autocrine activation of VEGF reactor 2.[47]

Among the cytokines, IL-6 is well known for inducing the liver synthesis of CRP and SAA in the acute phase of inflammation.[48] However, we have not found any metabolite significantly associated with IL-6 in this study.

In Figure 1, we observed that IL-1, IL-6, IL-8, IL-10, TNF-α, and VEGF are associated with each other. The

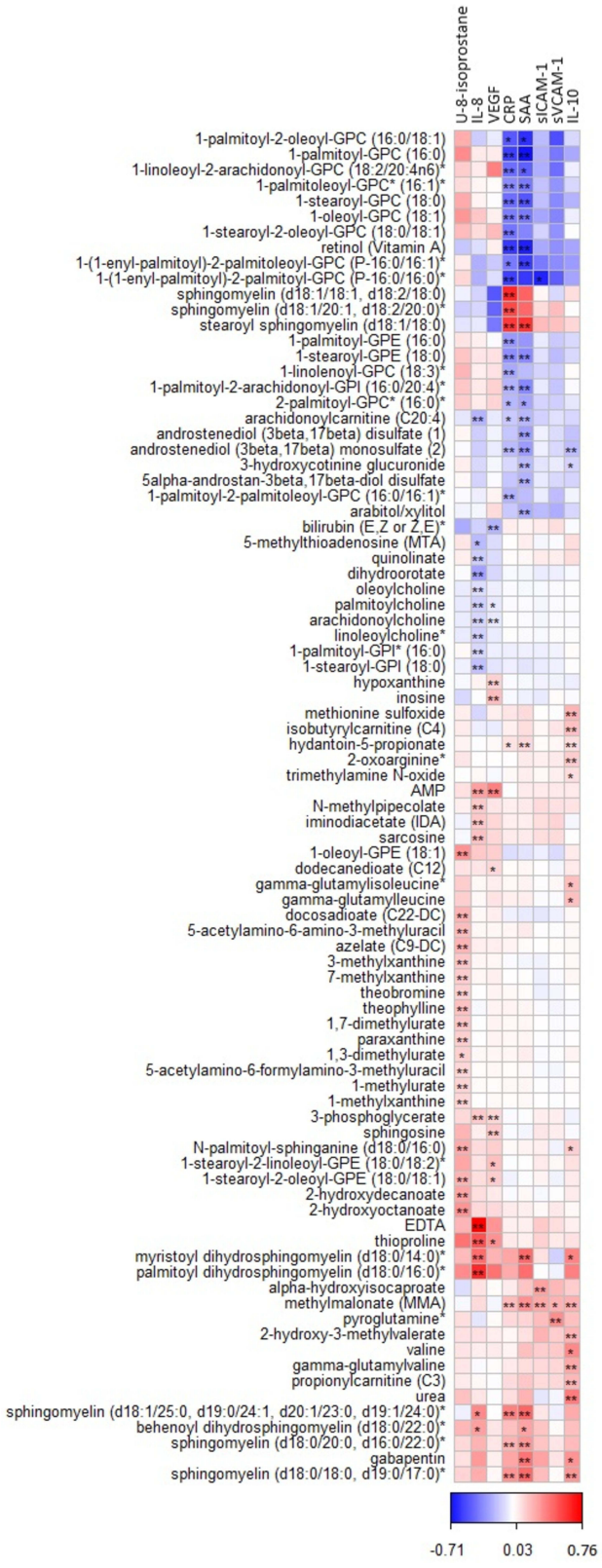

Figure 2 The hierarchical clustered feature-expression heat map of metabolite-inflammatory biomarker associations. Figure 2 is a feature-expression heat map depicting the longitudinal association between plasma metabolite concentration and plasma/urine inflammatory biomarker concentration. Statistical analysis was performed using the linear mixed-effect regression model. In the visualization, the color and color intensity shows the direction (red: positive, blue: negative) and effect size, respectively. The star marks represent the false discovery rate (FDR) adjusted statistical significance (*FDR P-value < 0.1; **FDR p-value < 0.05).

functional relationship may explain the correlation between inflammatory and oxidative stress biomarkers, and the similarity in association profiles, especially between CRP and SAA, as well as between VEGF and IL-8.

This study has several strengths. First, the repeated measurements from participants allowed us to assess the longitudinal association between inflammatory biomarkers and the metabolome, while previous epidemiological studies used cross-sectional designs. Second, large numbers of biomarkers and metabolites of each time point were measured at the same time, which ensures the same stage of metabolism and homeostasis. Third, we used linear mixed effects models to account for the unbalanced repeated measures and within-subject correlations.

There are also limitations of the study. First, many metabolites are functionally interrelated and statistically correlated, making it difficult to disentangle the effects. Second, immune responses may be delayed, and length of delay may vary across different inflammatory biomarkers. Such delays are not captured in the linear mixed model we used to quantify longitudinal associations, and therefore could bias the estimates. Third, because relatively few time points are available for each individual in the study, temporal precedence of changes in metabolites and inflammatory markers is not clear. Fourth, our analysis does not estimate causal effects, as longitudinal associations could be driven by causal effects in either direction or by omitted third variables that affect both metabolomics and inflammations.

In conclusion, in this longitudinal analysis in healthy male workers, we found novel associations between inflammatory and oxidative stress and metabolites including lysophospholipid, dihydrosphingomyelins, sphingomyelins, PC, PI, xanthine metabolism and fatty acid metabolism pathways. The mechanism underlying these associations should be investigated further to understand the etiology of inflammation-related diseases.

Abbreviations

IL-1β, interleukin 1 beta; IL-2, interleukin 2; IL-6, interleukin 6; IL-8, interleukin 8; IL-10, interleukin 10; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor; CRP, C-reactive protein; SAA, serum amyloid A; ICAM-1, intercellular adhesion molecule 1; sVCAM-1, circulating vascular cell adhesion molecule-1; 8-isoprostane, also known as 15F2t-IsoP or 8-iso-PGF2a; 8-OHdG, 8-Oxo-2′-deoxyguanosine; RP/UPLC-MS, reverse phase ultrahigh performance liquid chromatography-tandem mass spectroscopy.

Code Availability

The code of this study is not available to the public.

Data Sharing Statement

Data of this study are not available in a public open access repository.

Ethics Approval

All study procedures were approved by the institutional review board of the Harvard T H Chan School of Public Health. This study was conducted in accordance with the Declaration of Helsinki.

Consent to Participate

Signed informed consent was obtained from all study participants in each study period.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

There was no conflict of interested reported by any of the authors.

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