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Associations of circulating levels of phthalate metabolites with cytokines and acute phase reactants in a Spanish human cohort

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ABSTRACT

The associations between human phthalate exposure and the onset of chronic diseases with an immunological component (e.g., metabolic syndrome, cancer) remain unclear, partly due to the uncertainties in the underlying mechanisms. This study investigates cross-sectional associations of the concentrations of 10 phthalate metabolites with 19 cytokines and acute phase proteins in 213 serum samples of Spanish adults. The associations were explored by Spearman's correlation, multivariable linear regression, and weighted quantile sum regression analyses. In the multivariable analyses, levels of plasminogen activator inhibitor (PAI)-1 were positively associated with mono-n-butyl phthalate (fold-change per one IQR increase in phthalate levels, 95% Confidence Interval: 1.65, 1.45–1.88) and mono-iso-butyl phthalate (3.07, 2.39–3.95), mono-ethyl phthalate (2.05, 1.62–2.61), as well as categorized mono-iso-decyl and mono-benzyl phthalates. The same phthalates also were significantly associated with leptin, interleukin (IL)-18 and monocyte chemoattractant protein-1. Moreover, the proinflammatory markers IL-1β, IL-17, IL-8, IL-6, IL-12, tumor necrosis factor, and lipopolysaccharide-binding protein showed positive and negative associations with, respectively, mono-(2-ethyl-hexyl) and mono-methyl phthalates. Finally, phthalate mixtures were positively associated with PAI-1, leptin, IL-18, IL-12, IL-8 and IL-1β. Despite the cross-sectional design limitation, these associations point to relevant subclinical immuno-inflammatory actions of these pollutants, warranting confirmation in future studies.

1. Introduction

Phthalates are synthetic organic chemicals used as solvents, plasticizers and additives in polyvinyl chloride plastics or in personal care products (Wang et al., 2019). About one-two decades ago, phthalates with low molecular weight (LMWP), such as di-methyl phthalate (DMP), di-ethyl phthalate and di-n-butyl phthalate (DnBP) were widely used in personal care products, lacquers, coatings and varnishes, while high molecular weight phthalates (HMWP), including di-(2-ethyl-hexyl) phthalate (DEHP), di-n-octyl phthalate, di-iso-nonyl phthalate and di-isodecyl phthalate were generally used as plasticizers including food-packaging, building materials, furniture, and plastic toys (Schettler, 2006). HMWP used in commercial products can easily migrate to the surrounding environment through evaporation, leaching, and abrasion (Wang et al., 2019), specially under temperature and pH variations, since they are not covalently bound to the plastic matrix (Katsikantami et al., 2016). Phthalates are found ubiquitously, therefore exposure to them is virtually inevitable (Daniel et al., 2020).

Phthalates are classified as endocrine disrupting chemicals (EDCs)

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Abbreviations

IL	interleukin
IFN	interferon
TNF	tumor necrosis factor
MCP	monocyte chemoattractant protein-1
LBP	lipopolysaccharide-binding protein
PAI-1	plasminogen activator inhibitor-1
CRP	C-reactive protein
WQS	weighted quantile sum regression

(Katsikantami et al., 2016) and have been associated with hormone-mediated health outcomes, such as reproductive disorders (pathogenesis of early puberty, infertility, altered quality of sperm, etc.), metabolic diseases, cancer or neurodevelopmental problems (Baken et al., 2019; Benjamin et al., 2017a; Gore et al., 2015; Varshavsky et al., 2018). More recently, the immune system has been identified as another target of EDCs (Nowak et al., 2019). In particular, previous studies have related frequent HMWP exposure with chronic inflammation processes, oxidative stress and alterations in the secretion of various cytokines (Benjamin et al., 2017a; Braun et al., 2013). Consumption of plastic packaged high-temperature soup food has been linked to increased phthalate exposure resulting in an increase of interleukin (IL)-1β, IL-4 and tumor necrosis factor (TNF), and a decrease of IL-6 and interferon (IFN)-γ (Zhang et al., 2021). Another cross-sectional study reported a positive association of monocarboxynonyl phthalate concentrations with C-reactive protein (CRP) and IL-6, but inverse associations of monoethyl phthalate (MEP) and monobenzyl phthalate (MBzP) with CRP levels were found (Trim et al., 2021).

Nevertheless, the effect of phthalates on the release of adipokines (adipose tissue derived endocrine factors) has been less well studied. One study with a birth cohort has shown that prenatal phthalate exposure was not associated with changes in leptin levels in children, while the concentration of di-isononyl phthalate metabolites was negatively associated with adiponectin (Kupsco et al., 2021). Adipokines stimulate the production of several types of peptides, hormones and other molecules related to inflammation like IL-6, IL-8, TNF, monocyte chemoattractant protein 1 (MCP-1) (Amin et al., 2019), and adipose tissue-derived plasminogen activator inhibitor-1 (PAI-1) (Kaji, 2016). Emerging data also points to gut microbiota disruptions as a potential pathway to trigger inflammatory signals (Dubinski et al., 2021), particularly those related to endotoxemia, which might be evaluated using markers such as lipopolysaccharide binding protein (LBP).

We hypothesized that a potential mechanism linking phthalate exposure to chronic diseases is that frequent point exposures to phthalates might cause frequent concomitant changes in the inflammatory milieu. However, according to previous studies, the direction of that effect might depend on the specific phthalate and/or biomarker analyzed, with most studies reporting just few phthalates/endogenous biomarkers. Here we explore the cross-sectional associations between the serum levels of serum phthalate metabolites with a comprehensive set of immuno-inflammatory biomarkers in samples from adult residents in Southern Spain.

2. Methods

2.1. Design and study population

The present study is framed in a larger hospital-based study (n = 409), the GraMo cohort, that aimed to characterize the potential health effects of the exposure to environmental factors. Participants were recruited between 2003 and 2004 in two public hospitals: Clínico San Cecilio University Hospital, in Granada city (urban area) and Santa Ana Hospital in the town of Motril (semi-rural area). Participants were selected among patients undergoing non-cancer-related surgery (41% hernias, 21% gallbladder diseases, 12% varicose veins and 26% other conditions), in order to obtain an adipose tissue sample. More details of the cohort, including inclusion criteria have been published before (Arrebola et al., 2009; Donat-Vargas et al., 2021). Socio-demographic, lifestyle and clinical information was obtained from face-to-face interviews by trained staff during the hospital stay and by reviewing the clinical records of the patients. All participants signed their informed consent to participate in the study, which was approved by the Ethics Committee of each hospital in 2002 for the recruitment of patients and analysis of pollutants, and by the Ethics Committee of Granada in 2016 (Comité de Ética de Investigación Clínica de Granada 8/2016) for the analysis of cytokines.

2.2. Study samples

A 12h-fasting blood sample was collected in vacutainer tubes with inert gel from 405 patients before surgery, as 4 patients refused to donate blood. Serum was separated from the cellular fraction by centrifuging at 600 g for 10 min at room temperature, aliquoted, and stored at −80 °C. This study is a cross-sectional analysis of a subsample (n = 213, 52.1%) of the original cohort, and it includes all participants with enough serum volume to measure both the immuno-inflammatory biomarkers and phthalate metabolites. Supplementary Table 1 shows the main characteristics of the original cohort and the sub-cohort used in this study. No relevant differences were observed, except for the hospital of recruitment, as there were more participants from Motril in the sub-cohort than in the original cohort.

2.3. Laboratory analysis

2.3.1. Phthalate metabolite assessment

The concentrations of phthalate metabolites were measured by isotope diluted online-TurboFlow-liquid chromatography coupled with tandem mass spectrometry, as described in detail before (Donat-Vargas et al., 2021; Hart et al., 2018). Samples were randomly divided in 5 batches, each including 45 samples approximately plus calibration standards, three blanks, three serum pool controls, and three serum pool controls spiked with phthalate metabolite standards at low or high known concentration. The inter-day variation analyzed as the relative standard deviation between batches was < 21% for all analytes spiked in serum at low level and < 11% for all analytes spiked in serum at high level. This variation is in the range of previous reports (Hart et al., 2018).

Serum concentrations of a total of 32 metabolites from 15 phthalates were quantified (Donat-Vargas et al., 2021). Metabolites detected above the limit of detection (LOD) in less than 25% of samples were excluded from further analyses. Thus in this study five metabolites of five LMWP were included: mono-methyl phthalate (MMP), mono-ethyl phthalate (MEP), mono-iso-butyl phthalate (MiBP), mono-n-butyl phthalate (MnBP), mono-benzyl phthalate (MBzP) and five metabolites of three HMWP were included: mono-(2-ethyl-hexyl) phthalate (MEHP), mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono-(2-carboxymethyl-hexyl) phthalate (MCMHP), mono-iso-nonyl phthalate (MiNP) and mono-iso-decyl phthalate (MiDP). Values for their LOD are provided in Supplementary Table 2. For these 10 metabolites, values below the LOD were substituted with LOD/square root of 2. The distribution of phthalate metabolite concentrations and the correlation among them in this cohort has been previously published (Donat-Vargas et al., 2021).

2.3.2. Immuno-inflammatory biomarker assessment

Immuno-inflammatory biomarkers quantified in serum included a total of 13 cytokines (IL-1β, interferon –IFN–α, IFN-γ, TNF, MCP-1, IL-6, IL-8, IL-10, IL-12, IL-17 IL-18, IL-23, IL-33) in 213 samples; 4 adipokines

(PAI-1, leptin, resistin, adiponectin) in 192 samples, C-reactive protein (CRP) in 162 samples, and the endotoxemia marker LBP in 208 samples. The difference in sample size for the immuno-inflammatory biomarkers was due to sample availability. Cytokines were quantified in a flow cytometer with the LEGENDplex™ Multiplex Assay from BioLegend (San Diego, CA), whereas adipokines and CRP were measured in a Luminex machine using the Procartaplex™ Multiplex Immunoassay from Thermo Fisher Scientific (Vienna, Austria). Both kits were used following manufacturer's instruction. Briefly, 50 μL of serum samples were tested in duplicates in a filter plate in the case of cytokines, while 25 μL of serum were tested in single replicates in 96-well plates in the case of adipokines and CRP. Each plate contained duplicated serial dilutions (1:4) of a standard sample of known concentration for each analyte provided by the vendor, as well as two blank controls and a reference sample control in duplicate for quality control purposes. Standard curves were used to extrapolate the concentration of the samples, after fitting into a 5-parameter curve algorithm with the LEGENDplex™ Data Analysis Software. Values for their LOD are provided in Supplementary Table 3. Lipopolysaccharide binding protein (LBP) was measured following manufacturer's instructions of a commercial ELISA kit (RayBiotech, Catalog#: ELH-LBP). Briefly, samples were diluted 1000 times and tested in 96-well plates. Each plate contained duplicated serial dilutions of a standard sample of known concentration, used to extrapolate sample concentrations. Two blank samples were also used for quality control purposes. The range of the assay was between 0.82 and 200 ng/ml, and the intra- and inter-coefficient of variation were below 10 and 12%, respectively.

2.4. Statistical analyses

The descriptive analysis included the calculation of mean and standard deviation for continuous variables and percentages for the categorical variables. The Skewness-Kurtosis test for normality and graphical displays evidenced non-normal distributions for all phthalate metabolites and immuno-inflammatory biomarker distributions. Pairwise correlations were assessed using the Spearman's rank correlation coefficient (rho) and linear trends evaluated using Generalized Additive Models (GAM). The p-values were adjusted for multiple comparisons using the Benjamini-Hochberg method.

2.4.1. Regression analysis

The "phthalate-inflammatory" pairs with a higher magnitude of correlation (specified in the results section) were further explored using univariate (model 1) and multivariable (models 2, 3, 4 and 5) linear regression analyses. Dependent variables were the immuno-inflammatory biomarkers, while independent variables were phthalate metabolite concentrations. Three phthalates were categorized for the regression analyses. MBzP, which was detectable (>LOD) only in 25% of the samples was dichotomized into < LOD and ≥ LOD. MMP and MiDP, detectable in 26-66% of the samples, were divided into three categories, so-called "tertiles" from now on. The first tertile (established as the reference) comprised all samples below the LOD, and the rest of the values were dichotomized as the second and the third tertiles. Therefore, estimators for the second and third tertile represent the mean change versus the first tertile. In order to relax the linearity assumption in the regression models, concentrations of each phthalate metabolite and biomarker were natural-log transformed. Beta coefficients with corresponding 95% confidence intervals (CI) were estimated. To facilitate the interpretation of the results, antilogarithms of the coefficients were calculated, and effects expressed as the fold-change in biomarker concentration per one inter quartile range increase in phthalate metabolite levels (in continuous variables) or per category change in categorized phthalates.

2.4.2. Covariates

The whole list of the questionnaire variables analyzed in this study as well as their definitions are provided in Supporting Information. To select the covariates for the multivariable adjustment, we took into account biological (Benjamin et al., 2017b; Gangemi et al., 2016; Shivappa et al., 2018; Zatterale et al., 2020) as well as statistical considerations. First, we did a selection based on biological plausibility and literature search. This selection included many variables related to diet and prevalent diseases, which overall but not each specifically are known to be potential confounders in our study. Thus, we decided to include in the multivariable models only those with a p-value<0.2 in the univariate regression analysis with the biomarkers studied. We used a conservative p-value in the selection of confounders in order to minimize the potential interference of residual confounding. Finally, we excluded those covariates with high collinearity (variance inflation factor >2.5). Sex and age were always forced into the models.

The covariates finally included in model 2 were: 1) diet variables: quantity of milk, quantity of canned food, frequency of legumes intake, frequency of meat intake, frequency of butter intake, frequency of vegetable intake, frequency of fruit intake, frequency of egg intake, frequency of bread intake, type of water (tap/bottled), type of fat used for cooking (olive oil versus any other type); 2) alcohol intake and smoking habit; 3) body mass index (BMI); 4) proximity to industrial green houses, proximity to industry and proximity to agriculture area; and 5) type of surgery performed at inclusion on the study.

Initially, we hypothesized that the immuno-inflammatory biomarkers may be mediators of chronic diseases and therefore, the inclusion of the later in the models might represent an over-adjustment. Nevertheless, prevalent chronic conditions might be causally associated with the immuno-inflammatory biomarkers as well. Thus, sensitivity analyses were performed adjusting for prevalent chronic health conditions present at recruitment that had a p-value<0.2 in the univariate regression analysis with the biomarkers studied, excluding those with high collinearity (variance inflation factor >2.5). The variables included in the model 3 were those of model 2 plus the following diseases: diabetes, cataract, embolism, hypercholesterolemia, depression/anxiety, cardiovascular disease, arthritis, peptic ulcer, skin disorders, heart diseases, asthma, varicose veins, any other disease.

Dietary variables might be considered a confounder but also as a source of phthalate exposure. In the latter scenario, adjustment for diet might cause an overfitting of the models. In this regard, we fitted the models without adjustment for diet (models 4 and 5).

2.4.3. Multipollutant exposure model

Finally, we assessed whether there was a mixture effect of the phthalate metabolites on biomarkers' concentration by means of weighted quantile sum (WQS) regression (Carrico et al., 2014). WQS estimates a weighted index based on the combination of several exposures, considering their individual associations with the outcome. Then this index was included as the independent variable in a single linear regression with the concentration of the biomarkers as the dependent variable and adjusting for the same covariates as the individual associations in model 2. The individual weight of each phthalate in the mixture effect was also calculated in percentage. Considering that WQS regressions need to set a priori the expected direction of the association, we calculated two models (positive and negative) for mixture effects for each outcome. All WQS analyses were performed with tertile-scored pollutant concentrations, using a training set defined as a 40% random sample of the dataset, being the remaining 60% used for model validation. The final weights were calculated using a total of 1000 bootstrap steps.

The level of statistical significance was set at p-value = 0.05 for the Spearman's correlation. Regression models were interpreted based on the confidence intervals. The statistical software STATA/SE version 14.2 (Stata Corp LP, College Station, TX) was used to manage the database of the study and to perform statistical analyses whereas R statistical computing environment v 4.1.0 was used to elaborate the boxplots and to perform WQS analyses (Team RC, 2018), with gWQS package v2.0.1

(Renzetti et al., 2020).

3. Results

3.1. Characteristics of the study population

The characteristics of the study population are shown in Table 1. There was a similar distribution among sexes in the study population, with 106 (49.8%) being males. The average age was 48.9 years (standard deviation, SD: 17.4) and the mean BMI was 27.4 kg/m^2 (SD: 5.5). Regarding occupation, the majority were manual workers (72.8%). Besides, 73 (34.3%) of them had the surgery in the hospital of Granada and 140 (65.7%) in Motril. More than half of the participants reported regular alcohol consumption and were smokers or former smokers (Table 1).

3.2. Cytokines, adipokines and CRP's concentrations

As shown in Fig. 1A, cytokine's concentrations were generally low except for MCP-1 and IL-18, which also had all values above the LOD. On the other hand, IL-33 presented a high percentage (44%) of non-detected samples and was excluded from further analyses. In addition, CRP, LBP and the adipokines (Fig. 1B) were detected in virtually 100% of the samples, with relatively high median concentrations.

As expected, the concentrations of the classical pro-inflammatory cytokines such as TNF, IL-1β, IL-6, and IL-12, presented a moderate to strong correlation among them and with the concentrations of lymphocyte T_H1-derived IFN-γ and lymphocyte T_H17-derived IL-17. Of note, the levels of the aforementioned cytokines showed mostly an inverse although weak correlation with those of MCP-1, Leptin, IL-18 and PAI-1 (Supplementary Fig. 1).

3.3. Association of phthalates with immuno-inflammatory biomarkers

Spearman's rho values of the correlation between phthalate metabolites and immuno-inflammatory biomarkers are shown in Table 2. We observed two patterns of associations. First, a group formed by the adipokines PAI-1 and leptin, and the cytokines IL-18 and MCP-1, showed positive significant correlations with primarily the group of LMWP metabolites, i.e., MMP, MEP, MiBP, MnBP and MBzP but also with the HMWP metabolite MiDP. Among them, PAI-1 showed the highest associations, including strong (rho value > 0.6) correlations with MiBP and MnBP. Second, various proinflammatory markers, i.e., IL-12, IL-6, TNF, IL-8, IL-17, IL-1β, and LBP presented significant positive correlations with MEHP, while showing inverse correlations with MMP. This second pattern of associations with MEHP and MMP presented weaker magnitudes of correlation.

In a second step, we estimated lineal regression models to analyse multivariable associations between phthalate and immuno-inflammatory biomarkers. The presence of non-linearity in the models was discarded graphically by displaying GAM models (Supplementary Fig. 2). To minimize multiple comparisons, we focused on the two aforementioned patterns, with most pairs of phthalate-biomarkers showing the strongest correlations. As described in the methods section, univariable and four multivariable models were estimated. With few exceptions, no relevant differences existed among the four adjustment levels.

Focusing on the first pattern of associations (Table 3 and Supplementary Table 4), the two metabolites of the two dibutyl phthalate isomers, MiBP and MnBP, were positively and significantly associated with PAI-1, leptin, IL-18 and MCP-1 after adjusting for potential confounders in all the models. For instance, compared to individuals with low MiBP levels (percentile 25th), those with higher (an interquartile range higher, percentile 75th) levels had 3.07 times (207%) more PAI-1, 65% more IL-18, 41% more MCP-1 and 133% more leptin. The association of these 4 immuno-inflammatory markers with MEP, MiDP and

Table 1
Population description.

Variables	
N	213
Male [n (%)]	106 (49.8)
Age, years [mean (SD)]	48.9 (17.4)
Body mass index, kg/m^2 [mean (SD)]	27.4 (5.5)
Normal weight.	75 (35.2)
Overweight/obesity	138 (64.8)
Occupation [n (%)]	
Non-manual worker	45 (21.1)
Manual worker	155 (72.8)
Retired	13 (6.1)
Surgery hospital [n (%)]	
Granada	73 (34.3)
Motril	140 (65.7)
Regular alcohol consumption [n (%)] [c]	115 (54.0)
Smoking habit [n (%)]	
Never smoked	88 (41.3)
Former smokers	48 (22.5)
Current smokers	77 (36.2)
Proximity to commercial greenhouses [n (%)]	
Yes	45 (21.2)
No	167 (78.8)
Proximity to industry [n (%)]	
Yes	65 (30.7)
No	147 (69.3)
Proximity to agriculture area [n (%)]	
Yes	121 (57.1)
No	91 (42.9)
Quantity of milk[a] **[mean (SD)]**	1.49 (1.30)
Quantity of canned food[b] **[mean (SD)]**	1.71 (2.09)
Frequency of legumes intake [n (%)]	
≥2 times per week	158 (74.5)
<2 times per week	52 (24.5)
Frequency of meat intake [n (%)]	
>1 time per week	185 (88.1)
1 time per week	25 (11.9)
Frequency of butter intake [n (%)]	
<1 time per day	177 (84.3)
≥1 time per day	33 (15.7)
Frequency of vegetable intake [n (%)]	
≥2 times per week	147 (70.0)
<2 times per week	63 (30.0)
Frequency of fruit intake [n (%)]	
≥2 times per week	189 (90.)
<1 time per week	21 (10.0)
>2 times per week	60 (28.3)
≤2 times per week	150 (70.8)
Frequency of bread intake [n (%)]	
≥1 time per day	193 (91.0)
<1 time per day	17 (8.0)
Type of water [n (%)]	
Tap water	110 (52.13)
Bottled water	65 (30.81)
Both	36 (17.06)
Type of fat used for cooking [n (%)]	
Olive oil	174 (82.9)
Others	36 (17.1)
Type of surgery performed at inclusion on the study [n (%)]	
Hernias	81 (41.3)
Gallbladder diseases	39 (19.9)
Varicose veins	21 (10.7)
Others	55 (28.1)
Hypertension [n (%)]	50 (23.5)
Diabetes [n (%)]	17 (8.0)
Allergy [n (%)]	66 (31.1)
Embolism [n (%)]	5 (2.4)
Cataract [n (%)]	16 (7.6)
Hypercholesterolemia [n (%)]	48 (22.6)
Depression/anxiety [n (%)]	56 (26.4)
Cardiovascular disease [n (%)]	62 (29.2)
Arthritis [n (%)]	58 (27.4)
Peptic ulcer [n (%)]	22 (10.4)
Skin disorders [n (%)]	37 (17.4)
Heart diseases [n (%)]	22 (10.4)
Asthma [n (%)]	18 (8.5)

(continued on next page)

Table 1 (*continued*)

Variables	
Urinary tract disorder [n (%)]	55 (26.1)
Chronic constipation [n (%)]	64 (30.3)
Chronic bronchitis [n (%)]	17 (8.0)
Varicose veins [n (%)]	77 (36.5)
Any other disease [n (%)]	108 (50.9)

SD: Standard deviation.
[a] Glasses per day.
[b] Canned food units per week.
[c] regular alcohol intake (yes, i.e. ≥1 drink/week or no).

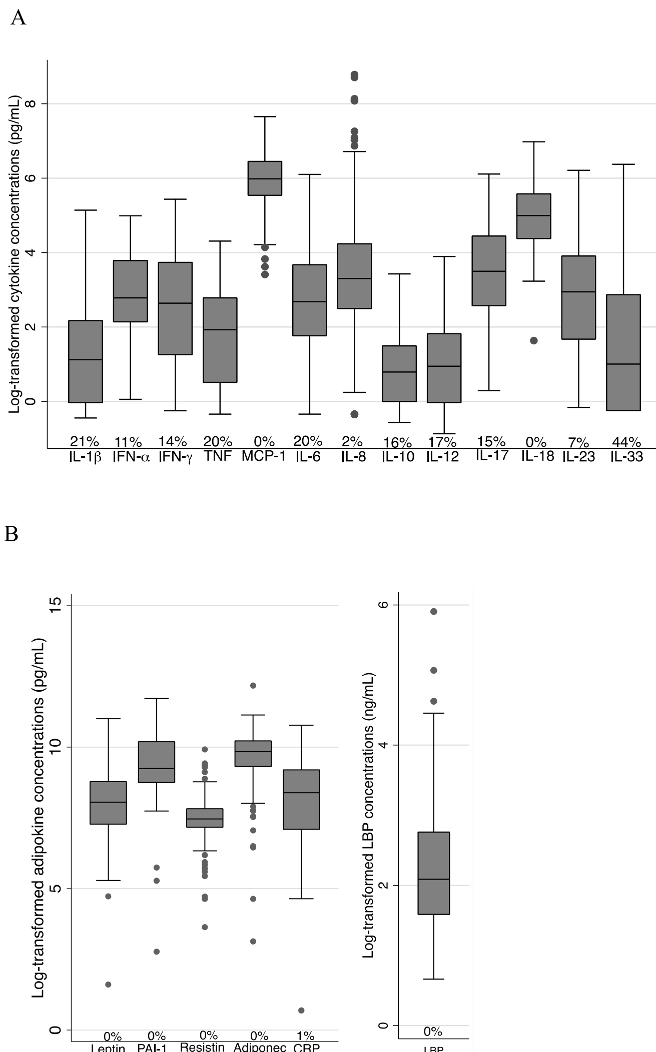

Fig. 1. Immuno-inflammatory biomarker serum concentrations. Log-transformed concentration of A) cytokines and B) adipokines, C-reactive protein (CRP), and lipopolysaccharide-binding protein (LBP) are represented as box plots, showing median (black line), and 25th and 75th percentiles (lower and upper hinge respectively). Numbers under the boxes represent the percentages of samples below the limit of detection. IL, interleukin; IFN, interferon; TNF, tumor necrosis factor alpha; MCP-1, monocyte chemoattractant protein 1; PAI-1, plasminogen activator inhibitor-1; Adiponec, adiponectin.

MBzP was weaker and in some of the adjusted models, lost statistical significance. However, the direction of the association remained the same.

We performed stratified analyses for PAI-1, to check for specific sex or BMI group effects (Supplementary Table 5). Although the associations seemed to be a bit higher in males than females, and in people with overweight than those with normal weight, the directions and statistical significance were the same in the groups.

With regards to the second pattern (Table 4 and Supplementary Table 6), MEHP was positively and MMP was negatively associated with IL-1β, IL-17, IL-8, IL12, IL-6, TNF and LBP in all models, with the exception of the model MEHP-LBP adjusted for diseases (model 3), which was borderline non-significant (Table 4). Thus, one interquartile range increase in MEHP levels was associated with a 49% increase in IL-1β, a 110% increase in IL-8, a 59% increase in IL-17, a 62% increase in IL-6, a 25% increase in LBP, a 35% increase in IL-12, and a 42% increase in TNF concentrations. Inversely, compared to individuals with undetected MMP levels (T1 reference), those with the highest levels (T3) had 56% less IL-1β, 73% less IL-8, 68% less IL-17, 61% less IL-6, 46% less LBP, 49% less IL-12, and 50% less TNF (Table 4).

3.4. Multi-pollutant models

Next, we calculated a positive and a negative WQS index as a measure of the combined effect of all the phthalates. Similar to the linear regression models, we only assessed the mixture effect on PAI-1, leptin, IL-18, MCP-1, IL-1β, IL-17, IL-8, IL-6, IL-12, TNF, and LBP.

Looking at the first pattern of associations, in the multivariable linear regression model the "positive" WQS index was significantly associated with PAI-1 concentrations ($\beta = 1.115$; $p < 0.001$), being MiBP (27%) and MiDP (16%) the metabolites driving the index; with leptin concentrations ($\beta = 0.947$; $p = 0.003$), being MMP (25%) and MiBP (20%) the metabolites with a higher contribution to the index; and with IL-18 concentration ($\beta = 0.806$; $p = 0.001$), being MiDP (30%) and MMP (16%) the metabolites contributing the most (Fig. 2). The "positive" WQS index was not significantly associated with MCP-1 ($\beta = 0.303$; $p = 0.232$). On the other hand, the "negative" WQS index did not converge for PAI-1, IL-18 nor for leptin, and it was not significantly associated with MCP-1 ($\beta = -0.192$; $p = 0.479$).

With regards to the second pattern of associations (with pro-inflammatory biomarkers), the "positive" WQS index was significantly associated with IL-1β concentration ($\beta = 0.821$; $p = 0.032$), being MEHP (28%) and MiNP (20%) the metabolites driving the index; with IL-8 concentration ($\beta = 1.187$; $p = 0.019$), being MiDP (25%) and MEHP (24%) the metabolites with a higher contribution to the index; and with IL-12 concentration ($\beta = 0.684$; $p = 0.048$), being MEHP (43%) and MMP (18%) the metabolites contributing the most (Fig. 2). Moreover, there were borderline non-significant association between the "positive" WQS index and the concentrations of IL-17 ($\beta = 0.835$; $p = 0.056$) and LBP ($\beta = 0.390$; $p = 0.095$), and non-significant associations with the concentrations of IL-6 ($\beta = 0.624$; $p = 0.216$) and TNF ($\beta = 0.548$; $p = 0.166$). The "negative" WQS index was not significantly associated with the concentrations of any of the proinflammatory cytokines (data not shown).

As MiDP, MBzP and MMP had a low detection rate, we performed a sensitivity analysis excluding those three phthalates. Although some differences in the coefficients and individual chemical contributions were observed, the direction and significance of the associations remained similar (Supplementary Fig. 3).

4. Discussion

In this cross-sectional study, we evidenced associations of phthalate metabolites in serum with several immuno-inflammatory biomarkers. We observed two patterns of results: i) PAI-1, leptin, IL-18, and MCP-1 showed positive weak to strong significant correlations with several phthalate metabolites (mainly LMWP); ii) various known proinflammatory markers, i.e., IL-12, IL-6, TNF, IL-8, IL-17, IL-1β and LBP, presented a significant although weak inverse correlation with MMP (LMWP), and a positive correlation with MEHP (HMWP). Moreover, based on the multi-pollutant analyses, a potential mixture effect of the combined exposure to several phthalates with converging mechanisms

Table 2
Correlation of between phthalate metabolite and biological biomarkers measured in serum samples.
Cells display the Spearman's correlation test rho value, whose value ranges between 1 (blue) and −1 (red). Bold indicates adjusted p-value <0.05. The p values were adjusted using the Benjamini-Hochberg method. MW; molecular weight; PAI-1, plasminogen activator inhibitor-1; MCP-1, monocyte chemoattractant protein 1; IL: Interleukin; CRP, C-reactive protein; IFN, Interferon; TNF, tumor necrosis factor alpha, MnBP, mono-n-butyl phthalate; MiBP, mono-iso-butyl phthalate; MEP, mono-ethyl phthalate; MBzP, mono-benzyl phthalate; MiDP, mono-iso-decyl phthalate; MMP, mono-methyl phthalate; MECPP, mono-(2-ethyl-5-carboxypentyl) phthalate; MCMHP, mono-(2-carboxymethyl-hexyl) phthalate; MiNP, mono-iso-nonyl phthalate; MEHP, mono-(2-ethyl-hexyl) phthalate.

	-				MW					+
	MMP	MEP	MiBP	MnBP	MBzP	MEHP	MiNP	MiDP	MECPP	MCMHP
PAI-1	**0.289**	**0.542**	**0.633**	**0.629**	**0.479**	-0.139	-0.074	**0.430**	0.217	0.132
IL-18	0.043	**0.298**	**0.379**	**0.417**	**0.399**	0.059	0.080	**0.335**	0.103	0.031
MCP-1	0.137	**0.250**	**0.352**	**0.352**	**0.421**	-0.064	-0.004	**0.244**	0.144	0.115
Leptin	0.039	**0.345**	**0.345**	**0.318**	0.247	-0.026	-0.027	0.212	0.182	0.105
Resistin	-0.012	0.257	0.142	0.164	0.063	0.014	-0.079	0.078	0.172	0.079
Adiponectin	0.267	0.126	0.086	0.079	0.049	-0.236	-0.236	-0.042	-0.005	0.041
CRP	0.137	0.204	0.250	0.356	0.316	-0.056	0.150	0.150	0.118	0.160
IFN-α	-0.165	0.003	0.049	0.081	0.085	0.168	0.111	0.093	0.037	0.078
IFN-γ	-0.063	-0.082	-0.126	-0.098	0.004	0.123	0.110	-0.068	-0.067	-0.004
IL-10	-0.123	-0.001	0.014	0.041	0.004	0.138	0.087	0.052	0.004	0.028
IL-23	0.059	0.146	0.219	0.217	0.259	0.021	0.005	0.128	0.029	0.086
IL-12	**-0.271**	-0.128	-0.101	-0.068	-0.104	**0.288**	0.166	0.044	0.035	0.052
TNF	**-0.293**	-0.202	-0.208	-0.134	-0.067	**0.288**	0.157	-0.002	-0.053	-0.010
LBP	**-0.316**	-0.179	-0.106	-0.109	-0.089	**0.255**	0.163	0.012	0.027	-0.029
IL-6	**-0.275**	-0.064	-0.009	0.044	0.098	**0.301**	0.186	0.034	0.002	0.027
IL-8	**-0.303**	-0.120	0.006	0.089	0.145	**0.394**	0.175	0.178	0.067	0.034
IL-17	**-0.311**	-0.026	0.055	0.108	0.092	**0.322**	0.211	0.168	0.026	0.058
IL-1β	**-0.337**	**-0.245**	-0.223	-0.163	-0.175	**0.328**	0.209	-0.063	-0.094	-0.011

Cells display the Spearman's correlation test rho value, whose value ranges between 1 (blue) and -1 (red). Bold indicates adjusted p-value <0.05. The p values were adjusted using the Benjamini-Hochberg method. MW; molecular weight; PAI-1, plasminogen activator inhibitor-1; MCP-1, monocyte chemoattractant protein 1; IL: Interleukin; CRP, C-reactive protein; IFN, Interferon; TNF, tumor necrosis factor alpha, MnBP, mono-n-butyl phthalate; MiBP, mono-iso-butyl phthalate; MEP, mono-ethyl phthalate; MBzP, mono-benzyl phthalate; MiDP, mono-iso-decyl phthalate; MMP, mono-methyl phthalate; MECPP, mono-(2-ethyl-5-carboxypentyl) phthalate; MCMHP, mono-(2-carboxymethyl-hexyl) phthalate; MiNP, mono-iso-nonyl phthalate; MEHP, mono-(2-ethyl-hexyl) phthalate.

of action may exist.

The strong and positive associations of PAI-1 with MiBP and MnBP (among other phthalate metabolites) levels are worth mentioning. PAI-1 is an adipokine produced by adipocytes and endothelial cells. Besides its classical pro-coagulant properties, PAI-1 is an acute-phase reactant involved in cell migration and proliferative programs (Higgins et al., 2011). PAI-1's expression increases in adipose tissue during acute systemic inflammation (Ekström et al., 2017), and this might explain its linkage with inflammation-related systemic metabolic changes. Thus, PAI-1 has been associated with the development of diabetes, cardiovascular disease, dyslipidaemias, cancer and chronic inflammation (reviewed in Kaji, 2016). Importantly, several epidemiological studies have shown that phthalates may be considered cardiometabolic risk factors for insulin resistance, dyslipidaemia, hypertension and obesity (Darbre, 2017; Mariana and Cairrao, 2020). Furthermore, *in vivo* and *in vitro* studies have confirmed the association between phthalates and PAI-1. For instance, the DBP isomers, DiBP and DnBP (parental phthalates for MiBP and MnBP) have been shown to increase the expression of PAI-1 in rat testis (Kobayashi et al., 2003). Besides, di-(2-ethylhexyl) phthalate (parental phthalate MEHP, MECPP and MCMHP) promoted human macrophages activation *in vitro* via a transduction pathway which involves PAI-1 (Yamaguchi et al., 2019). Altogether, these data allow us to hypothesise that PAI-1 may be in the causal pathway between phthalate exposure and cardiometabolic risk. Nevertheless, more epidemiological and toxicological studies are necessary to confirm this hypothesis.

To a lesser extent, another adipokine, leptin, as well as IL-18 and MCP-1, also presented a positive correlation mainly with LMWP. Leptin is an anorexigenic adipokine which usually shows increased plasma levels in obese individuals due to a resistance of the body to its actions. Our results contrast with a previous cross-sectional study in which phthalate metabolites were analyzed in urine samples (Carlsson et al., 2018) and with two birth cohorts (Ashley-Martin et al., 2014; Minatoya et al., 2018). The differences in the specimen used for biomonitoring and the developmental windows investigated may account for the differences with our results.

With regards to IL-18 and MCP-1, they are both pro-inflammatory cytokines produced mainly by monocytes/macrophages among other cells. Our results about IL-18 are somehow contradictory with a previous study showing no increased expression of IL-18 after *in vitro* incubation

Table 3
Associations of serum phthalate metabolite with PAI-1, IL-18, MCP-1 and Leptin. Multivariable linear regression.

Phthalate metabolite		MEP	MiBP	MnBP	MBzP* C1: ref	MiDP* T1: ref
PAI-1 (pg/ml)	**Model 1 n=192**	**2.43 (1.91; 3.02) [p < 0.001]**	**3.14 (2.62; 3.77) [p < 0.001]**	**1.71 (1.56; 1.86) [p < 0.001]**	**C2: 2.77 (1.99; 3.86) [p < 0.001]**	**T2: 1.58 (1.11; 2.27) [p=0.013]** **T3: 2.92 (2.05; 4.22) [p < 0.001]**
	Model 2 n=169	**2.05 (1.62; 2.61) [p < 0.001]**	**3.07 (2.39; 3.95) [p < 0.001]**	**1.65 (1.45; 1.88) [p < 0.001]**	**C2: 2.20 (1.49; 3.25) [p < 0.001]**	**T2: 1.54 (1.06; 2.25) [p=0.033]** **T3: 2.48 (1.57; 3.9) [p < 0.001]**
	Model 3 n=169	**2.01 (1.58; 2.61) [p < 0.001]**	**2.93 (2.23; 3.69) [p < 0.001]**	**1.60 (1.42; 1.82) [p < 0.001]**	**C2: 1.95 (1.27; 3.03) [p=0.003]**	T2: 1.36 (0.93; 2.03) [p = 0.110] **T3: 2.29 (1.42; 3.71) [p=0.001]**
IL-18 (pg/ml)	**Model 1 n=213**	**1.40 (1.21; 1.66) [p < 0.001]**	**1.65 (1.41; 1.94) [p < 0.001]**	**1.30 (1.20; 1.39) [p < 0.001]**	**C2: 1.92 (1.51; 2.41) [p < 0.001]**	**T2: 1.35 (1.04; 1.72) [p=0.026]** **T3: 1.88 (1.46; 2.44) [p < 0.001]**
	Model 2 n=190	**1.27 (1.07; 1.54) [p=0.006]**	**1.65 (1.35; 1.99) [p < 0.001]**	**1.28 (1.18; 1.42) [p < 0.001]**	**C2: 1.88 (1.43; 2.46) [p < 0.001]**	**T2: 1.34 (1.02; 1.75) [p=0.032]** **T3: 1.65 (1.20; 2.25) [p=0.002]**
	Model 3 n=190	**1.30 (1.07; 1.58) [p=0.009]**	**1.65 (1.35; 2.03) [p < 0.001]**	**1.28 (1.16; 1.40) [p < 0.001]**	**C2: 1.72 (1.26; 2.34) [p=0.001]**	**T2: 1.36 (1.04; 1.80) [p=0.020]** **T3: 1.58 (1.14; 2.23) [p=0.006]**
MCP-1 (pg/ml)	**Model 1 n=213**	**1.24 (1.07; 1.47) [p=0.005]**	**1.51 (1.32; 1.73) [p < 0.001]**	**1.22 (1.14; 1.30) [p < 0.001]**	**C2: 1.92 (1.55; 2.39) [p < 0.001]**	T2: 1.00 (0.79; 1.27) [p = 0.982] **T3: 1.6 (1.27; 2.03) [p < 0.001]**
	Model 2 n=190	1.15 (0.98; 1.37) [p = 0.105]	**1.41 (1.17; 1.73) [p < 0.001]**	**1.19 (1.09; 1.30) [p < 0.001]**	**C2: 1.87 (1.42; 2.46) [p < 0.001]**	T2: 0.98 (0.76; 1.26) [p = 0.875] T3: 1.35 (1.00; 1.82) [p = 0.053]
	Model 3 n=190	1.10 (0.91; 1.33) [p = 0.299]	**1.35 (1.10; 1.65) [p=0.006]**	**1.15 (1.06; 1.27) [p=0.002]**	**C2: 1.70 (1.27; 2.33) [p < 0.001]**	T2: 0.97 (0.75; 1.27) [p = 0.837] T3: 1.23 (0.90; 1.70) [p = 0.196]
Leptin (pg/ml)	**Model 1 n=191**	**1.96 (1.47; 2.55) [p < 0.001]**	**2.13 (1.65; 2.80) [p < 0.001]**	**1.40 (1.24; 1.60) [p < 0.001]**	**C2: 2.08 (1.38; 3.1) [p=0.001]**	T2: 1.40 (0.09; 2.20) [p = 0.136] **T3: 2.03 (1.30; 3.16) [p=0.002]**
	Model 2 n=168	**1.91 (1.43; 2.49) [p < 0.001]**	**2.33 (1.69; 3.22) [p < 0.001]**	**1.48 (1.27; 1.72) [p < 0.001]**	**C2: 1.73 (1.11; 2.69) [p=0.018]**	T2: 1.42 (0.93; 2.18) [p = 0.110] **T3: 1.79 (1.04; 3,00) [p=0.032]**
	Model 3 n=168	**1.79 (1.27; 2.32) [p=0.001]**	**2.23 (1.5; 3.14) [p < 0.001]**	**1.45 (1.24; 1.71) [p < 0.001]**	C2: 1.57 (0.95; 2.59) [p = 0.090]	T2: 1.23 (0.79; 1.92) [p = 0.376] T3: 1.63 (0.93; 2.83) [p = 0.101]

Cells display the fold-change in biomarker concentration per one inter quartile range increase in phthalate metabolite level or * per category change in categorized variables, and between parentheses, the 95% confidence interval. Bold indicates confidence interval does not include value “1” (statistical significance). MiDP and MBzP were categorized into 3 tertiles (T) and 2 categories (C) respectively, as described in the methods' section, and T1 and C1, which comprised the non-detected samples, were used as the reference category. All variables were log-tranformed. MnBP, mono-n-butyl phthalate; MiBP, mono-iso-butyl phthalate; MEP, mono-ethyl phthalate; MiDP, mono-iso-decyl phthalate; MBzP, mono-benzyl phthalate; PAI-1, plasminogen activator inhibitor-1; IL-18, interleukin 18; MCP-1, monocyte chemoattractant protein 1. Model 1: Univariable regression model. Model 2: Multivariable linear regression model adjusted for quantity of milk, quantity of canned food, quantity of legumes, frequency of meat intake, frequency of butter intake, frequency of vegetable intake, frequency of fruit intake, frequency of egg intake, frequency of bread intake, type (tap/bottled) of water, type of fat used for cooking (olive oil versus any other type), alcohol intake, smoking habit, BMI, proximity to industrial green houses, proximity to industry, proximity to agriculture area, and type of surgery performed at inclusion on the study. Model 3: Multivariable linear regression model adjusted for those of model 2 plus the following diseases: diabetes, cataract, embolism, hypercholesterolemia, depression/anxiety, cardiovascular disease, arthritis, peptic ulcer, skin disorders, heart diseases, asthma, varicose veins, any other disease.

of a keratinocyte cell line with DnBP(Lourenço et al., 2015). Nevertheless, it is possible that *in vivo*, other cell types may react to DnBP (or its metabolites) by producing IL-18. Furthermore, a very recent epidemiological study has shown a positive association between phthalate urine MBP, MEP and MBzP levels and serum MCP-1 concentration, in accordance with our study (Liu et al., 2022).

We also observed various significant phthalate mixture associations for PAI-1, leptin, and IL-18. However, while PAI-1 and IL-18, shared the metabolite which contributed most to the index (MiDP), for leptin MMP and MiNP contributed more. This suggests a different underlying mechanism relating the phthalate metabolites to the immuno-inflammatory process. These models clearly emphasize the need for consideration of mixture effects, that complement single-chemical associations and give a more realistic scenario. Despite the promising results, it is worth to mention that these models are based on statistical variance and not on biological processes and, therefore, coherence with other epidemiological and experimental research designs warrants further assessment.

We observed a second clear pattern of correlations between phthalate metabolites and the classical pro-inflammatory cytokines IL-12, IL-

Table 4
Association of serum phthalate metabolites with pro-inflammatory cytokines. Multivariable linear regression.

Phthalate metabolite		MMP* T1: ref	MEHP
IL-1β (pg/ml)	**Model 1 n=213**	**T2: 0.42 (0.29; 0.63) [p < 0.001]** **T3: 0.39 (0.26; 0.58) [p < 0.001]**	**1.70 (1.40; 2.08) [p < 0.001]**
	Model 2 n=190	**T2: 0.48 (0.30; 0.79) [p=0.004]** **T3: 0.44 (0.27; 0.73) [p=0.002]**	**1.49 (1.19; 1.88) [p=0.001]**
	Model 3 n=190	**T2: 0.42 (0.25; 0.73) [p=0.002]** **T3: 0.49 (0.29; 0.84) [p=0.009]**	**1.46 (1.15; 1.85) [p=0.002]**
IL-8 (pg/ml)	**Model 1 n=213**	**T2: 0.26 (0.16; 0.42) [p < 0.001]** **T3: 0.35 (0.22; 0.57) [p < 0.001]**	**2.23 (1.76; 2.83) [p < 0.001]**
	Model 2 n=190	**T2: 0.24 (0.13; 0.43) [p < 0.001]** **T3: 0.27 (0.15; 0.50) [p < 0.001]**	**2.10 (1.59; 2.76) [p < 0.001]**
	Model 3 n=190	**T2: 0.23 (0.12; 0.45) [p < 0.001]** **T3: 0.28 (0.14; 0.53) [p < 0.001]**	**2.08 (1.58; 2.78) [p < 0.001]**
IL-17 (pg/ml)	**Model 1 n=213**	**T2: 0.32 (0.20; 0.50) [p < 0.001]** **T3: 0.34 (0.21; 0.53) [p < 0.001]**	**1.80 (1.42; 2.27) [p < 0.001]**
	Model 2 n=190	**T2: 0.36 (0.20; 0.63) [p < 0.001]** **T3: 0.32 (0.18; 0.57) [p < 0.001]**	**1.59 (1.22; 2.08) [p=0.001]**
	Model 3 n=190	**T2: 0.33 (0.18; 0.61) [p=0.001]** **T3: 0.32 (0.17; 0.59) [p < 0.001]**	**1.51 (1.14; 2.01) [p=0.005]**
IL-6 (pg/ml)	**Model 1 n=213**	**T2: 0.39 (0.25; 0.63) [p < 0.001]** **T3: 0.41 (0.26; 0.65) [p < 0.001]**	**1.73 (1.37; 2.16) [p < 0.001]**
	Model 2 n=190	**T2: 0.38 (0.22; 0.67) [p=0.001]** **T3: 0.39 (0.22; 0.70) [p=0.002]**	**1.62 (1.25; 2.10) [p < 0.001]**
	Model 3 n=190	**T2: 0.30 (0.16; 0.58) [p < 0.001]** **T3: 0.33 (0.18; 0.63) [p=0.001]**	**1.70 (1.29; 2.27) [p < 0.001]**
LBP (ng/mL)	**Model 1 n=208**	**T2: 0.58 (0.44; 0.76) [p < 0.001]** **T3: 0.58 (0.44; 0.75) [p < 0.001]**	**1.27 (1.12; 1.46) [p < 0.001]**
	Model 2 n=185	**T2: 0.53 (0.38; 0.73) [p < 0.001]** **T3: 0.54 (0.39; 0.75) [p < 0.001]**	**1.25 (1.07; 1.46) [p=0.006]**
	Model 3 n=185	**T2: 0.61 (0.43; 0.87) [p=0.007]** **T3: 0.64 (0.44; 0.90) [p=0.013]**	1.16 (0.99; 1.36) [p = 0.066]
IL-12 (pg/mL)	**Model 1 n=213**	**T2: 0.51 (0.34; 0.76) [p=0.001]** **T3: 0.48 (0.32; 0.70) [p < 0.001]**	**1.51 (1.23; 1.83) [p < 0.001]**
	Model 2 n=190	**T2: 0.59 (0.36; 0.98) [p=0.041]** **T3: 0.51 (0.31; 0.84) [p=0.009]**	**1.35 (1.08; 1.70) [p=0.011]**
	Model 3 n=190	**T2: 0.47 (0.27; 0.78) [p=0.008]** **T3: 0.42 (0.24; 0.73) [p=0.002]**	**1.42 (1.11; 1.83) [p=0.005]**
TNF (pg/mL)	**Model 1 n=213**	**T2: 0.49 (0.32; 0.76) [p=0.001]** **T3: 0.43 (0.28; 0.67) [p < 0.001]**	**1.58 (1.26; 1.95) [p < 0.001]**
	Model 2 n=190	**T2: 0.52 (0.31; 0.87) [p=0.013]** **T3: 0.50 (0.30; 0.85) [p=0.011]**	**1.42 (1.12; 1.81) [p=0.004]**
	Model 3 n=190	**T2: 0.45 (0.26; 0.80) [p=0.007]** **T3: 0.53 (0.30; 0.93) [p=0.028]**	**1.44 (1.12; 1.85) [p=0.005]**

Cells display the fold-change in biomarker concentration per one inter quartile range increase in phthalate metabolite level or * per tertile change in categorized variables, and between parentheses, the 95% confidence interval. Bold indicates confidence interval does not include value "1" (statistical significance). MEHP (mono-(2-ethyl-hexyl) phthalate) was analyzed as a continuous variable and MMP (mono-methyl phthalate) was categorized into three tertiles (T), as explained in the methods section. IL: interleukin. Model 1: Univariable regression model. Model 2: Multivariable linear regression model adjusted for quantity of milk, quantity of canned food, quantity of legumes, frequency of meat intake, frequency of butter intake, frequency of vegetable intake, frequency of fruit intake, frequency of egg intake, frequency of bread intake, type (tap/bottled) of water, type of fat used for cooking (olive oil versus any other type), alcohol intake, smoking habit, BMI, proximity to industrial green houses, proximity to industry, proximity to agriculture area, and type of surgery performed at inclusion on the study. Model 3: Multivariable linear regression model adjusted for those of model 2 plus the following diseases: diabetes, cataract, embolism, hypercholesterolemia, depression/anxiety, cardiovascular disease, arthritis, peptic ulcer, skin disorders, heart diseases, asthma, varicose veins, any other disease.

6, TNF, IL-8, IL-17 and IL-1β. Despite the weak nature of the associations, the consistency of the results is remarkable, with all those redundant (with similar physiological functions) cytokines showing a negative association with MMP and positive association with MEHP, and only with them. The negative association of MMP with those cytokines was unexpected, as phthalates are hypothesized to stimulate inflammatory processes (Benjamin et al., 2017a; Braun et al., 2013; Nappi et al., 2016). However, while the evidence obtained so far implies a proinflammatory role for HMWP such as DEHP (MEHP) (reviewed in Hansen et al., 2015), as observed here, the role in inflammation of LMWP like DMP (MMP) has not been previously established in epidemiological studies. Interestingly, a very recent publication has reported that DMP administration to rats results in decreased peripheral blood concentration of IL-6, IFN-γ or IL-4 (Chi et al., 2022), reinforcing our epidemiological finding. The authors demonstrate that DMP exerts immunotoxicity via oxidative damage and apoptosis, which might explain our results as well. With diet being the main entrance route for DEHP exposure at the time for sample collection, this may also explain the positive association observed between MEHP and LBP, a potential marker of gut permeability leading to metabolic endotoxemia (Mohammad and Thiemermann, 2021). Indeed, a recent *in vivo* study showed that DEHP exposure was linked to microbiota disturbances in mice, including gut hyperpermeability and higher serum lipopolysaccharide concentrations (Tian et al., 2019).

The novelty and relatively large number of phthalate metabolites assessed, and the consistency of results throughout the different approaches used, are among the main strengths of the present study. In addition, the measurement of inflammation markers as the main outcome allowed us to estimate potentially subclinical effects.

Our study has also some limitations. First, the cross-sectional design of our study may prevent us from reaching causative conclusions. However, it should be noted that both phthalate metabolites and inflammatory biomarkers have a short half-life, hampering the ascertainment of causality in longitudinal studies. Moreover, it is noteworthy that there are no evidences supporting a potential reverse-causality, so

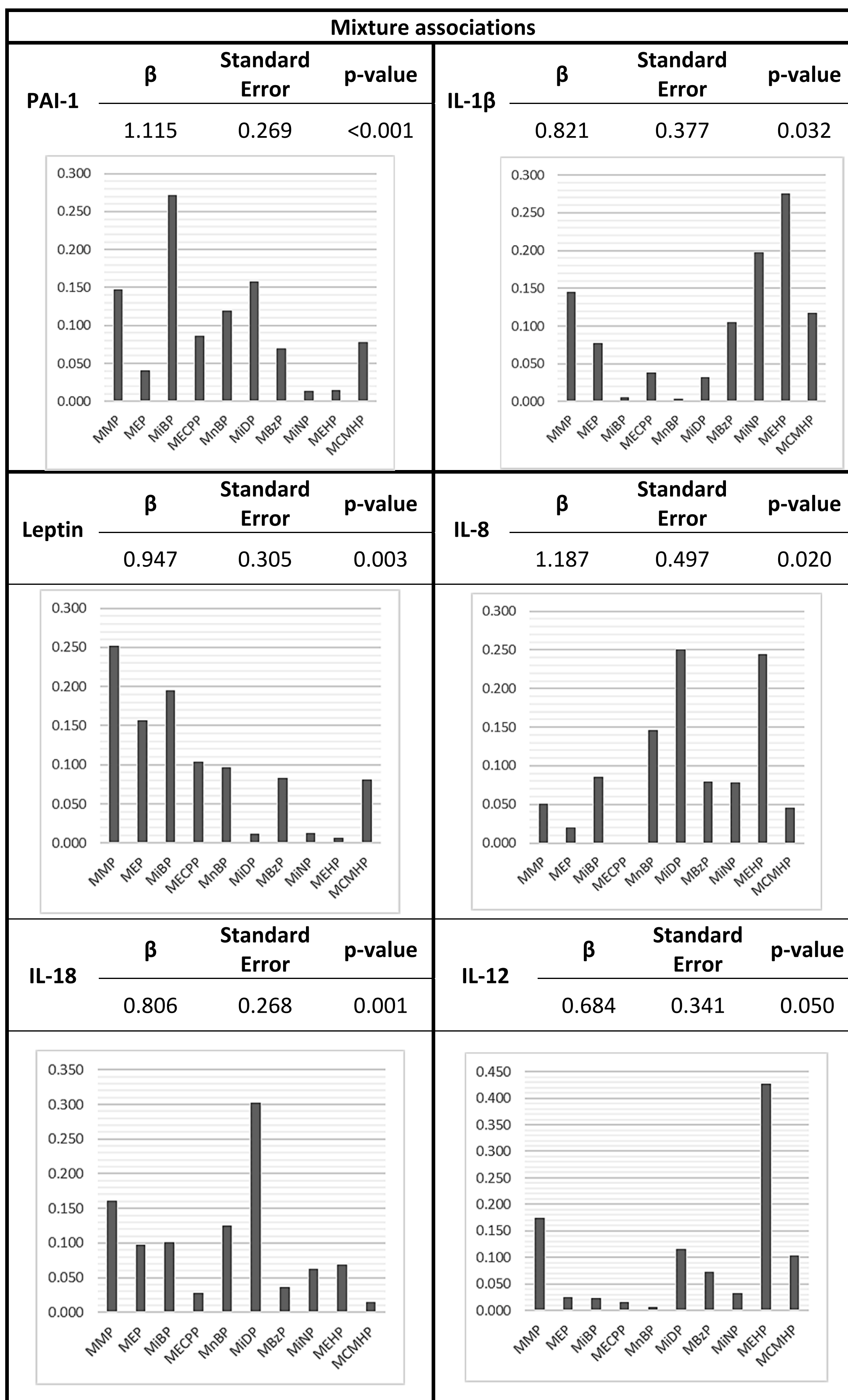

Fig. 2. Estimation of the mixture associations of phthalate metabolites with different immuno-inflammatory biomarker levels. Weighted quantile sum regression (WQS) models.

that inflammatory molecules would affect phthalate concentrations. Second, phthalate exposure was estimated by using a spot serum sample. Although phthalate metabolites are frequently detected at higher concentrations in urine and are probably more representative of a wider time lapse, serum concentrations are closer to the biologically effective dose, and moderate to strong correlations have been found between the two matrices (Henriksen et al., 2020; Hines et al., 2009). Moreover, our hypothesis is that point exposure to phthalates might cause point (and probably subclinical) concomitant changes in the inflammatory milieu, and, therefore, exposure assessment using serum concentrations seems adequate. Third, sample size was not estimated for this study and this may have prevented us from finding some associations due to a lack of power. Lastly, in our study we evidenced several associations with inflammatory cytokines, although we need to bear in mind that phthalate exposure might not be directly related to every biomarker since they are related among them and present feedback loops (Turner et al., 2014).

5. Conclusions

Our study offers novel findings: i) positive associations of PAI-1, MCP-1, IL-18 and leptin with individual and mixtures of phthalate metabolites, ii) positive and negative associations of high and low molecular weight phthalates respectively with proinflammatory biomarkers. These results open the door to future research since, to our best knowledge, no previous study has deepened into the mentioned relationships. Further research on the potential health implications of phthalate exposure, together with other pollutants is currently being performed within the GraMo cohort.

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Author contributions

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Juan Pedro Arrebola reports financial support, article publishing charges, and equipment, drugs, or supplies were provided by Carlos III Health Institute.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2022.114470.

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