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Transcriptional analysis in peripheral blood cells of individuals with elevated phthalate exposure – Results of the EuroMix study

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ABSTRACT

Exposure to phthalates is widespread in Europe. Phthalates are considered endocrine disrupting compounds and are classified as toxic for reproduction. However how phthalates affect the transcriptome in humans remains largely unknown. To investigate the effects of phthalate exposure on the transcriptomic profile we conducted RNA sequencing on peripheral blood samples from the Norwegian EuroMix cohort. We compared gene expression changes between participants with high, medium, and low exposure of six phthalates and 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH). Comparing high and low exposure groups, DINCH was the compound that showed the highest number of differentially expressed genes (126 genes) followed by mono-n-butyl phthalate (MnBP; 89 genes) and mono-iso-nonyl phthalate (MiBP; 70 genes). Distributions between up- or down-regulated genes were similar across the different phthalates and DINCH. All phthalates including DINCH shared common differentially expressed genes ranging from 3 to 37 overlaps. Enriched Gene Ontology (GO) and biological pathway analysis revealed that most of the differentially expressed genes were associated with general cellular metabolism GO terms. MnBP and DINCH, particularly, showed a marked enrichment in various immunological function pathways including neutrophil degranulation, adaptive immune system and signaling by interleukins. Furthermore, the association between genes involved in the peroxisome proliferator activated receptor (PPAR) signaling pathway and phthalates, including DINCH, was evaluated. In total, 15 genes showed positive or negative associations across 5 phthalates and DINCH. MnBP and MiBP were the phthalate metabolites with the highest number of associations: 8 and 4 PPAR signaling pathway genes, respectively. Overall, we have performed an association study between phthalate exposure levels and modulation of transcriptomic profiles in human peripheral blood cells. DINCH, which is often mentioned as a substitute for phthalates, had comparable effects on differential gene expression in peripheral blood cells as phthalates.

1. Introduction

Phthalates are diesters of phthalic acid (1,2-benzenedicarboxylic acid) used as plasticizers to soften and confer flexibility to a wide variety of daily-use products (such as polyvinyl chloride plastics) including food wrappers, toys, paints, personal care products (cosmetics) and medical equipment (Lorz et al., 2007). It is estimated that around one million tonnes of phthalates are manufactured per year in Western Europe (ECVM, 2021). Phthalates are not covalently bound to the polymer matrix and are susceptible to leaching (Andjelković et al., 2021; Wooten and Smith, 2013). Numerous biomonitoring studies have detected the presence of phthalate metabolites in human urine demonstrating the wide exposure of these compounds to the general population (HBM4EU, 2022). Typical concentrations across the globe range from 10 to 200 ng/ml found in urine, with the low molecular weight phthalates showing the highest urine concentrations (Zhang et al., 2021). Phthalate

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metabolites have been also detected in other body matrices such as semen, cord blood, breast milk and endometrium (Silva et al., 2022; Zhang et al., 2021) albeit at significant lower levels.

Phthalates have been shown to act as endocrine-disrupting chemicals (EDCs) targeting, particularly, the reproductive system (Arcadi et al., 1998; Foster et al., 2006; Swan et al., 2005). These chemicals were reported to decrease testosterone production by dysregulation of key enzymes of the testosterone biosynthetic pathway, leading to perturbations in testosterone signaling (Foster et al., 2006). Indeed, under the Classification, Labelling and Packaging regulation (EC 1272/2008) several phthalates, including DEHP, DBP, DiBP and BBP, are classified as toxic to reproduction category 1 B, and are currently restricted from children's toys use and medical equipment. Phthalates have been associated with alterations in other hormone-regulated systems such as those controlling glucose and lipid metabolism (Stahlhut et al., 2007) mediated by glucocorticoid activation (Klopčič et al., 2015; Leng et al., 2020; Sargis et al., 2010).

Furthermore, phthalates have been linked to perturbations of intracellular metabolic sensors such as peroxisome proliferator-activated receptors (PPARs) (Lapinskas et al., 2005). PPARs, including PPARα, PPARδ and PPARɣ, are nuclear receptors containing a DNA-binding domain and a ligand-binding domain expressed across multiple tissues including immune cells. Several endogenous compounds including polyunsaturated fatty acids, eicosanoids and oxidized lipid components have been shown to interact and modulate PPARs nuclear receptor activity (Berger and Moller, 2002). It is not known, however, whether there is any endogenous compound that interacts with high affinity with PPARs. Rather, it is suggested that multiple unspecific binding could occur due to PPARs large binding pocket. This could be an explanatory hypothesis of phthalate's interaction with PPAR members (Bility et al., 2004): unspecific binding of phthalates into PPARs binding pocket and subsequent activation of its transcription factor activity.

While much research has been conducted at the population- (Hauser and Calafat, 2005), tissue- and cellular-scale level showing reproductive system (Sedha et al., 2021), metabolic (Stahlhut et al., 2007), immunological (Bølling et al., 2020) and neurodevelopment (Zheng et al., 2022) adverse effects, with mechanisms encompassing insulin resistance (Stahlhut et al., 2007), adipogenesis induction (Lapinskas et al., 2005) and immune cell variation (Nygaard et al., 2021), less is known about phthalate effects at the gene expression level. In addition, there is high variability of effects observed in epidemiological studies, yielding null (Hansen, 1999; Modigh et al., 2002), slight (Duty et al., 2004) or moderate associations with phthalate exposure. Using transcriptomic analysis in blood samples reflecting current environmental exposure levels of phthalates has the potential to detect early changes that can be linked to late physiological adverse effects onset. Thus, improving the characterization and monitoring of toxicological effects from common environmental contaminants.

In this cross-sectional study we explored the relationships between high, medium and low environmental phthalate exposure and differential gene expression from 139 healthy adults recruited for the EuroMix biomonitoring study (Husøy et al., 2019). We focused our analysis on the metabolites of 7 phthalates measured in urine (MEP, MiBP, MnBP, MBzP, DEHP, DiNP, oh-MPHP) and DINCH (a phthalate substitute). We performed RNA sequencing from peripheral blood to detect statistically differentially expressed genes across participants with high, medium, and low phthalate exposure. The identified differentially expressed genes for each phthalate were associated with biological pathways to explore the functional links between a gradient of phthalate exposure and gene expression changes. Finally, we investigated the associations between phthalate exposure and genes involved in the PPAR signaling pathway.

2. Methods

2.1. Study subjects and data collection

The Norwegian EuroMix biomonitoring study was a part of the "European Test and Risk Assessment Strategies for Mixtures" project (EuroMix, ID 633172–2) and funded by the H2020 program (Husøy et al., 2019). 144 healthy adults (44 men and 100 women) from the Oslo area with and average age of 43 ± 12 years old were recruited to participate in the EuroMix study. Urine samples were collected after need during 24 h with an average of 8 urine samples per participant (with mean, minimum and maximum volumes corresponding to: 304, 50 and 520 ml for each of the samples). A more detailed description of the urine collection is given in Husøy et al. (2019). Blood samples were withdrawn at the end of the study period from 139 participants; establishing the sample size used throughout this study. . The EuroMix study is approved by the Regional Committee for Medical Research Ethics (REK IS no 2015/1868), and all the participants gave their written informed consent.

2.2. Phthalate's analysis

Liquid chromatography coupled to tandem mass spectrometry was performed for the detection of phthalate metabolites as previously described (Husøy et al., 2019). In total, 13 phthalate metabolites were analyzed from the 24 h urine pools: monoethyl phthalate (MEP), mono-isobutyl phthalate (MiBP), mono-n-butyl phthalate (MnBP), monobenzyl phthalate (MBzP), mono-2-ethylhexyl phthalate (MEHP), mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-2-ethyl-5-oxohexyl (MEOHP), mono-2-ethyl 5-carboxypentyl phthalate (MECPP), mono-2-carboxymethyl hexyl phthalate (MMCHP), mono-4-methyl-7-hydroxyoctyl phthalate (oh-MiNP), mono-4-methyl-7-oxooctyl phthalate (oxo-MiNP), mono-4-methyl-7-carboxyoctyl phthalate (cx-MiNP), 6-hydroxy monopropylheptylphthalate (oh-MPHP). Additionally, 2 metabolites of DINCH were measured: (2-(((Hydroxy-4-methyloctyl) oxy)carbonyl)-cyclohexanecarboxylic Acid (oh-MINCH) and 2-(((4-Methyl-7-oxyooctyl)oxy)carbonyl)-cyclohexanecarboxylic Acid (oxo--MINCH)). Some metabolites originated from the same parent molecule and their concentrations (ng/ml) were summed together: sumDEHP (MEHP, MEHHP, MEOHP, MECPP, MMCHP), sumDiNP (oh-MiNP, oxo-MiNP, cx-MiNP) and sumDINCH (oh-MINCH, oxo-MINCH). The variable 'sumPhthalates' was used as a surrogate for total phthalate mixture and calculated as the total phthalates' metabolite sum, excluding DINCH metabolites, adjusted for molecular weight. Urine samples were normalized using specific gravity (Husøy et al., 2019) according to the formula developed in (Bertelsen et al., 2013). Phthalate concentrations, including DINCH, were divided in quintiles (Q1 - Q5). The quintiles were merged to give low (Q1 & Q2 = 55 samples), medium (Q3 & Q4 = 56 samples) and high (Q5 = 28 samples) with a total number of samples of 139. The concentration ranges (ng/ml) of the different phthalates including DINCH for the low samples (0.7–51), mid samples (1.4–76), and high samples (3.6–118) where MBzP had the lowest concentration while sumDEHP had the highest.

2.3. RNA isolation, library preparation and sequencing

Whole blood samples were collected into Tempus tubes (3 ml blood per tube) according to the manufacturer's instructions (ThermoFisher, MA, USA) from the EuroMix study participants (N = 139) and stored at −80 °C until processing. Total RNA was extracted as previously described (Aarem et al., 2016) using the MagMAX™ for Stabilized Blood Tubes RNA Isolation Kit, compatible with Tempus™ Blood RNA Tubes on KingFisher instruments (ThermoFisher, MA, USA), and the optional DNase treatments were included in accordance with each manufacturer's protocol. The concentration and the purity of extracted total RNA was measured using NanoDrop ND-1000 spectrophotometer

(ThermoFisher, MA, USA). The RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The isolated total RNA was stored at −80 °C until analysis. The blood RNA samples were shipped to Novogene (Novogene Co., Ltd., Cambridge, UK) and sequenced on Illumina NovaSeq 6000; with and 2 X 150 bp paired-end sequence strategy. A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) including ribosomal RNAs (rRNA) depletion using NEBNext® rRNA Depletion Kit (NEB, USA) following manufacturer's recommendations. Raw reads fastq-files were trimmed using Trimmomatic (version 0.39) and trimmed reads were mapped to the human reference genome (version GRCh38) using HISAT2 (version 2.2.0.) (Kim et al., 2019). Counting of mapped reads was done using featureCounts v1.6.2 (Liao et al., 2014) based on annotation from Ensembl release 84. Approximately 48 × 10^6 reads were generated per library, and the average mapping rate was >96%. Raw counts from Novogene were preprocessed before downstream analysis performed at NIPH (section 2.5). The raw and preprocessed sequences are stored on secure servers at NIPH and due to GDPR cannot be shared without respective permissions and agreements with the NIPH.

2.4. Quantitative real-time PCR

The transcript levels of *PPARA, PPARG and PPARD* in participants' blood samples with the highest and lowest DEHP and DiNP phthalate levels (N = 28) were investigated by analyzing their relative mRNA levels. For first-strand cDNA synthesis, 500 ng of total RNA was first reverse-transcribed into cDNA in a total volume of 10 μL, using the PrimeScript RT reagent Kit Perfect Real Time for RT-PCR (Takara Bio, Europe), and a combination of random hexamers/oligo dT primers according to the manufacturer's instructions. Specific primers for the selected genes were designed using Primer-BLAST tool (NIH). The oligonucleotide sequences of these specific primers are provided (Supplementary Table 3). To avoid amplification of genomic DNA sequences, primers and/or amplicons were designed to cross the exon/exon boundaries, if possible. All genes were amplified by real-time PCR in the Step One Plus detection system with StepOnePlus Software v2.3 (Thermo Fisher Scientific, Waltham, USA) using PowerUp SYBR as the detection dye. Each amplification reaction was carried out in a total volume of 20 μL containing 10 μL PowerUp SYBR Green Master Mix (Applied Biosystems), 0.3 μM of each primer and 2 or 10 ng cDNA depending on sample dilution for adequate measurement of gene expression levels. The reactions were cycled 40 times using the following parameters: 95 °C for 3 s and 60 °C for 30 s during which the fluorescence data were collected. Melting curves were generated to verify the specificity of the products. A non-template control was run with every set of primers to exclude any indication of PCR contamination. Expression levels of the target genes were normalized to the reference genes *ACTB* (Actin beta) and *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) which were confirmed to be stably expressed throughout samples. Each cDNA was analyzed at least in triplicate by real-time PCR. Relative gene expression of high phthalate concentration samples versus low phthalate concentration samples (control) was calculated using the ΔΔCT method (Livak and Schmittgen, 2001).

2.5. Statistical analysis

For the RNAseq data, prior to differential gene expression analysis, read counts were preprocessed, i.e., filtering out feature with zero read counts in all samples or very lowly expressed, i.e., count-per-million; CPM <1 in more than 30% of the samples were removed to reduce statistical bias in the differential expression analyses using the *filterByExpr* function in the edgeR R-package (Robinson et al., 2010). A gene is considered sufficiently expressed CPM >1 in at least 70% of samples. The preprocessed read counts were normalized with Trimmed Mean of M-values (TMM) method through one scaling normalized factor. Differential expression analysis of stratified phthalate metabolite concentration levels (low, mid, and high) was performed by linear regression model adjusted for sex, age, and BMI using the limma R-package (Ritchie et al., 2015) to identify differentially expressed genes (DEGs) where the mean expression value of a gene in any group is significantly different from other sample groups. The phthalate metabolite concentration levels were stratified in order to get enough samples size during statistical analysis. The following contrasts: high vs low; mid vs low; and high vs mid were used in the analysis. False discovery rate (FDR) was calculated to account for multiple testing using the Benjamini-Hochberg method (Yoav Benjamini, 1995). Genes with absolute fold change ≥1.3 and FDR <0.1 were considered as differentially expressed genes (DEG). To assess the robustness of the created model, we also performed a sensitivity analysis including to the same linear model the most correlated chemicals, as an additional covariate (Supp. Fig 3). The association between phthalate metabolite concentration levels and genes involved in the PPAR signaling related pathways (N = 75 genes) was analyzed by a linear regression analysis adjusted for sex, age, and BMI. The PPAR signaling pathway gene list was obtained from literature searches and from the Reactome database (Supp. Table 2). Statistical analyses were done using R statistical software (Version 4.2.0., R Core Team, https://www.r-project.org/) (Team, 2022).

For qPCR analysis, statistical significance of differences in expression (compared to control samples) was assessed by the non-parametric Mann-Whitney test, using GraphPad Prism v.8 (GraphPad 9 Software, La Jolla, USA). A p value of <0.05 was assumed to be statistically significant. The statistical calculation was based on 2-ΔCt values.

2.6. Enrichment analysis

Identification of the enriched biological processes and pathways was carried out using Metascape (http://metascape.org/) (Zhou et al., 2019). Metascape (Metascape 3.5; database last update date: 2022-10-01) is an annotation analysis tool for gene function, and it integrates functional databases including GO, KEGG, Reactome, Disease ontology (DisGeNET database) and Uniprot to analyze multiple gene-sets simultaneously. The Metascape was used to identify enriched functional pathways of the statistically differentially expressed genes from the RNAseq data analysis. Statistically significant DEGs, regardless of the fold change-ratio (i.e., $p < 0.05$ and FDR<0.1, applying no fold-change cut-off), were used in the Metascape.

3. Results

3.1. Phthalate metabolite levels

The study population from which blood samples were collected consisted of adults with a mean age of 43 years (Table 1). Women participants represented more than double of the study population compared to men (96 and 43, respectively). Approximately 95% of the study population had more than 4 years university education. Participant's weight and heigh were recorded and their body mass index (BMI) was derived. Men's mean BMI fell in the lowest interval of the overweight category whereas women's mean BMI corresponded to the healthy weight, according to the cut-offs set by the World Health Organization (Consultation, 1995).

DEHP metabolites (sumDEHP) were the most abundant phthalate class with a median value of 61,74 ng/ml in urine followed by MEP, sumDINP, MnBP and MiBP with mean values of 9.91, 7.84, 10.73, 7.7 ng/ml, respectively. DINCH metabolites (sumDINCH), MBzP and oh-MPHP were the least represented phthalates with median values of 2.90, 1.42 and 0.44 ng/ml, respectively (Fig. 1A, and Supplementary Fig. 1A, boxplot showing phthalate metabolites in urine stratified by sex). All phthalate metabolites except MEP were significantly higher in

Table 1
EuroMix cohort participant description for the 139 included participants with RNAseq data.

Description of the study population	Males (n = 43)	Females (n = 96)
Age (years)	43.6 ± 11.7	42.1 ± 12.0
Weight (kg)	82.3 ± 8.44	65.3 ± 8.99
Height (m)	1.81 ± 0.06	1.68 ± 0.06
BMI (kg/m2)	25.0 ± 2.34	23 ± 2.93
Education		
University/college <4 years	3	4
University/college ≥4 years	40	92
Smoking status		
Non-smokers	25	63
Ex-smokers	11	22
Occasional smokers	7	11

Note. Values are means ± SDs. Education and smoking status reported in counts.

Table 2
Significant differentially expressed genes.

Name of chemical or chemical group	Number of DEGs	Upregulated genes	Downregulated genes
sumPhthalates	42 (221)	22 (90)	20 (131)
sumDEHP	25(151)	11 (64)	14 (87)
sumDiNP	70 (259)	33 (143)	37 (116)
sumDINCH	126 (668)	60 (433)	66 (235)
MEP	37 (308)	22 (191)	15 (117)
MiBP	33 (321)	11 (157)	22 (164)
MnBP	89 (1734)	35 (922)	54 (812)
MBzP	66 (355)	23 (153)	43 (202)

Note. Significant differentially expressed genes shown for each phthalate metabolite compared across exposure groups (high vs low) adjusted p value < 0.05, * FDR <0.1 and FC ≥ ± 1.3 with sex, BMI, and age as covariates. Numbers in parentheses are genes without FC cut-off. High = 28 samples, Low samples = 55 samples.

men compared to women using the Wilcoxon rank sum test (Husøy et al., 2019), including the sum of all phthalates (sumPhthalates). Due to the low concentrations of oh-MPHP in our study, others (Porras et al., 2020) and reported low rate of metabolism (Schutze et al., 2015) we excluded oh-MPHP from further analysis. Correlation analysis showed that there were moderate correlations of phthalate exposure across our cohort. The highest correlations were found between MnBP/MBzP, MiBP/MBzP, and MiBP/MnBP with 0.48, 0.45 and 0.43 correlation coefficients, respectively. MEP was the phthalate showing the least correlation with the other measured phthalates. As expected, sumPhthalates showed the highest number of individual correlations between the different phthalates except with DINCH metabolites (Fig. 1B and Supplementary Fig. 1B, Correlation plots between phthalates metabolites across EuroMix participants for each sex).

3.2. Gene expression analysis

Principal component analysis (PCA) was run on all transcripts (N = 12109). The PCA plot has shown that 15% and 10% of the variation in the data is explained by PC1 and PC2. Furthermore, we found a clear pattern in how samples clustered that was attributed to sex (Fig. 2A). However, there was no pattern revealed by gradient of total phthalate (sumPhthalates), where the three groups (low, medium, high) show no separation (Fig. 2B). The PCA plots for the individual phthalates show similar patterns (Supplementary Fig. 2A-G).

We next conducted analysis to identify DEGs among individuals with high, medium, and low phthalate exposure. Here we focused on the contrast between high and low exposed groups (Fig. 3A-H), data and comparisons with the medium group are found in the supplementary material (Supplementary Fig. 3). sumDINCH, MnBP and sumDiNP showed 126, 89 and 70 significantly DEGs across individuals with high exposure compared to those with low phthalate exposure. MBzP, MEP, MiBP and sumDEHP showed 66, 37, 33 and 25 DEGs, respectively (Table 2, and Supplementary Table 1., gene name and fold change values for the top 20 up- or down-regulated genes was presented). There were approximately similar distributions for upregulated and downregulated genes across the DEGs for each phthalate, ranging approximately from 30% to 50% of total upregulated DEGs and 40%–67% of total downregulated genes (Fig. 3A-I). Interestingly, MEP showed the highest proportion of upregulated gene numbers (22/37, 59.5%) and MiBP showed the highest proportion of downregulated gene numbers (22/33, 66.7%), with respect to the total DEGs. We also performed a sensitivity analysis on MBzP DEGs adjusting for MnBP co-exposure, the most correlated phthalates in our cohort (Fig. 1B). This analysis showed that 66% of the genes from the initial gene list were recovered (Supp. Fig.3). Next, we investigated whether there were any common DEGs across phthalates. All phthalates showed subfractions of common DEGs across each other (Fig. 4A). We observed DINCH and DiNP metabolites

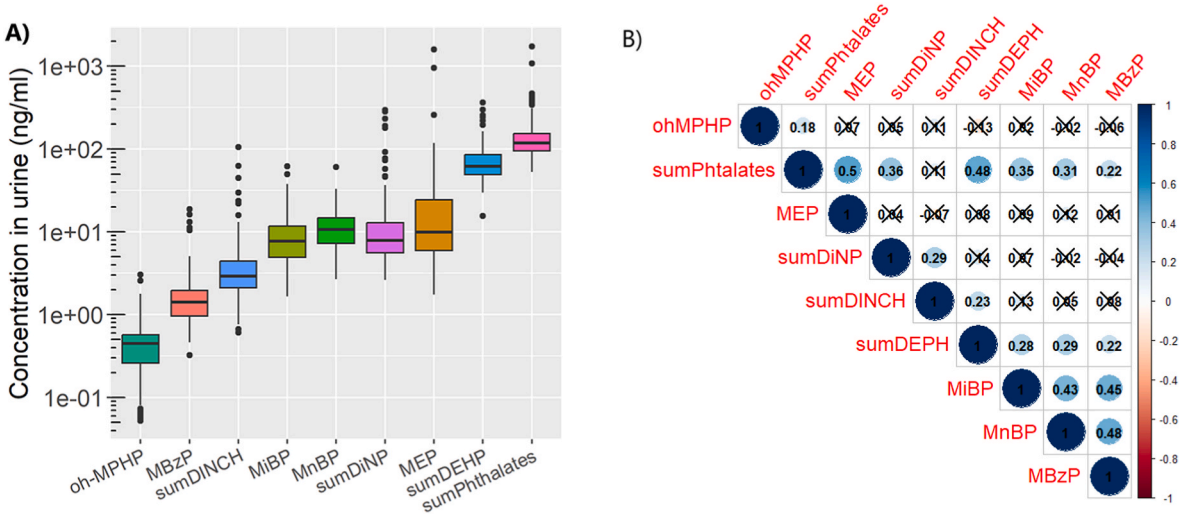


Fig. 1. Phthalates distribution and correlation in the EuroMix cohort. **A)** Boxplots representing total concentrations (ng/ml) of phthalate metabolites in urine from the EuroMix cohort (N = 139). Boxplot middle line represents median. Y-axis displayed in logarithmic scale. **B)** Correlation plot (co-occurrence) between phthalates across EuroMix participants. Color bar represents correlation coefficient. Blue represents positive correlation while red represents negative correlation. X = non-significant correlation.

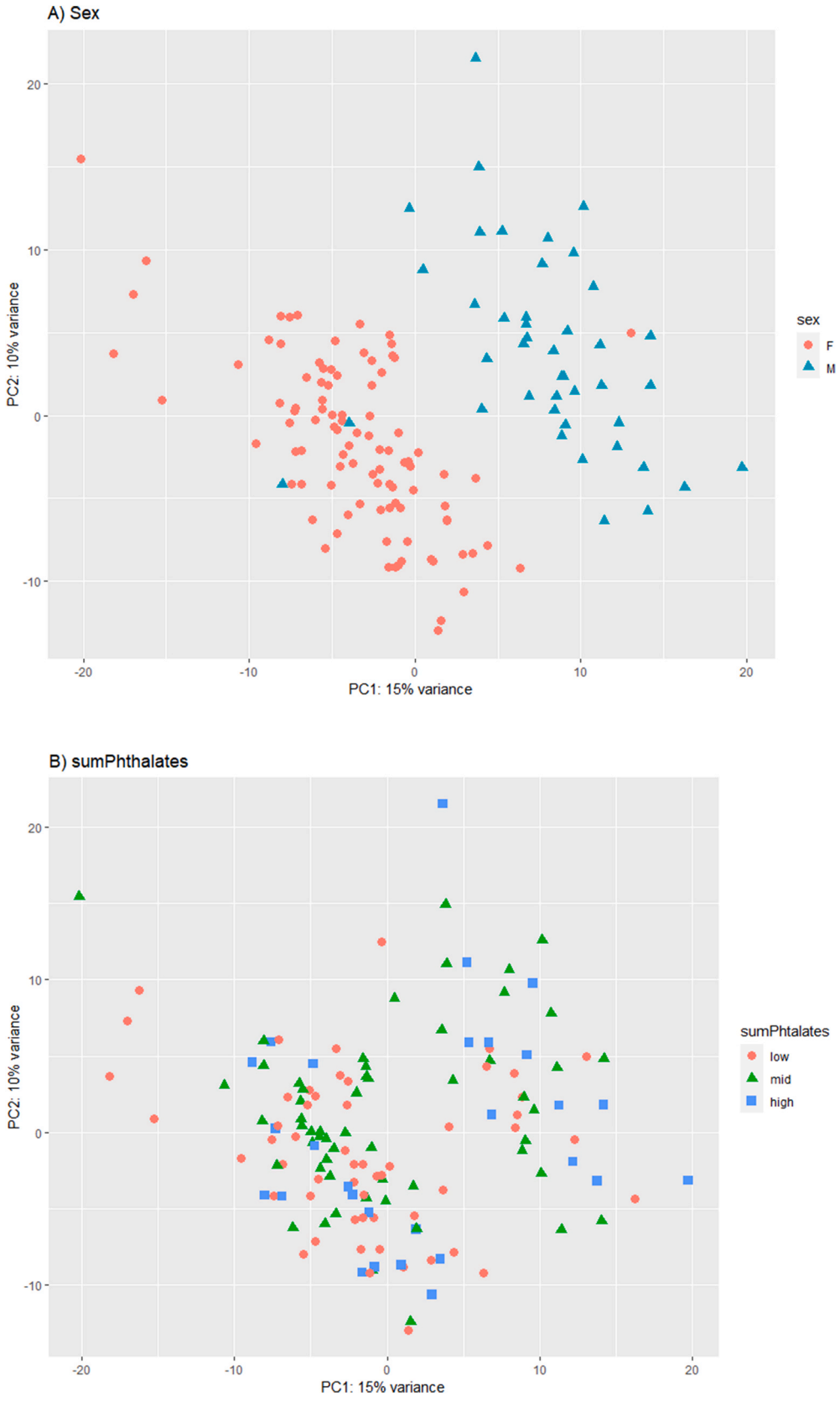


Fig. 2. PCA plot of the most variable genes of blood RNA-seq samples, based on 12109 transcripts. Each dot represents EuroMix participants transcription profiles transformed during PCA analysis to two dimensions colored by sex (Female = 96, Male = 43) (**A**) and phthalate exposure levels (high = 28 samples, mid = 56 samples, and low = 55 samples) (**B**).

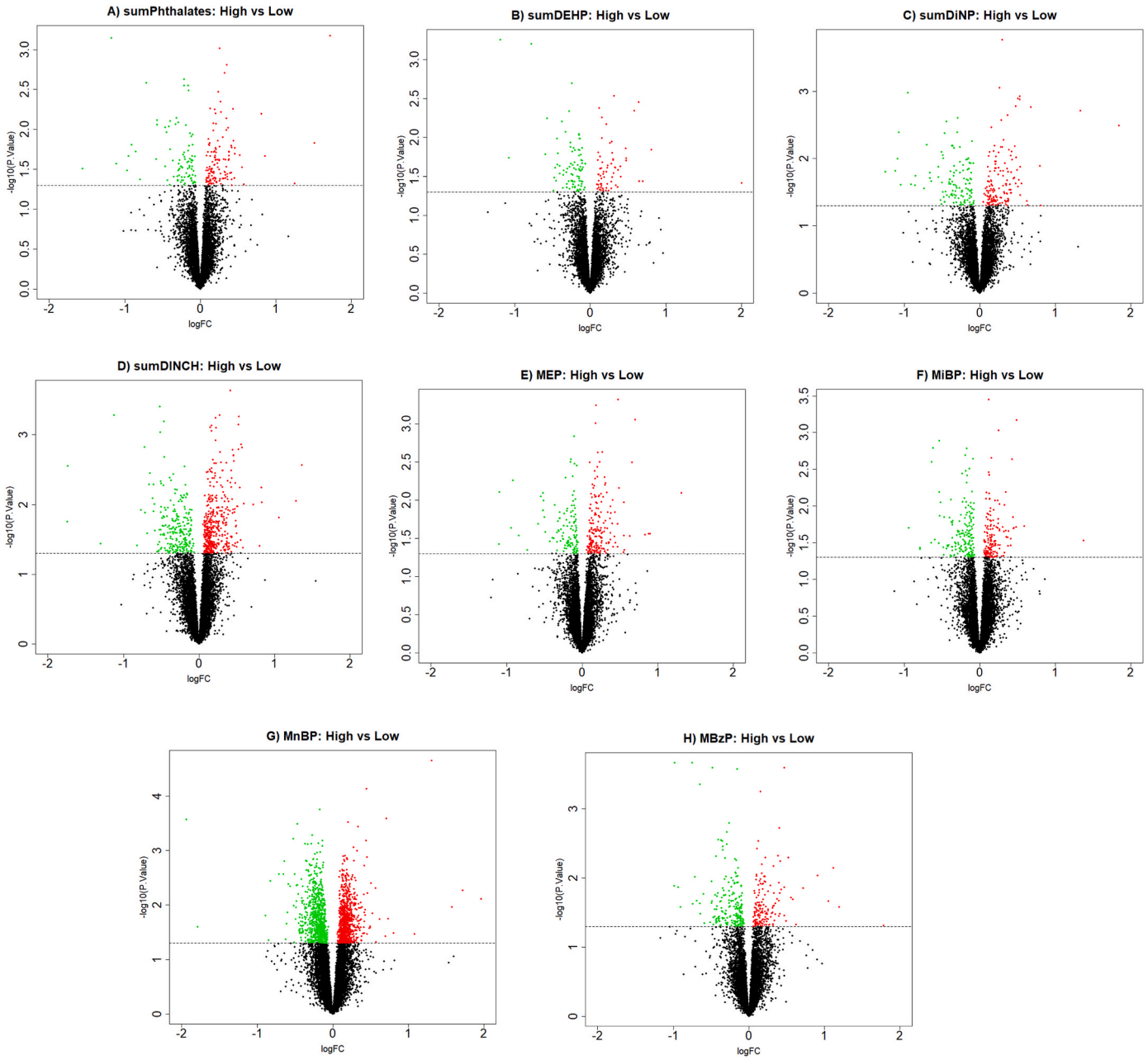
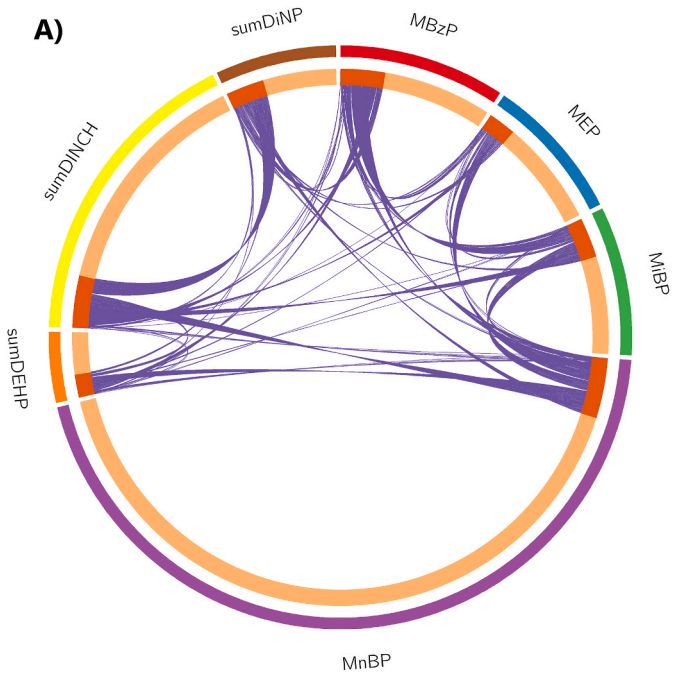


Fig. 3. Volcano plots of statistically significantly differentially expressed genes across high versus low groups. A) sumPhthalates; B) sumDEHP; C) sumDiNP, D) sumDINCH, E) MEP, F) MiBP, G) MnBP, H) MBzP. Marked features are genes with adjusted $p < 0.05$. Downregulated and upregulated genes shown in green and red, respectively.

share the highest fraction of common DEGs (37 genes), followed by DINCH and MnBP (28 genes). Conversely, MEP, MiBP and DEHP showed the least common DEGs (Fig. 4 B).

3.3. Identification of enriched biological pathways

Next, we selected the DEGs with absolute fold change above 1.3 (Table 2) and performed gene ontology (GO) enrichment analysis to explore molecular functions associated with the phthalate DEGs (Fig. 5A, Supplementary Table 2A.). This showed that most of the DEGs in phthalates belong to gene networks associated with general cellular metabolism including developmental processes, biological regulation, and response to stimulus (Fig. 5A). 6 out of 7 phthalates studied, showed enrichment of the GO-term immune system process. sumDINCH, MnBP and MBzP were the compounds with most significant enrichment for this GO term. In addition, MnBP and MBzP were highly co-occurring, and this was not the case for sumDINCH (Fig. 1B). Notably, sumDEHP (DEHP metabolites) was the only phthalate compound not showing enrichment for the immune response GO-term. In addition, viral process was enriched in MnBP, MBzP and sumDINCH. We next performed enriched biological pathway analysis and found four general categories corresponding to: immunological response (9 enriched pathways), general cell metabolism (8 enriched pathways), lipid metabolism (2 enriched pathways) and androgen response (1 enriched pathway) (Fig. 5B, Supplementary Table 2B.). MnBP was the phthalate that showed the greatest number of enriched pathways: neutrophil degranulation, transcriptional regulation by TP53, signaling by interleukins and adaptive immune system were the four most significantly enriched pathways. DINCH was the second phthalate with most significantly enriched pathways being adaptive immune system, signaling by interleukins and apoptosis the



B)

Names	# overlapping genes
MiBP sumDEHP	3
MEP sumDEHP	4
MEP sumDiNP	5
sumDEHP sumDiNP	5
MEP MiBP	6
MiBP sumDiNP	6
sumDEHP sumDINCH	7
MBzP MEP	8
MEP sumDINCH	11
MBzP sumDEHP	11
MBzP sumDINCH	11
MnBP sumDiNP	13
MBzP sumDiNP	14
MnBP sumDEHP	16
MBzP MnBP	17
MiBP sumDINCH	18
MEP MnBP	23
MiBP MnBP	24
MBzP MiBP	26
MnBP sumDINCH	28
sumDINCH sumDiNP	37

Fig. 4. Circos-plot showing how genes from the different phthalates or DINCH input gene lists (i.e., statistically differentially expressed genes) overlap. **A**) Overlapping differentially expressed genes across the different phthalates (including sumDINCH) in high versus low individuals are coded with colour, i.e., the outside arc represents the identity of each gene list, while the inside orange colour arc represents the genes that appear in multiple lists, and the light orange colour represents genes that are unique to that gene list. Purple lines link the same genes shared by the gene lists. The greater the number of purple links and the longer the dark orange arcs imply greater overlap among the input gene lists. **B**) Number of common differentially expressed genes between pairs of phthalates.

three most significantly enriched pathways. Interestingly, phthalates showing pathways enriched in lipid metabolism, including regulation by PPARα, were MnBP, sumDEHP, MiBP and MEP. Furthermore, disease ontology enrichment analysis was performed on the identified DEGs, i.e., DEGs with absolute fold change above 1.3 and FDR <0.1. In Fig. 5C, the top 20 enriched disease ontology terms were presented, and diseases terms related immune system and infection were among the enriched disease ontologies (Fig. 5C, Supplementary Table 2C.).

3.4. Analysis of PPAR signaling pathway genes

We evaluated genes involved in the PPAR signaling pathway and compare the effects of phthalates and DINCH exposure on their expression levels (Fig. 6). We observed statistically significant (both negative and positive) associations between 15 genes involved in the PPAR signaling pathway and the phthalate metabolites levels (Fig. 6). Noticeably, the phthalate with most association with genes involved in the PPAR signaling pathway was MnBP (N = 8 genes) followed by MiBP (N = 4 genes). Interestingly, no association between MEP and PPAR signaling pathway genes was found, and only MMP1 and RORC genes were positively associated with sumDEHP and sumDINCH, respectively.

To evaluate whether the observed changes in the PPAR signaling pathway genes (Fig. 6) were due to concomitant gene expression changes in the PPAR genes, we evaluated the expression levels of *PPARα*, *PPARδ* and *PPARγ* by qPCR analysis. The expression level of *PPARγ* showed a slight decrease between high and low phthalate groups and a non-significant similar trend was observed for *PPARδ* (Fig. 7). No statistically significant differences in gene expression were obtained for PPARα (Fig. 7).

4. Discussion

The Euromix study population showed phthalates levels ranging from 0.05 to 1587.54 ng/ml measured in 24 h urine samples. The phthalate oh-MPHP showed the lowest levels (median = 0.45 ng/ml) while DEHP (median = 61.74 ng/ml) was the one with the highest levels across participants. In general, the median value for the total measured phthalate levels from this cohort (118.9 ng/ml) was slightly lower compared to two recent biomonitoring studies from Czech Republic and Finland (median 160 ng/ml, 168 ng/ml, respectively) (Stuchlík Fišerová et al., 2022) (Porras et al., 2020) suggesting national differences in exposure. In addition, the EuroMix population consist mostly of highly educated participants. Two exceptions from this pattern were DiNP and DEHP which showed higher exposure levels in our cohort; however, as we measured more metabolites of DEHP than the two other reported studies direct comparison is difficult. The EuroMix data (Husøy et al., 2019) is consistent with several biomonitoring studies performed across the world: compared to the global mean, our cohort shows 4–6 times lower exposure levels for MEP, MnBP and MiBP with the exception of DEHP where levels are similar (Wang et al., 2019)[45]. Previously we have reported sources of exposure to DEHP in our study. The main contributors to dietary exposure are diary, grain, fruits and vegetables, meat, and fish. Body lotion contributed most to the exposure of DEHP from personal care products (Husøy et al., 2020).

The immune system is a complex, multicellular network present in numerous tissues across the body. Blood is one of the main conduit channels for most immune cells circulating the body and can reflect immune system status. How phthalates affect gene expression in blood immune cells, especially considering the immunomodulation effects exerted by sex hormones (Ortona et al., 2019; Wick et al., 1993), is less known. Using RNA-seq, we studied gene expression changes in peripheral immune blood cells from individuals with elevated phthalate exposure compared to low exposure. The greatest number of DEGs compared to individuals with low exposure was found in participants with high concentration of DINCH metabolites (126 genes) followed by MnBP (89 genes). Conversely, the lowest number of DEGs was found in individuals with high DEHP levels (25 genes). Notably, individuals with high DINCH levels, a substitute of DEHP, showed five times more DEGs than DEHP (126 vs 25). To gain a more detailed biological understanding, we performed GO analysis of the obtained DEGs. We found

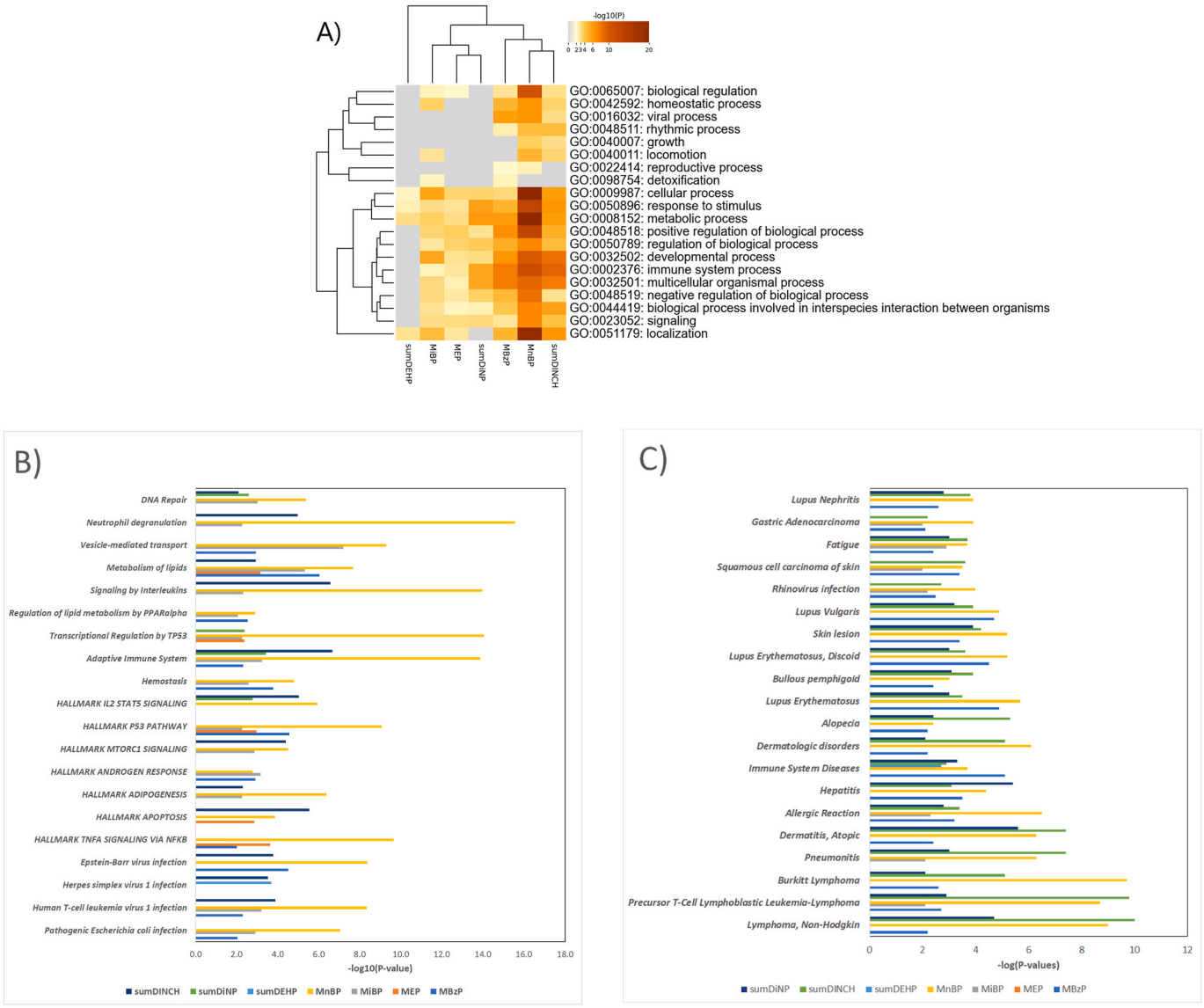


Fig. 5. Functional enrichment analysis of differentially expressed genes. **A**) Metascape enrichment analysis of statistically enriched GO-terms; the heatmap shows the top 20 enriched GO-terms across differentially expressed gene lists. **B**) Metascape enrichment analysis of all statistically enriched biological pathways; the top 20 enriched pathways are presented. Differentially expressed genes (adjusted $p < 0.05$) for each phthalate metabolite including DINCH were used in the Metascape analysis. **C)** Disease ontology enrichment analysis; the top 20 statistically significantly enriched disease terms are presented.

enrichment of multiple GO categories associated with general cellular regulation including 'response to stimulus', 'metabolic process', and 'detoxification' for most phthalate species analyzed. Compounds that showed highly significant enrichment in immune-related pathways including 'immune system process' and 'viral process' were MEP, MBzP, MnBP and DINCH. This suggests that MnBP, DINCH and, to a lesser extent, MBzP and MEP, display greater immune gene regulatory effects than DEHP, DiNP and MiBP. MnBP and MBzP were phthalates that correlated among our participants, and these were not correlated to DINCH. This could suggest that although all these 3 chemicals alter immune responses, as visible in gene expression changes, the mode of action of these two groups could be slightly different. Campioli et al. administered DINCH to rats *in utero* and observed testicular changes including differences in testicular gene markers and macrophage infiltration (Campioli et al., 2017). Similarly, zebrafish treated with DINCH showed alterations of their transcriptional profile (Saad et al., 2021). This, together with our gene expression results, suggests that DINCH shows equal or greater biopotency compared to other chemicals, concerning immunotoxicity effects, than the original plasticizers it was intended to substitute; raising the question whether chemical alternatives are innocuous enough (Qadeer et al., 2022).

PPARs are a protein family of ligand-activated nuclear receptors and paracrine regulators expressed in various cells of the immune system (Christofides et al., 2021; Daynes and Jones, 2002; Glass and Ogawa, 2006). In fact, phthalate metabolites have been shown to interact *in vitro* with PPARγ (Kratochvil et al., 2019) including several of their chemical substitutes (Schaffert et al., 2022) and to activate, in rat liver extracts (Lapinskas et al., 2005) and different cell lines (Hurst and Waxman, 2003), various members of the PPAR nuclear receptor family. We wondered, therefore, whether some of the reported immunotoxicity actions of phthalates (Bølling et al., 2020; Nygaard et al., 2021) might be mediated by modulation of PPAR signaling pathway genes. Notably, we found statistically significant upregulation of the pro-inflammatory gene TNF-α as well as CCL5 and RORC and downregulation of NFKB1, IL-1β, and IL4. MnBP was the phthalate member showing the highest number of associations between high exposure levels and significant up- or down-regulated of PPAR signaling pathway genes (*TNF*, *CCL5*, *NFKB1*, *IL1B*, *ARG1*). TNF-α and IL-1β are potent inflammatory mediators

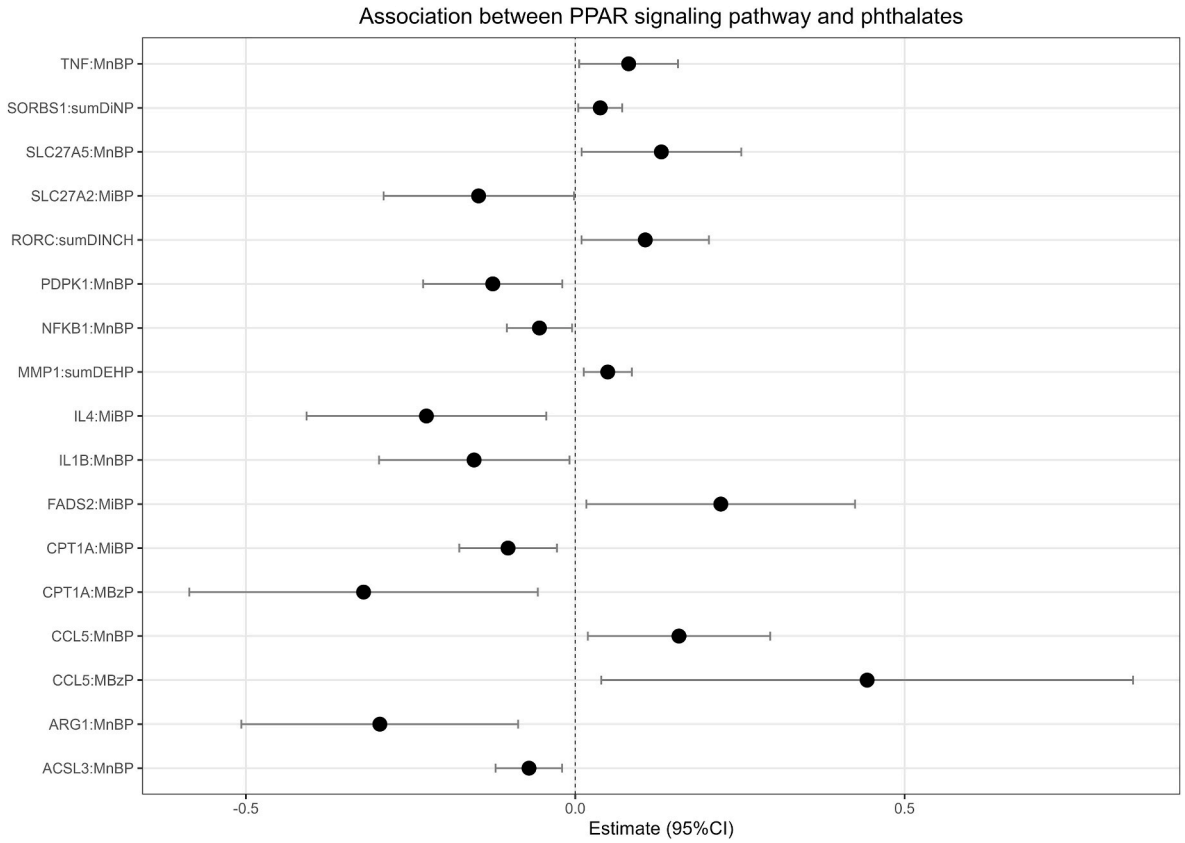


Fig. 6. Association between genes involved in PPAR signaling pathway and phthalates. Linear regression estimates for genes involved in PPAR signaling pathway and phthalates including DINCH. Data are shown as effect estimate (β-coefficient) and 95% confidence intervals as error bars. Analyses were adjusted for sex, age, and BMI.

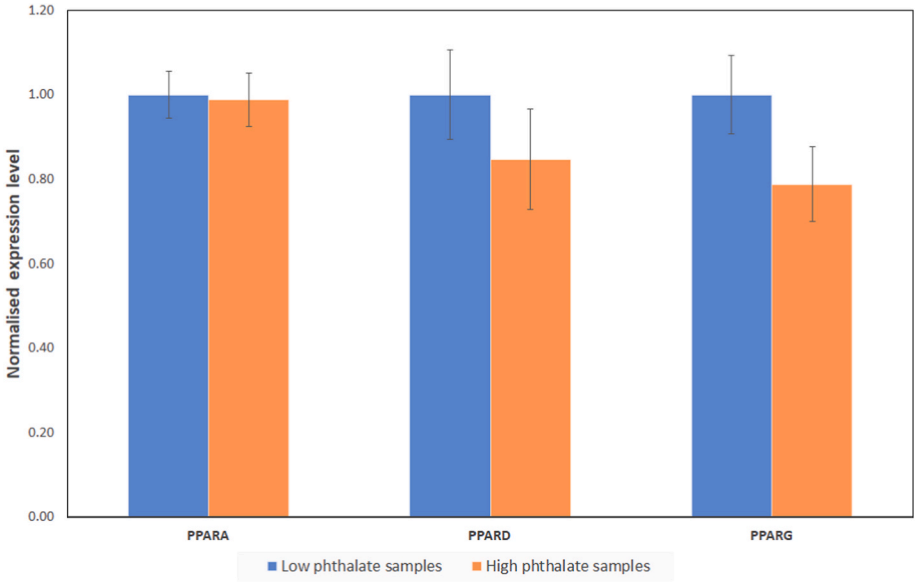


Fig. 7. qPCR analysis of selected genes on high versus low phthalate samples. Values represent average normalized expression level. Error bars represent SEM. $p < 0.05$. $N = 14$.

secreted by macrophages upon infection (Turner et al., 2014). Requena and colleagues explored the associations between phthalates exposure in humans and cytokine levels (Requena et al., 2022). From the 13 different pro-inflammatory cytokines studied, they found the strongest positive correlation between PAI-1, an adipokine, with MiBP and MnBP. They observed moderate negative correlations between MnBP levels and IL-1β and a positive correlation between MnBP and TNF-α, showing similar trends as our results. We observed upregulated expression of CCL5, a chemokine used to recruit leukocytes to sites of infection, in individuals that showed high levels of MzBP in addition to MnBP, and

upregulation of RORC, a lineage specification marker of T helper cells (Th17), in individuals with high exposure to DINCH. We have not found any other study that evaluates immunomodulatory effects of phthalates in blood from humans. Other experimental settings show opposing results regarding DnBP (MnBP) effects on TNF-α and IL-1β gene expression. Some studies found increased levels of IL-1β (Kassab et al., 2019; Teixeira et al., 2016) while other studies (Kim et al., 2015), including ours, found decreased levels. All the previous studies, except one (Kim et al., 2015), observed increased TNF-α, in line with our results. Additional studies have explored the role of phthalates and cytokines in other tissues including brain, testis and placenta (Alsubaie et al., 2021; Kassab et al., 2019; Zhou et al., 2020). Albeit the distinct functional and microenvironmental contexts, these studies observed increased TNF-α and IL-1β levels upon DnBP treatment. Some of the discrepancies across the studies may be explained by the different model systems and experimental designs used, in addition to cell- and context-specific modes of action. Collectively, this data shows differential gene expression associated to phthalate exposure of important modulators of the immune response which belong to the PPAR signaling pathway.

From all the phthalate metabolites analyzed in our study, DINCH had the highest number of differentially expressed genes (126 DEGs) followed by MnBP (89 DEGs). This suggests that DINCH and MnBP may have stronger effects on gene expression in peripheral blood cells. A recent study reported enhanced airway inflammation in mice models of asthma exposed to MnBP (Quoc et al., 2022). A mother-child cohort in China which measured 8 phthalate metabolites in the urine of pregnant women found significant negative associations between MnBP exposure and psychomotor development in 2-year-old children (Yu et al., 2022). This suggests that MnBP, and its parent phthalate (DnBP), display considerable toxic activity across diverse tissues. It would be highly interesting to further elucidate which nuclear receptors do different phthalates interact with highest affinity. This could provide a clearer view and approximation to putative phthalate-dysregulated genes and pathways. In addition, by knowing which tissues express phthalate nuclear receptor interactors we could better predict susceptible organs to phthalate toxicity. Interestingly, the parent molecule of MnBP (DnBP) and MINCH (a metabolite of DINCH) have been shown to interact with PPARγ in preadipocytes (Schaffert et al., 2022). As indicated by our data, MnBP and DINCH metabolites might be putative PPAR interactors in blood immune cells as well, although this should be empirically verified.

In vitro and animal-based toxicity studies typically use significantly higher phthalate exposure levels (100–500 times higher) (Sicińska et al., 2020) compared to the reported environmental concentrations from biomonitoring studies. In addition, experimental studies use more direct routes of compound administration compared to real exposure routes. It is not surprising, therefore, the moderate to mild nature of reported adverse associations in human biomonitoring studies including ours. Despite this, human studies clearly show a trend towards cellular and molecular changes upon phthalate exposure confirming animal and *in vitro* findings. A constant exposure to these family of chemicals throughout all life-stages including those with highest susceptibility (i.e., gestation) might increasingly exacerbate the adverse effects of phthalates in addition to permanently affect the establishment of important organ systems, such as the immune system, as evidenced by the increasing number of allergic and immunomodulatory conditions.

Given that: i) immune cells are present in virtually all tissues of the human body and ii), we and others (Bølling et al., 2020; Nygaard et al., 2021) show associations of phthalate exposure with immune dysregulation, it might be interesting to explore the interplay of tissue-specific immune cells with tissue-resident cells. This might be an underexplored area in the development of organ-specific phthalate toxicity. In this study we explored the expression of genes in peripheral blood immune cells. Blood is an important source in biomonitoring studies for its relatively low invasiveness, high richness of biological information and direct access to immune cells.

While most governmental and environmental agencies focus is directed towards the endocrine disruption activity of common chemicals, such as phthalates, other equally serious health hazards remain considerably understudied. The importance of a well-balanced immune system is clearly exemplified by the wide spectrum of autoimmune diseases affecting all parts of the body including the pancreas (type 1 diabetes), the musculoskeletal system (multiple sclerosis), and the small intestine (inflammatory bowel disease), among others. Although there is no definitive cause to the onset of autoimmune diseases, it is thought that up to 70% of autoimmune diseases are due to environmental factors. Considering the steep rise in allergic, autoimmune, and other chronic conditions of the immune system in the industrialized countries, evaluating the immunotoxicity effects of pollutants merits greater attention, especially in light of hypothesis that point towards the reduction of the epithelial barrier defense system against external factors (Akdis, 2021).

This chemical association study has some limitation that should not be disregarded. The study has small sample size. The EuroMix participants consisted of relatively healthy adults with high educational backgrounds overrepresented with females. These characteristics may alter the outcomes of the analysis, particularly the high educational background which is strongly associated with improved living conditions. Despite this, it was not included as a confounder in the analysis since 95% of the EuroMix population had either university or college education. Although we did not state it explicitly, most of the participants worked at various Norwegian national institutes (including the NIPH) in the day of the collections. The groups with lower education were under similar occupational conditions as the highly educated participants. Therefore, we decided not to stratify by this characteristic in the analysis. Important susceptible stages such as gestation and early-life development, where xenobiotic disturbances can permanently alter physiological mechanisms, could not be captured within the framework of this study. Factors such as phthalate co-exposure were addressed performing a sensitivity analysis on the two most correlated phthalates in this cohort and using sumPhthalates as a proxy for total exposure across all our analysis. However, we cannot rule out totally that co-exposures can further influence the outcome of the models. In addition, in this study we had not enough power to include all co-exposures as covariates. One of the ways of overcoming this problem for future studies is to increase further the sample size. In addition, inter-individual immune cell profile variability was not accounted for in the regression analysis. This could result as a potential confounder if participants show marked immunological profile differences. In addition, the effect size of transcription levels was small and might make result interpretation difficult. Such findings are common in environmental studies; however, it is worth noting that small changes in transcription levels can have biological effects if multiple genes belonging to the same biological pathway are affected. In addition, transcription levels were studied in blood samples which is not the primary effect target of phthalates. While connecting transcriptomic changes with future disease outcome is highly valuable, our data was not collected in a prospective manner, and it would be difficult to connect with later disease onset. However, we have performed a disease ontology enrichment analysis where the top enriched categories are misfunctions related to the immune system (Supplementary Table 2C).

5. Conclusion

Here we performed RNA sequencing from total peripheral blood cells as an initial proof-of-concept into the potential of phthalates to cause genome-wide expression changes. In individuals with high exposure compared to low exposure, we found DINCH metabolites and MnBP showing the highest number of DEGs with FC $\geq$ 1.3 (126 and 89 genes, respectively). DEHP metabolites showed the lowest number of DEGs. All metabolites analyzed except for DEHP showed immune response as enriched GO-term. MnBP was the phthalate metabolite that displayed

the greatest association across almost all the enriched biological pathways including neutrophil degranulation, transcriptional regulation by TP53, signaling by interleukins and adaptive immune system being the four most associated pathways. Adaptive immune system, signaling by interleukins, apoptosis and neutrophil degranulation were the most enriched biological pathways for DINCH metabolites. Finally, we focused on PPAR signaling pathway genes and explored associations between gene expression and phthalate levels. By exploring genome-wide transcriptional changes using RNA sequencing, this study suggests that the chemical substitute, DINCH, and MnBP show the greatest changes in gene expression compared to other common phthalate species. These changes appear to be associated with gene networks involved in general cell metabolism and various immunological-related biological pathways. This is relevant for environmental science and human health since DINCH which is a chemical substitute has comparable effects as the original phthalates regarding gene expression. Expanding the knowledge of phthalates and immunomodulatory effects considering their putative PPAR interactions is highly encouraged.

Credit author statement

Conceived and designed the experiments: GLS, TH, BL, HD, ND. Data analysis and interpretation: GLS, ND. Laboratory analysis: HH, AS, ESK, PB, KK, KM. Writing and reviewing of the manuscript: GLS, TH, BL, HD, MW, KK, ND. Acquiring grant: TH, HD. All authors have read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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.2023.115377.

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