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HBM4EU E-waste study – An untargeted metabolomics approach to characterize metabolic changes during E-waste recycling

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ABSTRACT

E-waste contains hazardous chemicals that may be a direct health risk for workers involved in recycling. We conducted an untargeted metabolomics analysis of urine samples collected from male e-waste processing workers to explore metabolic changes associated with chemical exposures in e-waste recycling in Belgium, Finland, Latvia, Luxembourg, the Netherlands, Poland, and Portugal. Questionnaire data and urine samples were obtained from workers involved in the processing of e-waste (sorting, dismantling, shredding, pre-processing, metal, and non-metal processing), as well as from controls with no known occupational exposure. Pre- and post-shift urine samples were collected and analysed using ultrahigh-performance liquid chromatography-mass spectrometry (UPLC-MS). A total of 32 endogenous urinary metabolites were annotated with a Variable Importance in Projection (VIP) above 2, indicating that e-waste recycling is mainly associated with changes in steroid hormone and neurotransmitter metabolism, energy metabolism, bile acid biosynthesis, and inflammation. The highest VIP was observed for dopamine-o-quinone, which is linked to Parkinson's disease. These and other changes in metabolism in workers employed in the processing of e-waste need further verification in targeted studies.

1. Introduction

The use of electrical and electronic equipment is one of the determinants of widespread global economic development. These devices have become essential in modern societies and improve the standard of living, but after use, they are disposed of and generate a huge stream of waste (e-waste) that contains hazardous chemicals. According to the Waste Electrical and Electronic Equipment Forum, households across the EU have, on average, 74 electronic products, of which 17 are not used [https://www.euronews.com/]. Most of them are small consumer electronics such as cables, external drives, and phones. From old laptops to washing machines, Europeans generate the most e-waste per capita in

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the world. In 2020, approximately 4.7 million tons of e-waste was discarded in the EU. This corresponds to 10.5 kg per capita. E-waste is one of the fastest-growing sources of waste in the EU, and only around 40 % of this waste is recycled [https://www.destatis.de/Europa/EN/Topic/Environment-energy/E_Waste]. Therefore, the proper recycling of e-waste is a huge challenge for the EU. However, this is not an easy task because e-waste contains many potentially harmful materials that not only pollute the environment but may also pose a direct health hazard risk to those involved in recycling. Exposure to hazardous components of e-waste may arise through inhalation, ingestion, and skin contact. However, urine samples were chosen as the monitoring medium in our study, considering their non-invasive nature, ease of collection, and effectiveness in indicating human exposure to harmful substances. The routes of exposure may vary depending on the substance as well as the recycling methods. E-waste components may contain toxic substances such as persistent organic pollutants (brominated flame retardants, polybrominated diphenyl ethers, polychlorinated biphenyls), dioxins (polychlorinated dibenzodioxins and dibenzofurans, dioxin-like polychlorinated biphenyls, perfluroalkyls), polyaromatic hydrocarbons as well as elements (barium, beryllium, chromium or hexavalent chromium, cadmium, cobalt, indium, lead, lithium, mercury, nickel, zinc), (Grant et al., 2013; Julander et al., 2014).

Within the European Human Biomonitoring Initiative (HBM4EU, Ganzleben, 2018), we performed a targeted study to explore the exposure of e-waste workers to various metals and organic compounds (Scheepers et al., 2021). According to initial results, these workers may be exposed to higher levels of hazardous metals, especially lead, cadmium, and mercury, when compared to controls (Leese et al., in preparation). Also, higher exposure to phthalates, some polychlorinated biphenyl compounds, and organophosphate flame retardants was suggested (Cleys et al., 2024; Cseresznye et al, 2024, Duca et al., 2023; https://www.eu-parc.eu/news/). Many studies have examined the adverse health effects of exposure to single substances or mixtures of similar substances. For example, several phthalates have been classified as endocrine disruptors (Schug et al., 2016), and exposure to these chemicals has also been associated with such health outcomes as urothelial (Chou et al., 2021) and thyroid cancers (Liu et al., 2020), cardiovascular disease (Fu et al., 2020), as well as other cardiometabolic disorders (Giuliani et al., 2020), including insulin resistance (Shoshtari-Yeganeh et al., 2019), diabetes mellitus (Zhang et al., 2022), high blood pressure, and higher levels of total cholesterol (Zhang et al., 2018). Polychlorinated biphenyls exposure can compromise cognitive functions, particularly verbal competence, learning, short-term memory (Schantz et al., 2003), and a high concentration of these compounds in brain tissue may potentially disrupt the nigrostriatal dopamine system (Caudle et al., 2006). Organophosphate flame retardants can induce adverse effects in the immune system, and disrupt the metabolism, genome, and endocrine function (Blum et al., 2019; Hou et al., 2016). Further possible adverse effects include cardiovascular, neurological, reproductive and developmental toxicity, as well as cancer (Blum et al., 2019; Doherty et al., 2019; Wang et al., 2021, Zhang et al., 2021).In the case of metals, their harmful health effects are related to the induction of DNA damage, protein modification, and lipid peroxidation, among others (Fu and Xi, 2020). In particular, depending on metals, their toxicity is related to damage to such organs as the brain, lungs, and kidneys, as well as to degenerative processes that may lead to the development of diseases such as multiple sclerosis, Parkinson's disease, Alzheimer's disease, and muscular dystrophy. Repeated, long-term exposure to some metals may cause cancer (Järup, 2003). In addition to those substances already identified and shown to result in occupational exposure during the recycling process, there might be many other less well-known substances that may be released or formed during the management of e-waste. (Robinson, 2009; Noel-Brune et al., 2013). Thus, e-waste workers are exposed to a mixture of metals and other chemicals and the net effects of exposure are not the simple effects of the sum of exposure to each individual chemical compound. Therefore, untargeted analyses can illustrate the final effect of exposure to a mixture of these various substances, and identification of effect markers may constitute the basis for further targeted studies and clinical trials in the aspect of early diagnostic and prevention of irreversible organ damage in e-waste workers.

Summing up, although the toxicity of the original components might be known, workers are likely to be exposed to complex mixtures with unknown health effects. This study aimed to explore the potential metabolic changes associated with e-waste recycling through an untargeted metabolomics analysis of urine samples obtained from a cohort of male workers from that sector and controls.

2. Materials and methods

2.1. Study population and data collection

The HBM4EU Occupational Biomonitoring Study on e-Waste included workers and controls from the majority of participating countries, as follows: Belgium, Finland, Latvia, Luxembourg, the Netherlands, Poland, and Portugal. Detailed study protocol was developed and applied in these European countries to ensure standardized data collection including questionnaire data and urine sampling (Scheepers et al., 2021). Questionnaire data and urine samples were obtained from a cohort of male workers (W) employed by companies involved in the processing of e-waste, including sorting, dismantling, shredding, and pre-processing for metal as well as non-metal components. The controls consisted of two groups: persons working in the same companies but with tasks not involving direct contact with e-waste (within company controls, WCC) and persons working in other jobs outside e-waste processing companies (outwith company controls, OCC), and were recruited from the same geographical region as the exposed workers. All workers and a part of the controls collected urine samples before starting the work week (pre-shift urine samples) and at the end of the work weekend of the shift (post-shift urine samples). Regarding the controls, the standard operation procedure specifies that only one urine sample should be collected. These samples were collected in most countries as pre- and/or post-shift spot samples. For a deeper analysis of control urine samples, we separated these groups for pre- and post-shift WCC. The number of women in the studied groups was very small (OCC n = 9; W pre-shift n = 9; W post-shift n = 9; WCC pre-shift n = 8; WCC post-shift n = 8), which carried the risk of drawing incorrect conclusions from metabolomics studies., and therefore these analyses were only performed in samples from male participants. All urine samples were collected between March 2021 and February 2022 and stored at −80 °C until analysis.

2.2. Urine sample preparation and metabolomics analysis

Formic acid (Chem-LAB NV, Zedelgem, Belgium), acetonitrile, and methanol (Avantor Performance Materials Poland S.A., Gliwice, Poland) were LC-MS grade. Ammonium formate (99.99 % purity) was sourced from Sigma-Aldrich (St. Louis, MO, USA). Internal standards (Benzoyl-D5, 98 %, and L-phenylalanine 3,3-D2, 98 %) were acquired from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). An R5 UV Hydrolab system (Wislina, Poland) was used to produce ultra-high-purity water. Following the manufacturer's instructions (Waters, UK), sodium formate (calibration solution) and leucine enkephalin (lock mass) were prepared.

Urine samples were randomized and divided into four batches. Samples were prepared using the latest protocol (Southam et al., 2020). Two extraction protocols were used: for nonpolar/semipolar (Assay 1) and polar (Assay 2) metabolites. The extraction procedure for nonpolar and semipolar metabolites was as follows: 300 µL of solvent consisting of ice-cold water and methanol (50:50 v/v) with internal standards (benzoyl-D5 and L-phenylalanine 3,3-D2) were added to 100 µL of urine thawed at 4 °C in ThermoMixerC (Eppendorf, Hamburg, Germany),

vortexed for 2 min, and centrifuged at 20,879 rpm for 20 min at 4 °C. The extraction procedure for polar metabolites was similar, except for the solvents, which were acetonitrile and methanol (50:50 v/v), with the same internal standards. Quality control (QC) samples were prepared by combining equal volumes of aliquots (100 μL) from every urine sampleand used to monitor system stability (injected every ten analysed samples). More detailed information on sample preparation and analysis is available in our previous study (Kozlowska et al., 2022).

Urine samples were analysed using a Waters AcquityTM Ultra Performance LC system (Waters Corp., Milford, MA, USA) connected to a Synapt G2Si Q-TOF mass spectrometer (Waters MS Technologies, Manchester, UK) equipped with an electrospray (ESI) source (Waters, Manchester, UK). In Assay 1, semi-polar and non-polar metabolites were separated using an ACQUITY UPLC HSS T3 column (1.8 μm, 2.1 × 100 mm) coupled with an ACQUITY UPLC HSS T3 VanGuard pre-column (1.8 um, 2.1 × 5 mm) from Waters (Milford, MA, USA). An injection volume of 2 μL was used for ESI + and ESI−, with separation conducted at a flow rate of 0.3 mL/min and a temperature of 40 °C. The mobile phases comprised 0.1 % formic acid in water (A) and acetonitrile (B). In both ion modes, the gradient elution program was set as follows: 0.0 – 0.5 min, 1 % B; 2.0 min, 10 % B; 4.0 min, 20 % B; 5.0 min, 30 % B; 6.0 min, 50 % B; 8.0 min, 99 % B; 11.0 min, 99 % B; 11.5 min 1 % B; 15.0 min 1 % B.

In Assay 2, polar metabolites were separated using an ACQUITY UPLC BEH Amide column (1.7 μm, 2.1 × 100 mm) with a VanGuard pre-column (1.7 μm, 2.1 × 5 mm) from Waters (Milford, MA, USA). An injection volume of 2 μL was used for ESI + and ESI−, with separation conducted at a flow rate of 0.5 mL/min and a temperature of 35 °C. The mobile phases consisted of acetonitrile and water in distinct ratios: A was 95 % acetonitrile and 5 % water, B was 50 % acetonitrile and 50 % water. Both phases contained 10 mM ammonium formate and 0.1 % formic acid. In both ion modes, the gradient elution program was set as follows: 0.0 – 1.0 min 1 % B; 3.0 min, 15 % B; 6.0 min, 50 % B; 7.5 min, 95 % B; 8.5 min, 99 %B; 10.0 min, 99 % B; 10.5 min, 1 % B; 13.0 min, 1 % B.

MS centroid, high-resolution mode was used for the Assays 1 and 2 analyses. The MS settings included a scan time of 0.3 sec, desolvation gas flow of 900 L/h at 350 °C, cone gas flow of 50 L/h, and source temperature of 120 °C. In Assay 1, the capillary voltages were set to 3.2 kV in ESI + and 2.4 kV in ESI−, while in Assay 2, they were set to 3.5 kV in ESI + and 2.7 kV in ESI − . To ensure accuracy and reproducibility, all data were collected using a lock mass system with leucine enkephalin, featuring a scan time of 0.5 s, an interval of 15 s, and a mass window of ± 0.5 Da. The obtained LC/MS data files were uploaded to the Progenesis QI v3.0 software application (Waters, Milford, MA, USA). Feature detection, retention time correction, alignment, and putative annotation of compound classes were performed using the default parameter settings for UPLC – High Resolution (Waters), achieving level 3 annotation according to the minimum reporting standards defined by the Metabolomics Standards Initiative (Sumner et al., 2007). For putative annotation of compounds, the Urine Metabolite Data Base was used (Bouatra et al., 2013). Following data acquisition, the raw intensity data from Progenesis QI v3.0 underwent a filtering process. Metabolite features were discarded based on criteria including a blank contribution greater than 5 %, missing values exceeding 50 % (across the entire dataset), and a QC relative standard deviation (RSD) surpassing 35 % (within each batch), (Evans et al., 2020). Signal drift correction, batch effect elimination, and normalization were performed on the remaining data using the MetaboGroupS software application (https://www.omicsolution.com/wukong/MetaboGroupS/) (Wang et al., 2018). Missing values in the data were imputed with the k-nearest Neighbor (KNN) algorithm within MetaboGroupS software, followed by log2 transformation to correct data skewness. Data normalization was performed using seven different methods. Among these, the EigenMS normalization method had the lowest coefficients of variation across all experiments datasets, and thus, this method was selected (Wang et al., 2018). These data were used for statistical and chemometric analyses (see section below).

The Fast Data Dependent Acquisition method was used to fragment significant compounds following statistical analysis. The chromatographic and spectrometer parameters, except the collision energy, were maintained as in the MS mode. The resulting fragmentation spectra were matched against those available in the Human Metabolome Database (Wishart et al., 2018) for level 2 putative annotation of compounds.

2.3. Statistical and chemometric analysis

The normality of the frequency distribution was checked using the Shapiro-Wilk test. Next, samples were analysed using the Student's *t*-test or Mann-Whitney *U* test, depending on the frequency distribution. For all analyses, p-values < 0.05 were considered statistically significant.

Principal components analysis (PCA) was performed to explore and visualize trends in collected samples. The results of PCA (performed with all detected features) were also analysed to check how the grouping of samples is affected by features related to individual variabilities like smoking pattern, alcohol consumption, age, body mass index (BMI), or related sample variability including country and urine sample collection time (3.00 a.m.–9.00 a.m.; 9.00 a.m.–12.00p.m.; after 12.00p.m.) All obtained plots are collected in supplementary materials (Fig. S1-S7). The above-mentioned PCA analyses were performed using self-prepared R scripts and RStudio software (R version 3.60) with mixOmics and ggplot2 packages (Lê Cao et al., 2009).

Next, the obtained data matrix was loaded into the Urine Metabolite Data Base (Bouatra et al., 2013), which contains detailed information about 3100 small metabolites found in human urine, to select metabolites that can be detected in this kind of specimen. After that, the obtained data matrix was analysed using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/).

First, two multivariable methods, unsupervised PCA and supervised partial least-squares discriminant analysis (PLS-DA), were applied to select the most significant metabolites differentiating analysed groups (worker pre-shift; worker post-shift, within company control, and outwith company control). Briefly, PLS-DA can be classified as a supervised version of PCA since PLS-DA uses information about the class labels during dimensionality reduction (Ruiz-Perez et al., 2020). The main differences between PCA and PLS-DA concern how the principal components are calculated. In PCA, PC1 presented the maximum possible variance in the original data, whereas, in PLS-DA, PC1 refers to the maximum covariance between the original data and its labeling. Based on the Variable Importance for Projection (VIP) value, the most critical unknown metabolites were selected for further identification.

Putatively annotated metabolites with VIP > 2 were also analysed by the 'Biomarker Analysis' module in MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) for the selection of biomarker panel. A classical univariate analysis was performed to generate the receiver operating characteristic (ROC) curve and calculate the area under the curve (AUC) as well as their 95 % confidence intervals (CI). The analysis of variance (ANOVA) and post-hoc Fisher test was used to check the impact of such factors as smoking status and time of sample collection on the signal intensity of analysed metabolites.

3. Results

3.1. General characteristics of the study population

Table 1 shows the number of male controls and workers involved in the processing of e-waste recruited from each country. The pre-shift and post-shift urine samples were planned to be collected from each exposed individual, but in some cases, workers did not consent or forgot to take them, e.g., the pre-shift sample, which is why the numbers do not always match.

In the area of general characteristics, no significant differences in

Table 1
Number of male controls and workers involved in the processing of e-waste recruited from each country.

Country/groups	OCC (n)	WCC (n)		E-waste workers (n)	
		Pre-shift	Post-shift	Pre-shift	Post-shift
Male workers (n)	**20**	**23**	**16**	**85**	**128**
Belgium	–	7	–	–	39
Finland	–	–	2	11	13
Luxembourg	5	1	–	9	7
Latvia	4	–	–	11	11
The Netherlands	–	13	13	27	27
Poland	–	–	3	–	8
Portugal	11	–	–	27	23

Abbreviations: OCC = outwith company controls; WCC = within company controls.

age, height, body mass, or BMI were observed in the examined groups of men. Additional information about smoking status and consumption of alcohol in the analysed groups of men is presented in Table 2.

3.2. Urine metabolic changes in the analysed groups of men

In addition to examining the influences of exposure on the metabolomic profiles, we used PCA plots to verify whether the division of groups is the effect of other factors. We checked the subsequent qualitative factors: the country of employment, smoking, alcohol consumption, age, BMI, and sample collection time. Results are summarized in supplementary materials as Figs. S1-S7. Based on the PCA results, we assume that almost all investigated factors do not significantly affect metabolomic profiles since no trends (between those factors and grouping patterns) were observed. The only two factors differentiating the examined groups of men were the time of urine sample collection and smoking status. In W (post-shift), 81.1 % of urine samples were collected after 12p.m. In the remaining groups, urine samples were collected mainly until 12p.m.

Table 2
General characteristics of the male workers involved in the processing of e-waste and included in the control groups (values are presented as mean ± SD or as percentage).

Parameters	OCC	WCC		E-waste workers	
		Pre-shift	Post-shift	Pre-shift	Post-shift
Male workers (n)	**20**	**23**	**16**	**85**	**128**
Age	41.6 ± 9.3	42.9 ± 9.9	41.8 ± 10.2	45.0 ± 10.4	43.0 ± 9.6
Height (cm)	178.2 ± 6.4	179.7 ± 8.9	181.6 ± 7.2	177.2 ± 6.3	176.8 ± 6.8
Body mass (kg)	83.2 ± 9.3	88.0 ± 17.5	88.4 ± 19.3	87.3 ± 13.8	83.9 ± 13.9
BMI (kg/m^2)	26.5 ± 3.0	27.3 ± 5.2	26.8 ± 5.9	27.9 ± 4.6	26.8 ± 4.3
Smoking status*					
No n (%)	11 (55.00)	18 (78.26)	11 (68.75)	37 (43.53)	60 (46.88)
Yes n (%)	6 (30.00)	2 (8.70)	3 (18.75)	37 (43.53)	50 (39.06)
Former smoker n (%)	3 (15.00)	3 (13.04)	2 (12.50)	11 (12.94)	18 (14.06)
Alcohol use**					
No n (%)	3 (15.00)	8 (34.78)	6 (37.50)	17 (20.0)	53 (41.41)
Yes, weekly use n (%)	11 (55.00)	9 (39.13)	5 (31.25)	32 (37.65)	42 (32.81)
Yes, monthly use n (%)	4 (20.00)	6 (26.09)	5 (31.25)	22 (25.88)	20 (15.63)
Yes, daily use n (%)	2 (10.00)	–	–	14 (16.47)	13 (10.16)

Abbreviations: BMI – body mass index; OCC = outwith company controls; WCC = within company controls * – value of Chi-square test in relation to smoking status, p = 0.0856; ** – value of Chi-square test in relation to alcohol consumption, p = 0.0309.

The results of PCA, both on the whole data matrix (Fig. 1S) and data selected based on the Urine Metabolite Data Base (Fig. 1A), indicated that while analyzing the male workers, unsupervised methods could distinguish four groups: W (pre-shift), W (post-shift), OCC and WCC (pre- and post-shift are mixed).

Using a supervised PLS-DA, five exposure groups were separated (Fig. 1B). Therefore, the list of metabolites with the highest value (≥2) of variable importance in the projection (VIP) was used for further identification of metabolites.

In general, putatively annotated metabolites with a VIP value above 2 can be divided into three groups: endogenous metabolites, whose changes may be the result of exposures during e-waste processing, metabolites of nicotine, and exogenous metabolites, mainly as food ingredients. The group of endogenous metabolites (n = 32) include metabolites belonging to the following metabolic pathways: hormones metabolism (11-hydroxyandrosterone, 6-dehydrotestosterone glucuronide, 2-methoxy-estradiol-17b 3-glucuronide, tetrahydrodeoxycortisol), primary bile acid biosynthesis (cholic acid glucuronide, cholic acid, 1b,3a,12a-trihydroxy-5b-cholanoic acid), fatty acid metabolism (phenylacetylglycine, capryloylglycine, 7-octenoylglycine, phenylpropionylglycine, isobutyrylglycine, hexanoylglycine, N-butyrylglycine), tryptophan metabolism (indoleacetic acid, 5-hydroxyindoleacetaldehyde, tryptophol), tyrosine metabolism (p-cresol glucuronide, vanillactic acid, *cis*-4-hydroxycyclohexylacetic acid, vanylglycol, norepinephrine sulfate, 3-methoxytyrosine, dopamine-o-quinone), metabolism of remaining amino acids (N-formyl-L-methionine, cystathionine ketamine, pyroglutamic acid, N6,N6,N6-trimethyl-L-lysine, 5-aminolevulinic acid, dimethylguanidino valeric acid), arachidonic acid metabolism (20-carboxy-leukotriene B4), and biotin metabolism (pimelic acid). Normalized signal intensities come from these metabolites, and VIP values are presented in Fig. 2, and additionally, the supplementary material contains box plots representing the distribution of the signal intensities in the analysed groups (Figs. S8–S39).

The second group of metabolites belongs to the pathway of nicotine metabolism: nicotine, cotinine, *trans*-3-hydroxycotinine glucuronide, nicotine-1′-N-oxide, nornicotine, and hydroxycotinine (Fig. 3). The signal intensity coming from these six metabolites was significantly higher in W (pre-shift) vs. WCC (pre-shift) as well as in W (post-shift) vs. WCC (post-shift) – in all cases, $p < 0.0000$. The third group of metabolites consists of 53 exogenous metabolites, which are mainly food ingredients. The list and characteristics of all putatively annotated metabolites are included in Table S1.

Taking into account the fact that factors such as smoking status and time of sample collection may influence the signal intensity of metabolites, an analysis of variance (ANOVA) was done. This analysis has been performed in pre-shift WCC because, in this group, sampling was conducted in 3 periods of time, and this group had a potentially small risk of exposure. In OCC, sample collection was only in 2 time periods, and therefore, in this group, these statistical analyses were not conducted. Time of sampling influenced only the signal intensity of 20-carboxy-leukotriene B4 (signal intensity in period 2 was significantly lower than in period 1, $p = 0.0233$, and in period 3, $p = 0.0363$) and isobutyrylglycine (signal intensity in period 2 was significantly lower than in period 1; $p = 0.0200$). In case of differences connected with smoking status, signal intensity was significantly higher in smokers vs. non-smokers only in 5-Hydroxyindoleacetaldehyde ($p = 0.0036$) and Dimethylguanidino valeric acid ($p = 0.0079$). Results of the ANOVA test in connection to smoking status and period of urine sampling are in Supplementary Material 1.

All the above-mentioned endogenous 32 metabolites (with VIP > 2) were assessed as potential biomarkers of exposure to e-waste as a comparison W (post-shift) vs. OCC. Classical univariate receiver operating characteristic (ROC) curve analysis was performed to generate

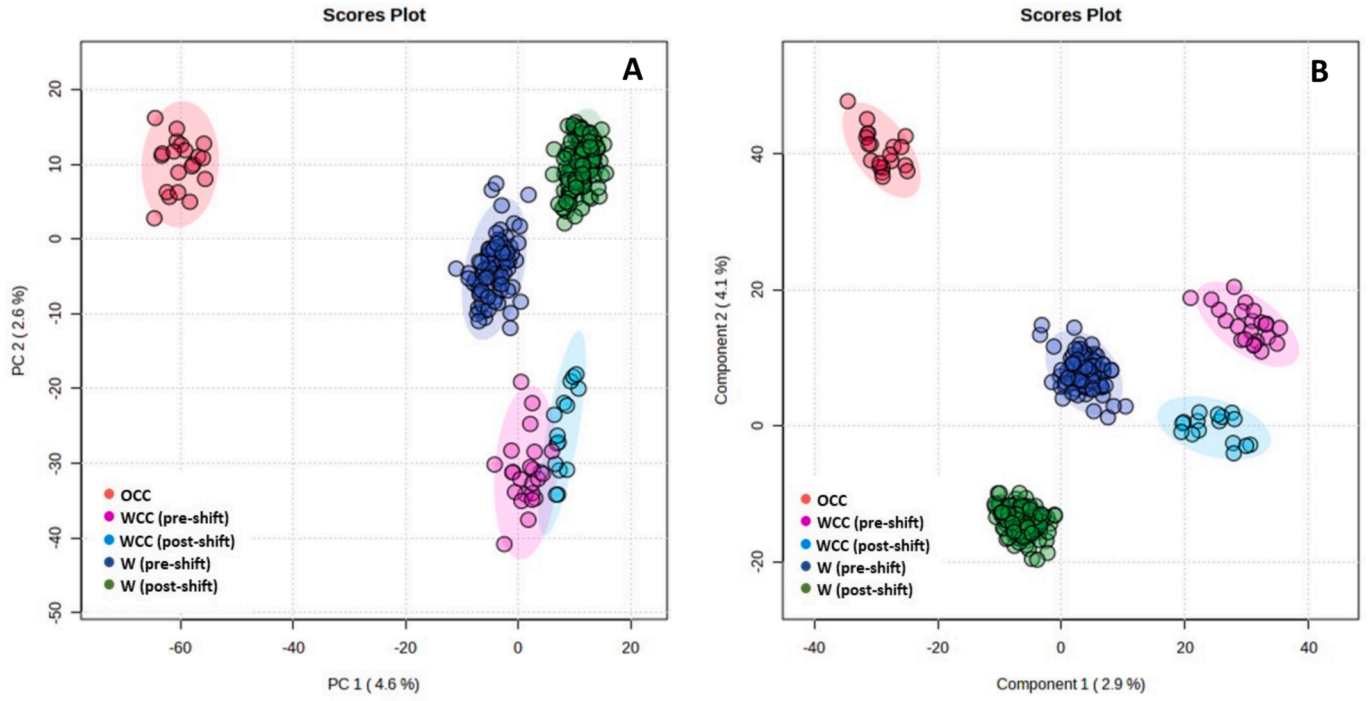

Fig. 1. A – Scatter plot representing the projection of two first principal components of PCA of metabolomics profiles of the investigated groups of men. B – Scatter plot representing the projection of two first principal components of PLS-DA of metabolomics profiles of the investigated groups of men. Abbreviations: OCC = outwith company controls; WCC = within company controls; W = workers.

ROC curves and calculate the AUC curves. Among investigated metabolites, similar predictive power with an AUC above 0.9 was related to the following compounds: 20-carboxy-leukotriene B4, indoleacetic acid, 3-methoxytyrosine, dopamine-o-quinone, phenylacetylglycine and N6, N6,N6-trimethyl-L-lysine (Fig. 4).

4. Discussion

To the best of our knowledge, this is the first untargeted metabolomics study in which the effect of working in e-waste processing was analysed in pre- and post-shift urine samples. The study cohort came from Belgium, Finland, Latvia, Luxembourg, the Netherlands, Poland, and Portugal within the HBM4EU Occupational Biomonitoring Study on e-Waste management and included male workers employed by companies involved in the processing of e-waste, within company controls (from the same company but with no known exposure to industrial recycling of e-waste) and outwith company controls. The same study protocol was applied in these seven European countries to ensure standardized data collection.

Annotated metabolites, in which significant differences were observed between the analysed groups of men, can be divided into two groups: exogenous metabolites, which come from the nicotine metabolism pathway, and compounds which are mainly food ingredients, as well as endogenous metabolites, which may be the result of work-related exposure in e-waste processing. In the case of a large group of exogenous metabolites with VIP values above 2, their significant differences in the signal intensity could be due to the fact that in W (post-shift), more than 80 percent of urine samples were collected after 12:00p.m., which is most likely a few hours after meal consumption.

In W (pre-shift) and W (post-shift), the percentage of cigarette smokers was higher than in the groups of controls. Tobacco smoke is a mixture containing an estimated 5,000 chemicals, and 98 of them are on the list of hazardous smoke components. This list includes components that are known as human carcinogens (IARC Group I carcinogens), that are probably carcinogenic to humans (IARC Group 2A carcinogens), and that are possibly carcinogenic to humans (IARC Group 2B carcinogens). Some of the chemicals included in this list are hydrogen cyanide, formaldehyde, ammonia, radioactive elements, benzene, carbon monoxide, tobacco-specific nitrosamines (TSNAs), polycyclic aromatic hydrocarbons (PAHs), as well as arsenic, cadmium, lead, Cr(VI), and Hg (Talhout et al., 2011). Nicotine, among others, acts on nicotinic cholinergic receptors and causes the release of neurotransmitters. (Okorare et al., 2023). Bearing in mind that in W (pre- and post-shift) vs. WCC (pre- and post-shift) signal intensities from six metabolites belonging to the nicotine metabolism pathway were significantly higher, it should be noted that the observed effect of e-waste exposure may also be influenced by a contribution from smoking.

Endogenous compounds, whose signal intensity was significantly different in the analysed groups of participants, belong to the following pathways: metabolism of hormones, fatty acids, arachidonic acid, biotin, tryptophan, tyrosine, and the remaining amino acids, as well as bile acid biosynthesis.

In the area of hormone metabolism, significant changes were observed among such steroid hormones as C18 steroids – estrogens contain 18 carbons (2-methoxy-estradiol-17beta 3-glucuronide), C19 steroids [11-hydroxyandrosterone belonging to 17-ketosteroids and 6-dehydrotestosterone glucuronide) and C21 Steroids (tetrahydrodeoxycortisol – a mineralocorticoid, the main urinary metabolite of 11-deoxycortisol belonging to 17-hydroxycorticosteroids). An important class of C19 steroid hormones are androgens, which control normal male development and reproductive function. The main circulating androgen is testosterone, which is produced in the Leydig cells of the testis and can also act as a pro-hormone after being metabolized to dihydrotestosterone (DHT) or estradiol. The alternative pathway for DHT synthesis can be done without going through testosterone, via androsterone (McEwan and Brinkmann, 2000). In our study, we observed higher intensity signals coming from 11-Hydroxyandrosterone (a metabolite of androsterone) in W and WCC in comparison to OCC, but signal intensity coming from the testosterone metabolite (6-dehydrotestosterone glucuronide) was significantly higher only in WCC (post-shift) vs. OCC.

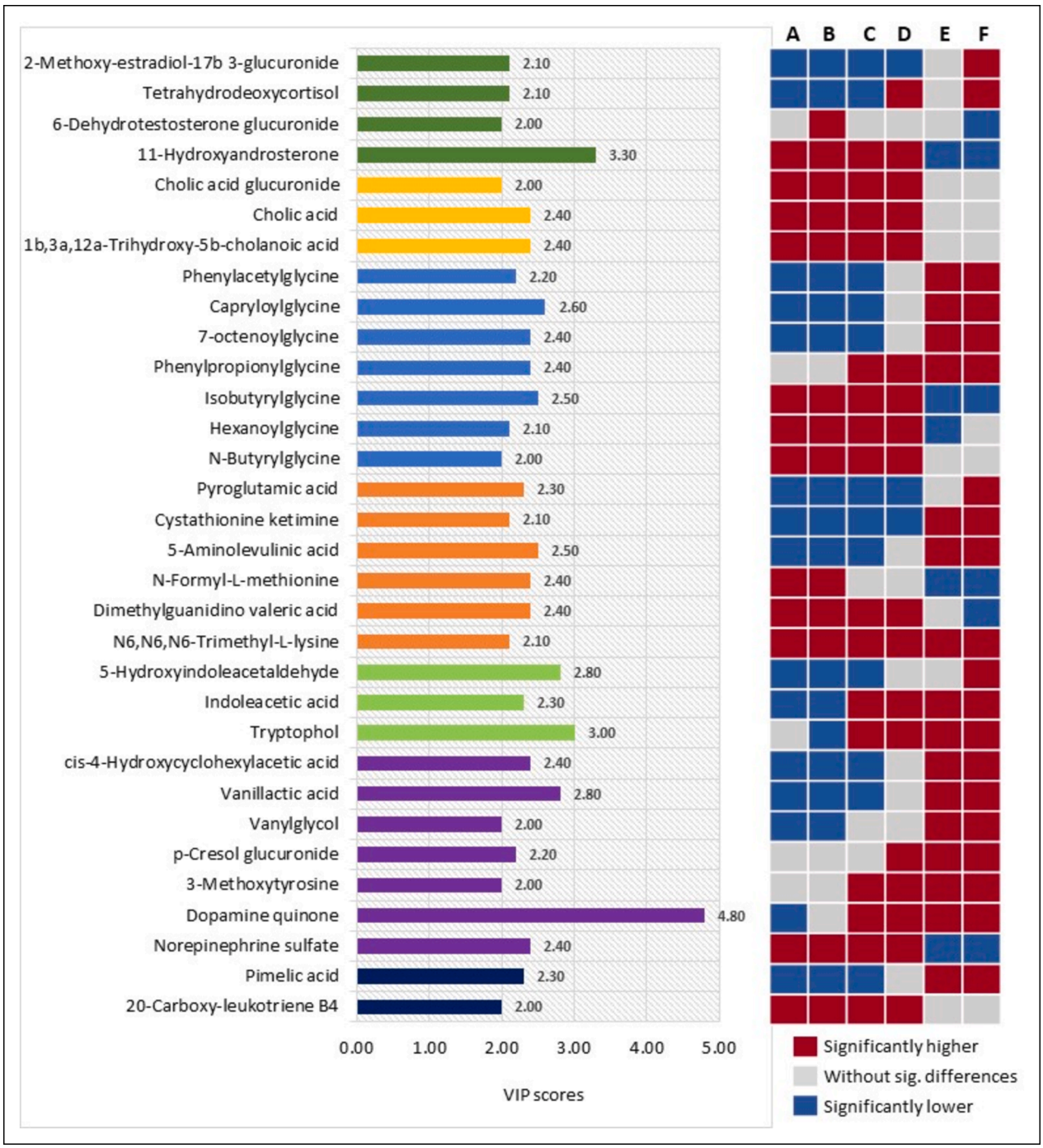

Fig. 2. Variable importance in projection (VIP) plot displays the top 32 most important metabolites identified by PLS-DA. VIP is a weighted sum of squares of the PLS-DA loadings considering the amount of explained Y-variable in each dimension. Colored boxes on the right indicate differences between analysed groups of men. Abbreviations: A – differences between WCC (pre-shift) vs. OCC; B – differences between WCC (post-shift) vs. OCC; C – differences between W (pre-shift) vs. OCC; D – differences between W (post-shift) vs. OCC; E – differences between W (pre-shift) vs. WCC (pre-shift); F – differences between W (post-shift) vs. WCC (post-shift) OCC – outwith company controls; WCC (pre-shift) – pre-shift within company; WCC (post-shift) – post-shift within company controls; W (pre-shift) – pre-shift workers; W (post-shift) – post-shift workers.

On the other hand, in relation to metabolites of C19 and C21 steroids, signal intensities in W and WCC were mainly lower than in OCC. These changes in steroid hormones can be connected with heavy metals exposure (Bhardwaj et al., 2021), other chemical compounds associated with e-waste such as polychlorobiphenyls used earlier as flame retardants (D'Errico et al., 2016), polybrominated diphenyl ethers (PBDEs) (Singh et al., 2022) and many other factors, either endogenous or related to lifestyle (Sarkola and Eriksson, 2003; Waring et al., 2008). Among them, cigarette smoking has been shown to increase plasma levels of cortisol and androgens (Kapoor and Jones, 2005). Exposure to Cd, Pb, and Hg is known to affect fertility (Massányi et al., 2020). Numerous studies on male model organisms describe the effects of heavy metal exposure that are connected with apoptosis in testicular tissue (exposure to Cd, Hg, and Pb), (Benoff, 2000, Telišman et al., 2007, Bjørklund et al., 2019), a decrease of testosterone and other androgen's levels (exposure to Pb and Hg), (Reshma Anjum et al., 2011; Tan et al.,

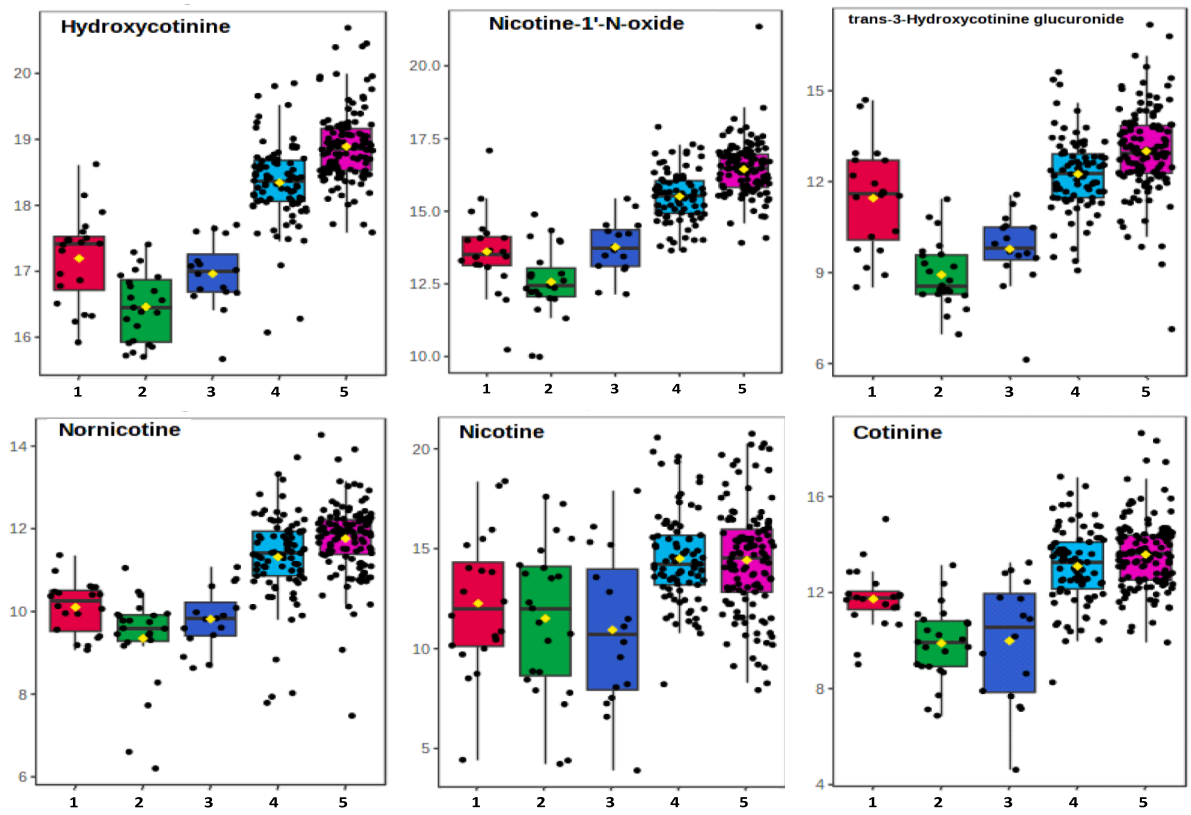

Fig. 3. Box plot representing the distribution of the normalised signal intensities come from hydroxycotinine, nicotine-1′-N-oxide, *trans*-3-hydroxycotinine glucuronide, nornicotine, nicotine, and cotinine. The boxes represent the interquartile range (difference between the upper 75% and lower quartile 25%); the thick black lines, the median. Abbreviations: 1 – outwith company controls; 2 – pre-shift within company controls; 3 – post-shift within company controls; 4 – pre-shift workers; 5 – post-shift workers.

2009) as well as decreased level of estrogen, luteinizing hormone, and follicle-stimulating hormone (Pb), (Huang et al., 2017, Igharo et al., 2018). In workers occupationally exposed to polychlorobiphenyls, the effect of smoking habits on the excretion of urinary steroids was analysed. Both the urinary concentrations of the total 17-ketosteroids and some single steroids, as well as their glucuronidated compounds, were significantly lower in the exposed workers than in controls, but higher in smokers than in the group of non-smokers and ex-smokers. Also, in the e-waste exposed population, the effect of PBDEs related to increased testosterone levels in men, but significant results reported for estradiol was inconsistent in men across studies, which could not be explained by differences in exposure levels (Singh et al., 2022).

The second group of metabolites with significant differences in signal intensity are acylglycines, normally minor metabolites of fatty acids that are produced through the action of glycine N-acyltransferase which catalyzes the reaction between acyl-CoA and glycine. Alterations in oxidative phosphorylation relate to the impairment of the mitochondrial β-oxidation of fatty acids, the accumulation of non-oxidized acyl-CoA esters, and the activation of alternative pathways of oxidation such as the synthesis of acylglycines (Adeva-Andany et al., 2019). Changes in signal intensity coming from acylglicines may related to Hg, Pb, Cd, and other chemicals associated with e-waste exposure. In the human embryonic kidney epithelial cell line (HEK-293 T) treated with inorganic mercury, the mitochondrial disorder of HEK-293 T cells was observed, which was mediated by downregulating the expression of silent information regulator two ortholog 1 and peroxisome proliferator-activated receptor coactivator-1α signaling pathway (Han et al., 2022). Pb can raise reactive oxygen levels and the accumulation of Ca ions within cells, which, in turn, leads to a decrease in mitochondrial potential and apoptosis (Moreira et al., 2001). Moreover, Pb can inhibit mitochondrial oxidative phosphorylation, thereby changing the D-aminolevulinic acid dehydratase activity (Kasperczyk et al. 2012). In our study, we also observed significant changes in the urinary 5-aminolevulinic acid signal intensity. Studies performed in animal models have found that Cd exposure also has an impact on mitochondrial energy metabolism, tricarboxylic acid cycle, amino acid metabolism, as well as on intestinal flora metabolism, bile acid metabolism, etc. (Chen et al. 2018; Hu et al. 2018).

In our study, we also found dysregulated diverse metabolic pathways connected with amino acid metabolism, particularly cysteine and methionine, glutamate, arginine, and lysine. A methylated form of lysine is detected in the current study as N6,N6,N6-trimethyl-L-lysine – a precursor of carnitine and a coenzyme in mitochondrial fatty acid oxidation. Mitochondrial 6-N-trimethyllysine dioxygenase converts 6-N-trimethyllysine to 3-hydroxy-6-N-trimethyllysine as the first step for carnitine biosynthesis (Vaz and Wanders, 2002). In addition, the downregulation of mitochondrial β-oxidation and the changes in signal intensity coming from the same amino acids may also be related to the observed changes in pimelic acid. A higher-intensity signal coming from this amino acid was detected in W (pre-shift) vs. WCC (pre-shift) and in W (post-shift) vs. WCC (post-shift). Pimelic acid is needed for the synthesis of biotin, which is important in fatty acid synthesis, branched-chain amino acid catabolism, and gluconeogenesis. Elevated urinary excretion of pimelic acid was detected in mitochondrial beta-oxidation disorders and peroxisomal beta-oxidation disorders (Bennett et al., 1992).

Our findings suggested that e-waste exposure was associated with significantly increased urinary excretion of bile acids (cholic acids and 1b,3a,12a-Trihydroxy-5b-cholanoic acid) in W and WCC vs. OCC. Bile acids are the final products of cholesterol catabolism and play an important role in the regulation of hepatic glucose and lipid metabolism, energy homeostasis, and the development of liver diseases (Chiang and

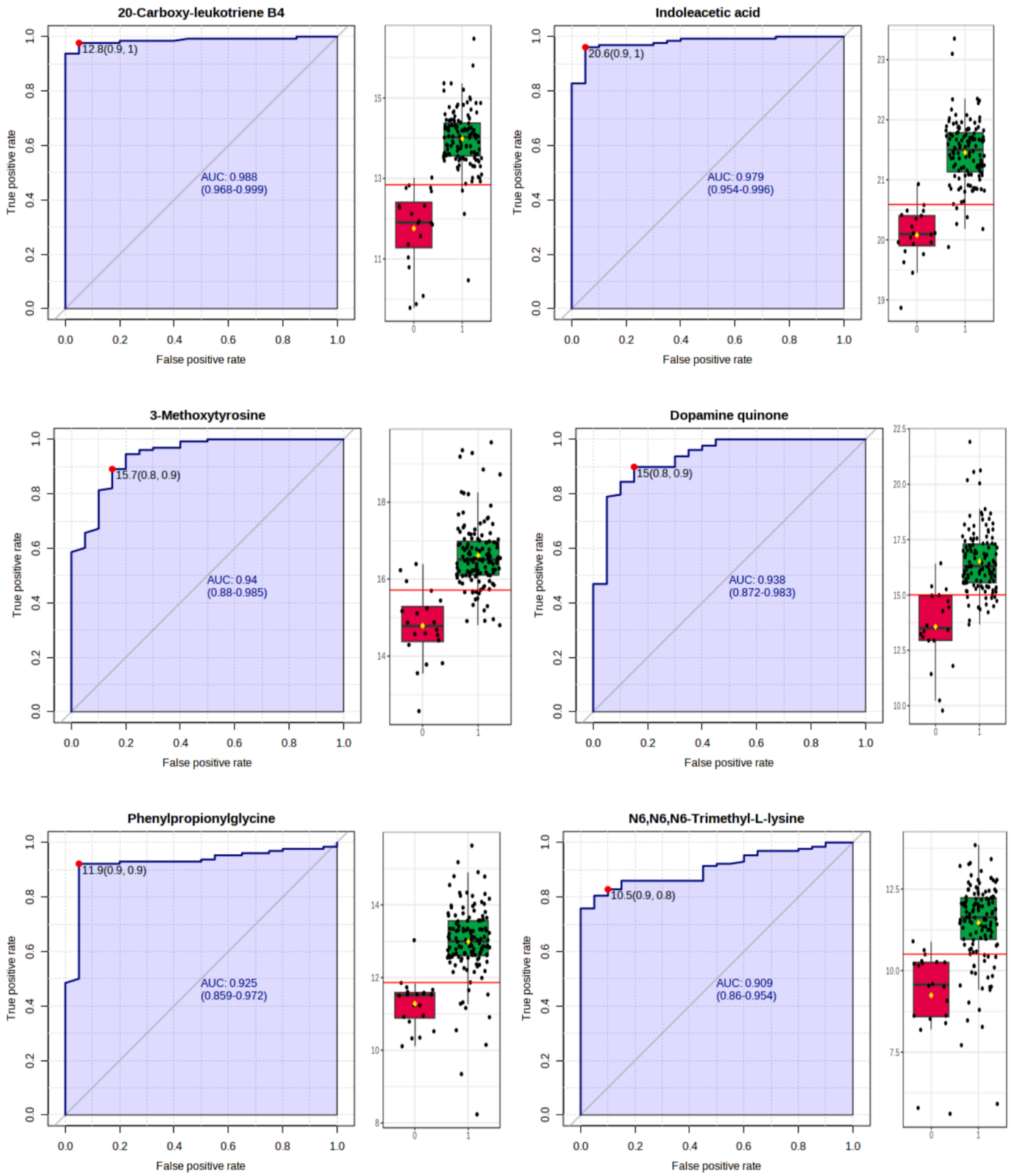

Fig. 4. The receiver operating curve (ROC curve) for the seven metabolites biomarker panel with the highest area under the curve value (AUC value) and boxplots of signal intensities come from these metabolites in outwith company controls (red box) and in post-shift workers (green box). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Ferrell, 2019; McGlone and Bloom, 2019). Bile acid toxicity is connected with membrane destruction (Davis et al., 2002) via binding with the cell membrane components, the inflammatory processes, and the destruction of cell membrane integrity, and is subsequently linked to the lysis of hepatocytes (Cao et al., 2017; Perez and Briz, 2009). As bile acid concentrations increased, binding to the membranes also increased. Studies in Zebrafish and rat models have shown that heavy metals, such as arsenic (As), manganese (Mn), and Cd, can cause liver injury by interfering with bile acid homeostasis (Li et al., 2016; Zhu et al., 2020). The study involved 3589 adults from the National Health and Nutrition Examination Survey was shown that exposures to Cd, uranium (U), and barium (Ba) were individually associated with multiple markers of liver injury as well as the total metal mixture exposure was positively correlated with these markers and Cd, U, and Ba were the main contributors to the combined effects (Tang et al., 2023). Therefore, observed by us the effect of high-intensity signals coming from bile acid may be connected to mixed metal exposure.

Metabolites belonging to the tyrosine and tryptophan pathways are another large group of metabolites whose signal intensity differed significantly between the studied groups of controls and workers. In our study, we have found that dopamine-o-quinone and 3-methoxytyrosine in W (pre- and post-shift) were significantly higher than in OCC. Dopamine is a member of the catecholamine family of neurotransmitters in the brain and is a precursor to adrenaline and noradrenaline. Dopamine is synthesized mainly by nervous tissue and the adrenal glands. The first step of synthesis is the hydration of the tyrosine to L-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase, followed by the decarboxylation of L-DOPA by aromatic-L-amino-acid decarboxylase (AADC) to dopamine, which is immediately transported into synaptic vesicles (Nagatsu and Stjärnet 1997; Asanuma et al., 2003). Free dopamine remaining in the cytoplasm can lead to toxicity, because it can be easily oxidized to dopamine-o-quinone, leading to oxidative stress and damage connected with protein-quinone modifications (Monzani et al. 2019; Sulzer et al. 2000) and consequently leading to mitochondrial and lysosomal dysfunction (Burbulla et al. 2017; Segura-Aguilar et al., 2014). Dopamine-o-quinone urinary excretion was consistently higher in workers (in both pre-shift and post-shift urine) compared to both control groups (WCC and OCC). With a VIP of 4.8 (on a scale of 0–5) this was the most prominent among the 32 metabolite changes observed (Fig. 3). The reactive oxygen or nitrogen species generated in the enzymatical oxidation or auto-oxidation of an excess amount of dopamine induce neuronal damage and/or cell death (Asunama et al., 2003). Dopamine oxidation is suggested to be an important link between mitochondrial and lysosomal dysfunction in the pathogenesis of Parkinson's disease (Aravindan et al. 2024). Furthermore, AADC deficiency leads to an increase in 3-methyloxytyrosine due to the alternative process of L-DOPA methylation by catechol O-methyl-transferase. AADC catalyses the decarboxylation reactions of L-DOPA to dopamine and 5-hydroxytryptophan to serotonin, and therefore AADC deficiency is also connected with reduced levels of dopamine and serotonin substrates: homovanillic acid and 5-hydroxyindoleacetic acid, respectively (Atwal et al., 2015). In our study, in WCC (pre-shift), WCC (post-shift), and W (pre-shift) vs. OCC, we observed a significantly lower signal intensity coming from 5-hydroxyindoleacetaldehyde and vanillactic acid. 5-hydroxyindoleacetaldehyde is an intermediate product of serotonin degradation to 5-hydroxyindoleacetic acid (Atwal et al., 2015). Vanillactic acid is an acidic catecholamine metabolite present in normal human urine and has also been linked to metabolic disorders, including AADC deficiency (Bräutigam et al., 2002).

The higher intensity signal observed coming from indoleacetic acid, tryptophol, and p-cresol glucuronide in W vs. OCC also may have a link to heavy metal exposure. Previous studies reported that exposure to toxic heavy metals such as Pb, Cd, and As affects the composition of the gut microbiota (Eggers et al., 2019; Li et al., 2019; Sitarik et al., 2020; Zeng et al., 2022). Indoleacetic acid and tryptophol are metabolites of tryptophan, and p-cresol glucuronide is a metabolite of tyrosine, which are all produced by the mammalian gut microbiota. Higher levels of indoleacetic acid are associated with bacteria from Clostridium species, including *C. stricklandii*, *C. lituseburense*, *C. subterminale*, and *C. putrefaciens* (Finegold et al., 2002). Tryptophol is a *Saccharomyces cerevisiae* metabolite (Roager and Licht, 2018), and p-cresol is mainly generated by anaerobic intestinal bacteria (Liabeuf et al., 2013).

In the studied groups of controls and workers, differences in signal intensity of norepinephrine sulfate and vanylglycol were also detected. Vanylglycol is synthesized from epinephrine and norepinephrine and is used to measure catecholamine turnover. Catecholamines play an important role in platelet activation and aggregation as well as can regulate the immune response by stimulating the synthesis and release of stress-induced proinflammatory cytokines (Johnson et al., 2005). The increase in signal intensity coming from vanylglycol and norepinephrine sulfate suggests that e-waste exposure may stimulate neuroendocrine responses and might be associated with increased inflammation. The increase in urinary vanylglycol concentration was also observed following exposure to fine particulate matter (PM2.5) coming from air pollution (Chen et al., 2019). Moreover, exposure to per- and polyfluoroalkyl substances (PFAS) was also associated with plasma vanylglycol concentrations (Goodrich et al., 2023).

The proinflammatory effect of e-waste exposure may also be associated to the leukotriene B4 (LTB4) signalling pathway. Both in W and WCC signal intensity coming from 20-carboxy-leukotriene B4 was significantly higher than in OCC. LTB4 is a metabolite of arachidonic acid and is the major metabolite in neutrophil polymorphonuclear leukocytes. LTB4 plays a prominent role in immunity through the induction of phagocyte recruitment, the release of antimicrobial effectors, and the ingestion and killing of pathogens. In humans, LTB4 has a short half-life and is rapidly metabolized into 20-oxo and 20-carboxy derivatives (20-oxo-leukotriene and 20-carboxy-leukotriene B4). These LTB4 metabolites bind to the BLT_1 receptor with high affinity, but they activate neutrophils to a much lesser degree than LTB4, and it was also shown that they dampen neutrophil functions connected with migration, degranulation, and leukotriene biosynthesis (Archambault et al., 2019). In cell culture studies, the LTB4 signalling pathway was consistently upregulated by Cr(VI), clearly demonstrating that this pathway is involved in Cr(VI)-induced inflammation (Kouokam et al., 2022). Also, in workers occupationally exposed to Cr(VI) vs. controls, a higher-intensity signal from metabolites of leukotriene B4 (12-keto-tetrahydro-leukotriene B4; 12-oxo-10,11-dihydro-20-COOH-LTB4; 20-carboxy-leukotriene B4) and leukotriene E4 (20-oxo-leukotriene E4) was observed (Kozlowska et al., 2022). It has been reported that the proinflammatory and pro-oxidative effects of Cd exposure on neutrophils and macrophages may also be connected with the LTB4 signaling pathway (Hossein-Khannazer et al., 2020).

The observed significant differences in the signal intensity of metabolites occurred not only in the group of e-waste workers vs. OCC but also in many cases in WCC vs. OCC. In our targeted study of e-waste workers within HBM4EU there were no significant differences in levels of measured contaminants (lead, cadmium, mercury, phthalates, some polychlorinated biphenyl compounds, and organophosphate flame retardants) in WCC vs. OCC (Cleys et al., 2024; Csereszny et al., 2024, Duca et al., 2023; https://www.eu-parc.eu/news/; Leese et al., in preparation) but in the PCA and PL-SDA models (Fig. 1) these groups are separated. These results suggest that people performing other tasks in these companies may be at risk of exposure to various other groups of contaminants. This aspect is worth attention in future research, and some preventive action may be needed.

Taking into consideration the six generated biomarkers with an AUC above 0.9, it can be assumed that in the analysed group of workers employed by companies involved in the processing of e-waste, the main metabolic changes were connected with inflammatory processes (high-intensity signal coming from 20-carboxy-leukotriene B4), disturbance in the metabolism of dopamine (3-methoxytyrosine, dopamine-o-quinone), changes in the composition of the gut microbiota

(indoleacetic acid), alterations in oxidative phosphorylation connected with the impairment of mitochondrial β-oxidation and the activation of alternative pathways such as the synthesis of acyl-glycines (phenylacetylglycine), as well as changes related to the synthesis of a precursor of carnitine and a coenzyme involved in mitochondrial fatty acid oxidation (N6,N6,N6-trimethyl-L-lysine).

Our study has several strengths, such as modern analytical techniques and a collection of urine samples in 7 countries, which gives a cross-section of the working conditions and, consequently also metabolic changes in this group of workers in the EU. Additionally, standardized procedures, e.g., for worker recruitment, sample collection, and transport, allowed for the elimination of many confounding factors. Nevertheless, this study has three main limitations. The first limitation is the small group of recruited women, which did not support strong conclusions regarding changes in metabolism and, therefore, these results are not presented. We planned to collect pre- and post-shift samples from all exposed workers, but this has not always been successful. Because collecting biological material is difficult, we anticipated that recruitment might not meet targets. Observed effects of exposure can also be dependent on the intake of food with higher contamination and dietary nutrients such as antioxidants or dietary fiber. Finally, we could not control for every possible lifestyle factor, and the observational nature of this design leaves the possibility of residual confounding factors. Despite these limitations, our study contributes valuable data on metabolic changes in e-waste workers and lays the groundwork for future targeted studies on the long-term health impacts of e-waste exposure.

5. Conclusions

We observed significant changes in urinary metabolites associated with e-waste exposure in a cohort of male workers employed by companies involved in the processing of e-waste. These changes in metabolite excretion suggested that e-waste exposure may be associated with disturbances in steroid hormone and neurotransmitter metabolism, energy metabolism, bile acid biosynthesis, and inflammation. The most prominent and consistent finding was an increased excretion of dopamine-o-quinone in the workers when compared to the controls. Dopamine oxidation links mitochondrial and lysosomal dysfunction in the pathogenesis of Parkinson's disease. These and other changes in the metabolism of workers employed in the processing of e-waste need further verification in targeted studies. In addition, a comprehensive approach is needed to improve occupational health and safety in this sector, including raising awareness among employers and workers, targeting guidelines for risk management, and biomonitoring programs for exposed workers.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and the study protocol was submitted to the Institutional Review Board (or Ethics Committee) in the participating countries.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

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CRediT authorship contribution statement

Lucyna Kozlowska: Writing – review & editing, Writing – original draft, Visualization, Resources, Methodology, Investigation, Formal analysis, Data curation. **Susana Viegas:** Writing – review & editing, Resources, Investigation, Conceptualization. **Paul T.J. Scheepers:** Writing – review & editing, Investigation, Conceptualization. **Radu C. Duca:** Writing – review & editing, Resources, Investigation. **Lode Godderis:** Writing – review & editing, Resources, Investigation. **Carla Martins:** Writing – review & editing, Resources, Investigation. **Krzesimir Ciura:** Software, Formal analysis. **Karolina Jagiello:** Software, Formal analysis. **Maria João Silva:** Writing – review & editing, Resources. **Selma Mahiout:** Writing – review & editing, Investigation. **Inese Mārtiņsone:** Investigation. **Linda Matisāne:** Writing – review & editing, Investigation. **An van Nieuwenhuyse:** Resources, Writing – review & editing. **Tomasz Puzyn:** Software, Formal analysis. **Monika Sijko-Szpanska:** Writing – review & editing. **Jelle Verdonck:** Writing – review & editing, Investigation. **Tiina Santonen:** Writing – review & editing, Resources, Project administration, Data curation, Conceptualization.

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.

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Affiliations of these authors can be found in Supplementary material S3.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2025.109281.

Data availability

Data will be made available on request.

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