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Epidemiology

A urinary metabolomics study of a Polish subpopulation environmentally exposed to arsenic

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ABSTRACT

Background: Almost every organ in the human body can be affected by arsenic (As) exposure associated with various industrial processes, as well as with contaminated food, drinking water and polluted air. Much is known about high exposure to inorganic As but there is little data on the metabolic changes connected to a low exposure e.g. in people living in smelter areas.

Objectives: The objectives of the study were: (1) characterise urinary concentration of total arsenic (AsT) in Polish inhabitants of the vicinity of a copper smelter area, (2) speciation analysis of various forms of arsenic in girls (GL), boys (BL), women (WL) and men (ML) with a slightly elevated AsT concentration and age/sex matched groups with a substantially higher AsT concentration, (GH, BH, WH and MH – respectively), (3) comparison of metabolomics profiles of urine between the age/sex matched people with low and high AsT concentrations.

Methods: Urine samples were analysed for total arsenic and its chemical forms (As^{III}; As^{V}, methylarsonic acid, dimethylarsinic acid, arsenobetaine) using HPLC-ICP-MS. Untargeted metabolomics analysis of the urine samples was performed using UPLC system connected to Q-TOF-MS equipped with an electrospray source. The XCMS Online program was applied for feature detection, retention time correction, alignment, statistics, annotation and identification. Potentially identified compounds were fragmented and resulting spectra were compared to the spectra in the Human Metabolome Database.

Results: Urine concentration of AsT was, as follows: GL 16.40 ± 0.83; GH 115.23 ± 50.52; BL 16.48 ± 0.83; BH 95.00 ± 50.03; WL 16.93 ± 1.21; WH 170.13 ± 96.47; ML 16.91 ± 1.20; MH 151.71 ± 84.31 μg/l and percentage of arsenobetaine in AsT was, as follows: GL 65.5 ± 13.8%, GH 87.2 ± 4.7%, BL 59.8 ± 12.5%, BH 90.5 ± 2.4%, WL 50.8 ± 14.1%, WH 90.4 ± 3.5%, ML 53.3 ± 10.0%, MH 74.6 ± 20.2%.

In the people with low and high AsT concentrations there were significant differences in the intensity of signal (is.) from numerous compounds being metabolites of neurotransmitters, nicotine and hormones transformation (serotonin in the girls and women; catecholamines in the girls, boys and women; mineralocorticoids and glucocorticoids in the boys, androgens in the women and men and nicotine in the boys, women and men). These changes might have been associated with higher is. from metabolites of leucine, tryptophan, purine degradation (in the GH, WH), urea cycle (in the WH and MH), glycolysis (in the WH) and with lower is. from metabolites of tricarboxylic acid cycle (in the BH) in comparison with low AsT matched groups. In the MH vs. ML higher is. from metabolite of lipid peroxidation (4-hydroxy-2-nonenal) was observed. Additionally, the presence of

Abbreviations: AsT, total arsenic; iAs^{III}, inorganic As^{III}; iAs^{V}, inorganic As^{V}; MMA, methylarsonic acid; DMA, dimethylarsinic acid; AsB, arsenobetaine; iMD, sum of inorganic As and its methylated form; low AsT, people with a slightly elevated concentration of AsT; high AsT, people with a substantially higher concentration of AsT; GL, girls with slightly elevated concentration of AsT; BL, boys with slightly elevated concentration of AsT; WL, women with slightly elevated concentration of AsT; ML, men with slightly elevated concentration of AsT; GH, girls with a substantially higher concentration of AsT; BH, boys with a substantially higher concentration of AsT; WH, women with a substantially higher concentration of AsT; MH, men with a substantially higher concentration of AsT; BMI, body mass index; DOWN, direction of change ↓; ESI, electrospray ionization; FC, fold change; Fit(%), score that indicates the percentage of peaks in the library MS/MS spectrum found in the query spectrum; HMDB, Human Metabolome Database; s.i., signal intensity; LOD, limits of detection; *m/z*, mas-to-charge ratio; MET, Metabolic Equivalent of Work; QC, quality control sample; RT, retention time; s/d, number of serving per day; TCA, tricarboxylic acid cycle; TPA, total physical activities; UP, direction of change ↑

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significant differences was reported in is. from food components metabolites, which might have modulated the negative effects of As (vitamin C in the girls, boys and men, vitamin B_6 in the girls, boys and women as well as phenolic compounds in the boys and girls). We hypothesize that the observed higher is. from metabolites of sulphate (in MH) and glucoronate degradation (in BH, WH and MH) than in the matched low AsT groups may be related to the impaired glucuronidation and sulfonation and higher is. from catecholamines, nicotine and hormones.

Conclusion: Our results indicated that even a low exposure to As is associated with metabolic changes and that urine metabolomics studies could be a good tool to reflect their wide spectrum connected to specific environmental exposure to As, e.g. in smelter areas.

1. Introduction

Human exposure to arsenic can occur due to the presence of various industrial processes, as well as intake of contaminated food, drinking water and polluted air [1]. Arsenic exists in many different organic and inorganic forms [2]. For example fish contain a high amount of organic arsenic compounds, especially arsenobetaine (AsB), while rice and drinking water in some regions of the world contain predominantly inorganic arsenic [3,4]. AsB is generally considered to be low-toxic in comparison to the inorganic forms of arsenic [5]. Taking into consideration complexity of the problem related to the presence of various sources of exposure to different arsenic forms, it is extremely difficult to assess health risk in a given population.

So far two works investigating urinary metabolic changes after As exposure have been published [6,7]. In a Chinese adult male cohort with high consumption of rice five potential biomarkers related to arsenic exposure (i.e., testosterone, guanine, hippurate, acetyl-*N*-formyl-5-methoxykynurenamine, and serine) have been identified. Also in pregnant women exposed to a low-dose As, nine urine potential biomarkers have been putatively identified (LysoPC (14:0), glutathione, 18-carboxydinor-LTE4, 20-COOH-LTE4, cystathionine ketimin, 1-(beta-d-ribofuranosyl)-1,4-dihydronicotinamide, thiocysteine, p-cresol glucuronide and vanillactic acid).

We hypothesized that Polish citizens living in the vicinity of a copper smelter had a high urinary concentration of total arsenic (AsT) and a specific profile of its species such as: inorganic As^{III} (iAs^{III}), inorganic As^{V} (iAs^{V}), methylarsonic acid (MMA), dimethylarsinic acid (DMA), and AsB. Young as well as adult people with a slightly elevated concentration of AsT (low AsT) in the urine as well as sex and age matched groups of people with a substantially higher concentration of AsT (high AsT) were qualified for the metabolomics studies. The aim of the study was to look for differences in the metabolite profile between people with low and high AsT concentrations. Results from metabolomic studies binding arsenic concentration with metabolic changes in groups of children and adults living in the vicinity of a copper smelter area has not been published before.

2. Material and methods

2.1. Site description and the study population

Questionnaire data and urine samples were obtained from a large cohort of 2000 inhabitants of the vicinity of a copper smelter. In the study group determination of total arsenic concentration in the urine was performed. In the case of individuals with a AsT concentration above 15 μg/L, speciation studies of arsenic forms were carried out and of this group in total 149 individuals were qualified for the metabolomics study: 74 individuals with the lowest AsT concentration (13 girls - GL, 16 boys - BL, 25 women - WL and 20 men - ML) and 75 individuals with the highest AsT concentration (16 girls - GH, 12 boys - BH, 25 women - WH, 22 men - MH). Data on the study subjects' age, height, body weight and general characteristics (health status, selected habits, duration of residence in copper smelter area) were also collected. Additionally, the study subjects filled in questionnaires on the frequency of food products consumption during the last month (Food Frequency Questionnaire) and International Physical Activity Questionnaire - IPAQ (in a short, lasting 7 days self-administered format) [8]. The collected information on the amount of time spent on vigorous, moderate physical activities as well as on walking was multiplied by a relevant value of the Metabolic Equivalent of Work - MET (8, 4 and 3.3 - respectively). The value of MET-min/week index was obtained by multiplying MET index attributed to a given activity by the number of days it was performed a week as well as its duration in minutes per day. Data on the consumption of products during the last month were calculated into the mean frequency of consumption on a day (number of servings per day - s/d). All the participants were informed about the aim of the study and they provided their informed consent. The study was approved by the Ethics Committee of the Nofer Institute of Occupational Medicine in Lodz, Poland (Nr. 10/2017).

2.2. Urine collection and determination of total As and its species

The urine samples were collected between September and December 2017. The metabolomics studies and determination of AsT concentration and its species were performed using the same urine samples. Urine samples (for speciation analysis) after collection were stored in −20 °C without additives and were determined in 4–5 weeks after collection. Samples for metabolomics analysis were stored in 80 °C and determined after 7 month. Urinary arsenic species such as: iAs^{III}, iAs^{V}, MMA, DMA and AsB were measured. The sum of inorganic As as well as its methylated form (iMD) was calculated as iAs^{III} + iAs^{V} + MMA + DMA. The limits of detection (LOD) were, respectively: AsT - 0.026; As^{III} - 0.045; As^{V} - 0.11; MMA - 0.12; DMA - 0.07 and AsB - 0.027 μg/L. ELAN DRC-e ICP-MS with a Dynamic Reaction Cell (Perkin Elmer, SCIEX, USA) was used for arsenic determination. The instrument Series 200 HPLC (Perkin Elmer, SCIEX, USA) was applied to separate arsenic chemical forms. An Anion Exchange, Hamilton PRP-X100 column (4.1 mm i.d. × 250 mm × 10 μm) was used under the following conditions: 5 mM (NH_4NO_3) ammonium nitrate/5 mM (NH_4H2PO_4) (dibasic), flow rate 1.5 ml/min, injection volume 100 μl. Certified reference material SRM 2669 (human urine) from the National Institute of Standard and Technology (NIST) were examined at the beginning and at the end of the analysis. The detailed methodology concerning analysis of urinary arsenic concentration was published in the paper of Janasik et al. [9].

2.3. Metabolomics analysis

Chemicals applied: formic acid LC–MS grade Sigma-Aldrich (St. Louis, MO, USA), acetonitrile and isopropanol UHPLC-MS grade ChemSolve™S.Witko (Lodz, Poland). Ultra-high purity water was prepared by the R5 UV Hydrolab system (Wislina, Poland). Sodium formate calibration solution and leucine encephalin lock mass solution (Waters, UK) were prepared according to the manufacturer's specifications.

Prior to UPLC-MS analysis, the urine samples were diluted with prechilled (4 °C) ultra high purity water 1 : 1, vortex mixed (10 min) and centrifuged at 16,000 rpm for 15 min at 4 °C. The supernatants were subjected to UPLC Q-TOF/MS system for analysis. A pooled

"quality control" (QC) sample was prepared by mixing equal aliquots (20 µL) from the extracted samples for optimization of the chromatographic and TOF/MS conditions. QC samples were prepared from the following groups of people: girls (GL & GH), boys (BL & BH), women (WL & WH) and men (ML & MH).

Analysis of the urine samples was performed using Waters Acquity™ Ultra Performance LC system (Waters Corp., Milford, USA) connected to a Synapt G2Si Q-TOF mass spectrometer (Waters MS Technologies, Manchester, UK) equipped with an electrospray (ESI) source (Waters, Manchester, UK). An ACQUITY UPLC HSS T3 column (1.8 um, 2.1 x 100 mm) was applied with a ACQUITY UPLC HSS T3 1,8 um, VanGuard Pre-Column 3/Pk 2.1 x 5 mm (Waters Corporation, Milford, USA). The injection volume was 5 µl and separation was performed at 0.4 ml/min and 50 °C. The gradient mobile phase was a mixture of 0.1% formic acid in water (A) and in acetonitrile (B). The gradient elution was performed in a following manner: 0–2 min 1% phase B; 2–8 min from 1% to 30% B; 8–11 min from 30% to 95% B; 11–13 min 95% B; 13–13.5 min from 95% to 100% B; 13.5–16.0 min 100% B; 16.0–16.5 min from 100% to 1% B; 16.5–20.0 min 1% B.

All the tests were performed in ESI^+ and ESI^- ionization modes. The profile data from *m/z* 50–1200 were recorded. Nitrogen gas was used as the cone and desolvation gas. The desolvation gas flow was set at 15 L/min at a temperature of 350 °C, the cone gas was set at 0,83 L/min and the source temperature was set at 120 °C. In ESI^+ ion mode, the capillary voltage was 3.2 kV and in ESI^- ion mode, the capillary voltage was 2.4 kV. All of the data were acquired using the lock mass to ensure accuracy and reproducibility. Leucine enkephalin was used as the lock mass at a scan time 0.1 s, interval 30 s and mass window ± 0.5 Da. MS method was used for the data collection with scan time of 0.2 s, data format centroid and high resolution mode. Prior to the analysis, the QC sample ran seven times to test stability of the instrument. During the analytical run, the QC sample was injected into every seven-eight experimental sample to monitor system consistency. Method for preparing the urine samples and procedure for their analysis were consistent with the protocol of urine metabolomics [10].

The collected data in a form of files with the extension. raw were converted using the MSConwert program to the mzXML format. In this format, the files were loaded into the XCMS Online program [11]. The default XCMS parameter set for UPLC – High Res (Waters) with G2S MS was used with: feature detection (method CentWave, maximal tolerated *m/z* deviation in consecutive scans of 15 ppm, minimum peak width of 2 s, maximum peak width of 25 s), retention time correction (method obiwarp, step size for profile generation 0.5 *m/z*), alignment (allowable retention time deviations of 2 s, width of overlapping *m/z* slices to use for creating peak density chromatograms and grouping peaks across samples of 0.01), statistics, annotation (ppm error 5, *m/z* absolute error 0.015), identification (tolerance for database search of 10 ppm, pathway deviation of 5 ppm). Integration of METLIN and MUMMIC-HOG to XCMS Online allowed a putative identification of metabolites.

After processing the data using the above mentioned parameters, among others, such results were generated: data files with the signal intensity of all potentially identified metabolites in each analysed urine sample; table from the results page with unique ID feature, fold change, p-value, mean feature signal intensity in the compared groups, adducts, extracted ion chromatogram and box-and-whisker-plot; table from the systems biology results page with predictive metabolites results and information on each metabolic feature (direction of dysregulation, fold change, p- value, *m/z*, retention time, and the XCMS feature ID number).

Potentially identified compounds obtained from the data processing in the XCMS Online were subjected to fragmentation using the average collision energy of 20 V in MS/MS mode. It was done to confirm their identification. The resulting fragmentation spectra of the compounds were compared to the spectra in the HMDB (The Human Metabolome Database). A QC sample used to fragment the compounds and the chromatographic and spectrometer parameters, except for collision energy and quadrupole parameters, were the same as for the MS mode. The quadrupole parameters were adjusted for fragmentation of each ion so that the ions with the accuracy of 0.5 *m/z* were subjected to fragmentation in the given retention time period.

2.4. Statistical analysis methods

Normality distributions of the continuous data were inspected with the Shapiro-Wilk W test. Categorical data were presented as numbers and percentages, while continuous data as the mean ± SD. Differences in the continuous variables were assessed using the Student's t-test. Values of $p < 0.05$ were accepted as statistically significant. These statistical procedures were completed by means of Statistica (StatSoft Inc., Tulsa, USA) version 13.1 software. The following statistical options were used in the XCMS program: the unpaired non-parametric Mann-Whitney test, *p*-value threshold of 0.05, fold change threshold of

Table 1
Overall characteristics of the study groups (age, anthropometric and demographic data and a physical activity level). Data are presented as the arithmetic mean and standard deviation.

Variable	Group/*p***	Girls n (13/16)*	Boys n (16/12)*	Women n (25/25)*	Men n (20/22)*
Age (years)	low AsT	10.6 ± 3.2	11.4 ± 2.2	49.8 ± 12.7	52.3 ± 15.8
	high AsT	10.6 ± 2.7	10.3 ± 2.1	51.5 ± 15.6	53.4 ± 15.8
	*p***	0.962	0.189	0.685	0.825
Body mass (kg)	low AsT	41.5 ± 18.1	51.2 ± 16.8	67.5 ± 10.5	88.9 ± 8.2
	high AsT	43.2 ± 16.2	42.4 ± 11.1	65.8 ± 10.8	90.3 ± 12.8
	*p***	0.804	0.145	0.571	0.689
Height (m)	low AsT	1.47 ± 0.18	1.58 ± 0.17	1.63 ± 0.07	1.76 ± 0.07
	high AsT	1.47 ± 0.18	1.49 ± 0.14	1.64 ± 0.06	1.78 ± 0.07
	*p***	0.999	0.185	0.697	0.467
BMI (kg/m^2)	low AsT	18.3 ± 3.8	20.0 ± 3.7	25.3 ± 3.9	28.6 ± 2.5
	high AsT	18.9 ± 4.1	18.9 ± 3.4	24.7 ± 4.8	28.5 ± 4.1
	*p***	0.680	0.411	0.593	0.984
Residence in a copper smelter area (years)	low AsT	9.0 ± 5.2	9.8 ± 3.8	30.0 ± 17.1	31.1 ± 14.6
	high AsT	6.9 ± 4.0	8.7 ± 2.3	28.5 ± 16.6	30.2 ± 14.7
	*p***	0.238	0.366	0.756	0.844
TPA (MET-min/week)	low AsT	1898 ± 1293	3339 ± 2596	1956 ± 1182	2443 ± 1871
	high AsT	1994 ± 1626	2083 ± 990	3102 ± 3653	4517 ± 7317
	*p***	0.882	0.145	0.190	0.329

*number of people with low AsT/high AsT concentrations; *p*** - *p* value unpaired *t*-test (low AsT vs. high AsT); BMI – body mass index; TPA - total physical activity; MET - metabolic equivalent of work.

1.2 and median fold change normalization. Due to the fact that the studied parameters depended to a large extent on gender and age, the statistical analysis of the obtained results was not performed between the groups of different sex and between young people and adults. The obtained results were compared in the following groups of individuals: girls (GL vs. GH), women (WL vs. WH), boys (BL vs. BH) and men (ML vs. MH).

3. Results

3.1. General characteristics of the study groups

In the compared groups of girls, boys, women and men with low and high AsT concentrations there were no significant differences regarding height, body weight, Body Mass Index (BMI), residence in a copper smelter area as well as in the total physical activity (Table 1). It was observed that a high percentage of the individuals both in the group with low as well as high AsT concentrations (from 25 to 56%) took various types of dietary supplements. Almost all the study subjects used tap water (aqueduct) to prepare meals. In the WL, WH and MH some part of the individuals smoked cigarettes and additionally, a high percentage of the girls, boys and especially men were exposed to tobacco smoke. In some girls and boys both with low and high AsT concentrations a habit of biting nails was observed, and in the BL and BH there was also a hand-to-mouth habit. Some part of the study subjects declared presence of diseases (Table 2).

The frequency of consumption of 169 products and food product groups was also subjected to an analysis. The frequency of consumption between all the study subjects with low AsT (n = 74) and high AsT (n = 75) was compared. Significant differences in the frequency of consumption of meat (low AsT 0.59 ± 0.51 s/day, high AsT 0.85 ± 0.82 s/day, p = 0.019), pate with liver (low AsT 0.09 ± 0.10 s/day, high AsT 0.17 ± 0.29 s/day, p = 0.033), fish (low AsT 0.23 ± 0.15 s/day, high AsT 0.43 ± 0.56 s/day, p = 0.005), dried fruit (low AsT 0.36 ± 0.60 s/day, high AsT 0.20 ± 0.43 s/day, p = 0.050) and sunflower seeds (low AsT 0.23 ± 0.30 s/day, high AsT 0.13 ± 0.21 s/day, p = 0.025) were indicated. There were no significant differences in the frequency of the remaining products consumption between all the study subjects.

3.2. Urinary arsenic concentrations

In all the groups iAs^{V} concentrations were below the limit of detection. Only in the men and women with a high AsT concentration, iAs^{III} was detected. In the GL and BL concentrations of MMA were significantly higher than in the GH and BH groups (p = 0.007 in both group). There were no significant differences between concentrations of MMA between the groups of men and women with high and low AsT concentrations. DMA concentrations were significantly higher in the GH, WH and MH in comparison with the corresponding GL, WL and ML (p = 0.000 in each group). In all the examined groups of individuals with a high AsT, concentrations of AsB were several times higher than in the compared groups with low AsT (p = 0.000 in all groups). Very high concentrations of AsB constituted the main component of AsT concentration in all the examined groups of girls, boys, women and men with high AsT (Fig. 1). The average percentage of AsB in the AsT concentration in each group was, as follows: GL 65.5 ± 13.8%, GH 87.2 ± 4.7%, BL 59.8 ± 12.5%, BH 90.5 ± 2.4%, WL 50.8 ± 14.1%, WH 90.4 ± 3.5%, ML 53.3 ± 10.0%, MH 74.6 ± 20.2%.

3.3. Differences in signal intensity of the urine metabolites

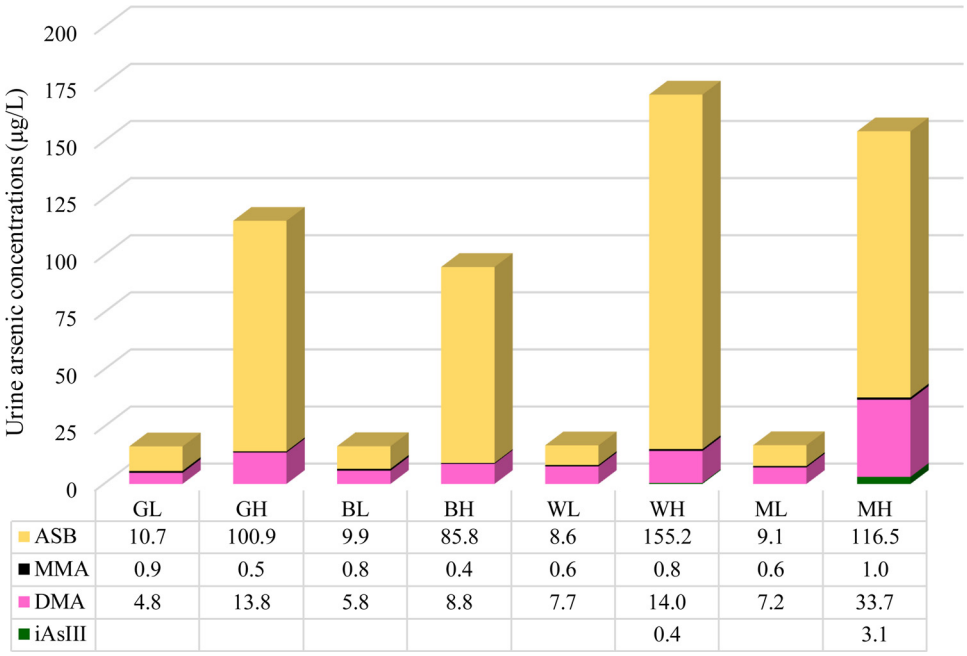
Obtained by using the optimal UPLC Q-TOF/MS conditions described above, the representative based peak intensity (BPI) chromatograms of the urine samples from the GL and GH in ESI + mode, the extracted ion chromatogram, the mass spectrum and the box-and-whisker plot are presented in Fig. 2A–E. In all the compared groups with low AsT and high AsT concentrations significant differences concerning signal intensity (s.i.) from numerous potentially identified compounds were observed. In the case of some compounds the changes were characteristic only in one compared group e.g. GH vs. GL. There were also compounds the changes of which were characteristic in many examined groups.

In the GH when compared to the GL there was significantly lower s.i. coming from potentially identified compounds that belong to the following pathways: serotonin degradation (5-hydroxytryptophol), L-dopachrome biosynthesis (leucodopachrome), phenylethylamine degradation I (phenylacetaldehyde, phenylacetic acid), tyrosine degradation (fumaric acid), and adenosine nucleotides degradation (xanthine); and significantly higher s.i. from the compound belonging to tryptophan degradation pathway (2-aminomuconic acid

Table 2
Health status and selected habits among the study participants. Data are presented as the number of observations and percentages.

Variable	Group	Girls n (13/16)*	Boys n (16/12)*	Women n (25/25)*	Men n (20/22)*
Diabetes	low AsT	0 (0.0%)	0 (0.0%)	0 (0.0%)	4 (20.0%)
	high AsT	0 (0.0%)	0 (0.0%)	2 (8.0%)	3 (13.6%)
Hypertension	low AsT	0 (0.0%)	0 (0.0%)	5 (20.0%)	7 (35.0%)
	high AsT	0 (0.0%)	0 (0.0%)	3 (12.0%)	7 (31.8%)
Renal disease	low AsT	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	high AsT	0 (0.0%)	0 (0.0%)	1 (4.0%)	1 (4.5%)
Diseases of the lungs and bronchi	low AsT	0 (0.0%)	0 (0.0%)	2 (8.0%)	1 (5.0%)
	high AsT	0 (0.0%)	0 (0.0%)	1 (4.0%)	0 (0.0%)
Consumption of supplements	low AsT	5 (38%)	5 (31%)	14 (56%)	11 (55%)
	high AsT	4 (25%)	6 (50%)	12 (48%)	11 (50%)
Preparing meals using tap water	low AsT	13 (100%)	15 (94%)	24 (96%)	18 (90%)
	high AsT	15 (94%)	12 (100%)	25 (100%)	21 (95%)
Current cigarette smoking	low AsT	0 (0%)	0 (0%)	3 (12%)	0 (0.0)
	high AsT	0 (0%)	0 (0%)	2 (8%)	8 (36%)
Exposure to tobacco smoke	low AsT	2 (15%)	5 (31%)	1 (4%)	4 (20%)
	high AsT	2 (13%)	1 (8%)	1 (4%)	6 (27%)
Biting nails	low AsT	2 (15%)	3 (19%)	0 (0%)	0 (0%)
	high AsT	7 (44%)	1 (8%)	0 (0%)	0 (0%)
Habit of hand-to-mouth	low AsT	0 (0%)	2 (13%)	0 (0%)	0 (0%)
	high AsT	0 (0%)	3 (25%)	0 (0%)	0 (0%)

* number of people with low AsT/high AsT concentrations.



	GL	GH	BL	BH	WL	WH	ML	MH
ASB	10.7	100.9	9.9	85.8	8.6	155.2	9.1	116.5
MMA	0.9	0.5	0.8	0.4	0.6	0.8	0.6	1.0
DMA	4.8	13.8	5.8	8.8	7.7	14.0	7.2	33.7
iAsIII						0.4		3.1

Fig. 1. Urine arsenic concentrations in the in the studied groups of people. Data are presented as the arithmetic mean. GL - girls with low total arsenic concentration; BL - boys with low total arsenic; WL - women with low total arsenic; ML - men with low total arsenic ; GH - girls with high total arsenic; BH - boys with high total arsenic; WH - women with high total arsenic; MH - men with high total arsenic; iAs^{III} - inorgnic As^{III}; MMA - methylarsonic acid; DMA - dimethylarsinic acid; AsB - arsenobetaine.

semialdehyde), (Fig. 3; Table S1).

The WH when compared to the WL was characterized by significantly higher s.i. coming from the compounds belonging to such pathways as: serotonin degradation (5-hydroxyindoleacetic acid), purine ribonucleosides degradation (adenine), glycolysis (D-lactic acid) and significantly lower s.i. from the compound coming from L-dopa degradation pathway (vanillactic acid), (Fig. 4; Table S2).

In the BH when compared to the BL there was significantly higher s.i. from the compounds belonging to the pathways of: glucocorticoid biosynthesis (cortisone) and mineralocorticoid biosynthesis (18-hydroxycorticosterone) and significantly lower s.i. from the compounds belonging, inter alia, to tricarboxylic acid cycle pathway (citric acid, succinic acid), (Fig. 5; Table S3).

The MH was characterized by significantly higher s.i. from the compounds belonging to the pathways of sulfate activation/oxidation, 4-hydroxy-2-nonenal detoxification (4-hydroxynonenal) and significantly lower s.i. from adenosine monophosphate - metabolite belonging to many pathways (Fig. 6; Table S4).

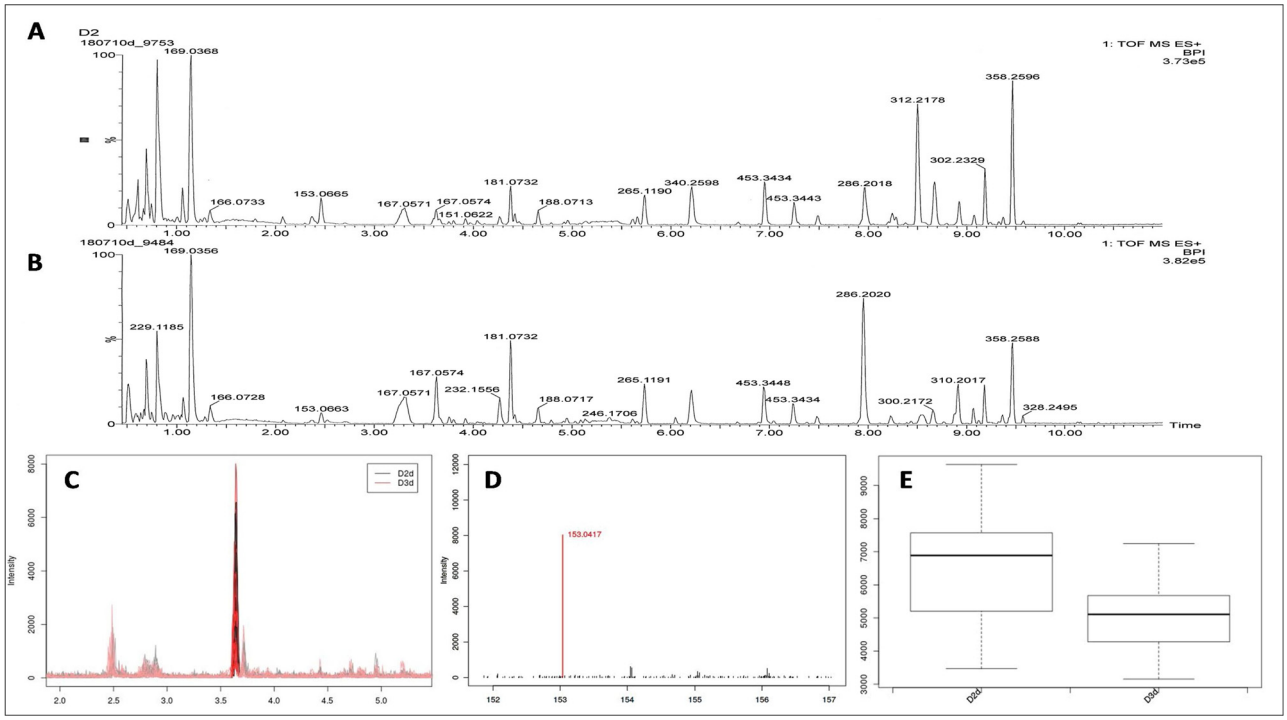


Fig. 2. Exemplary UPLC-QTOF MS profiling in ESI + mode and results obtained by XCMS Online. (A) - the base peak intensity chromatogram (BPI) of a urine sample of a girl with a slightly elevated concentration of total arsenic, (B) – BPI chromatogram of a urine sample of a girl with a substantially higher concentration of total arsenic, (C) – the extracted ion chromatogram: 153.0391–153.0429 m/z, (D) – the mass spectrum at 3.64 min selected to highlight the peak for 153.0417 *m/z*, which represent the protiated M + H[1 +] specie, (E) – the box-and-whisker plot for that metabolic feature.

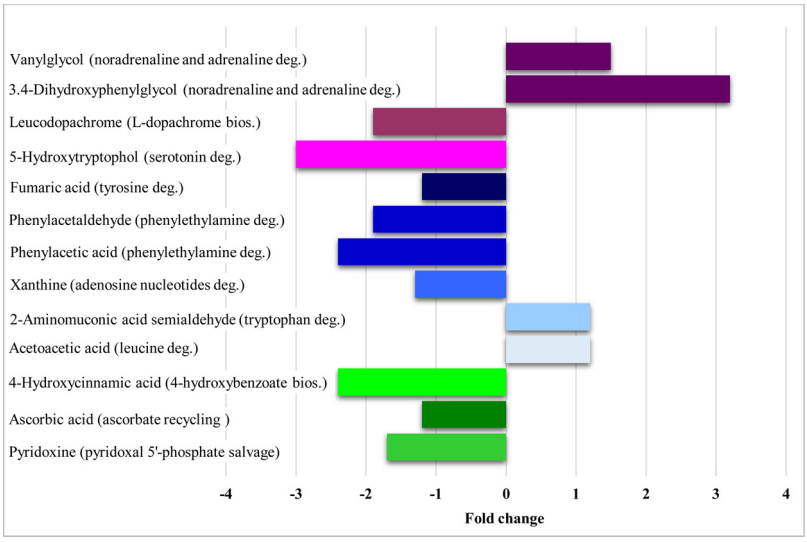


Fig. 3. Mean fold change of intensity signal from the potentially identified compounds belonging to the specific pathways (information in brackets) in the group of girls with high urinary concentration of total arsenic (AsT) in comparison with the corresponding group of girls with low AsT. bios. - biosynthesis; deg. - degradation.

In the GH, WH and BH when compared to the corresponding GL, WL and BL there was significantly higher s.i. from the compounds belonging to noradrenaline and adrenaline degradation pathways (vanylglycol in GH, 3.4-dihydroxyphenylglycol in GH, WH and BH), (Figs. 3–5; Table S1-S3). Significantly higher s.i. from the compounds belonging to leucine degradation pathway (acetoacetic acid in GH and ketoleucine in WH) was observed in the GH and WH in comparison with the corresponding GL and WL (Figs. 3–4; Table S1-S2).

In the BH and GH changes in 4-hydroxybenzoate biosynthesis were observed (s.i. from 4-hydroxycinnamic acid in the GH was significantly lower than in the GL but in the BH s.i. from 4-hydroxyphenylpyruvic acid and hydroxycinnamic acid in the BH was significantly higher than in the BL). In GH and BH there were also significant differences in terms of s.i. from the compounds that belonged to the vitamins transformation pathways. In ascorbate recycling pathway significantly lower s.i. was observed in the GH and BH (ascorbic acid) than in the GL and BL. Only in the MH s.i. from metabolites of ascorbate recycling pathway was significantly higher than in the ML. In the case of a pathway of pyridoxal 5′-phosphate salvage s.i. from pyridoxine in the GH was significantly lower than in the GL, while in the WH (pyridoxal) and BH (pyridoxal, pyridoxine) s.i. was significantly higher than in the WL and BL (Figs. 3–6; Tables S1-S4).

In a pathway of dopamine degradation the intensity of signal from 3.4-dihydroxyphenylacetaldehyde was significantly higher in the WH and BH compared with the WL and BL, (Figs. 4–5; Tables S2-S3). In the compared groups of men and women also changes in such pathways as: androgen biosynthesis (significantly higher s.i. from pregnenolone in the WH and dehydroepiandrosterone in the MH, WH), urea cycle (significantly higher s.i. from argininosuccinic acid in the WH and MH) were observed (Figs. 4 and 6; Tables S2, S4). In the WH, BH and in MH there was significantly higher s.i. from the compounds belonging to the pathways of D-glucuronate degradation (L-threo-2-pentulose) and nicotine degradation (4-hydroxy-4-(3-pyridyl)-butanoic acid in the WH, norcotinine in the GH and MH as well as 3-pyridylacetic acid in the BH), (Figs. 4–6; Tables S2-S4).

4. Discussion

4.1. General characteristics of the study groups and urinary arsenic concentrations

The study individuals lived in southwestern Poland, in an area with

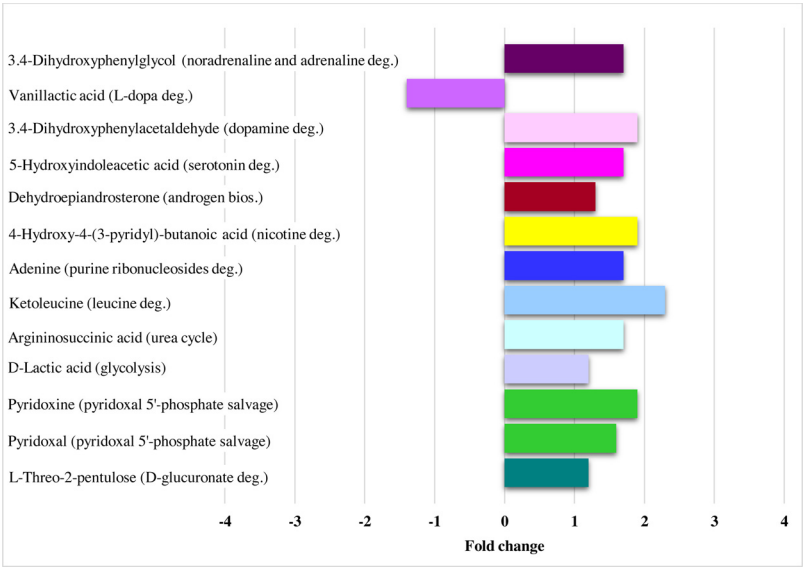


Fig. 4. Mean fold change of intensity signal from the potentially identified compounds belonging to the specific pathways (information in brackets) in the group of women with high urinary concentration of total arsenic (AsT) in comparison with the corresponding group of women with low AsT. bios. - biosynthesis; deg. - degradation.

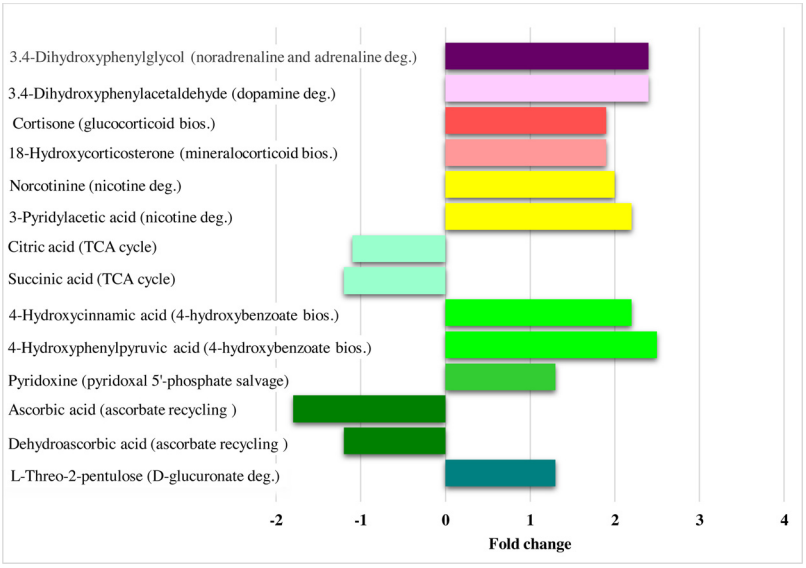


Fig. 5. Mean fold change of intensity signal from the potentially identified compounds belonging to the specific pathways (information in brackets) in the group of boys with high urinary concentration of total arsenic (AsT) in comparison with the corresponding group of boys with low AsT. TCA - tricarboxylic acid cycle; bios. - biosynthesis; deg. - degradation.

a documented environmental problem of As related to mining and copper smelters [12]. Potential sources of exposure to inorganic arsenic in this area may also include air, dust and water. Soil and food are yet another source of exposure to organic and inorganic arsenic forms. Current cigarette smoking and exposure to tobacco smoke was a significant problem occurring in the study individuals. Particularly worrying was the fact that passive smoking was observed also in girls and boys. Tobacco smoke contains many chemical compounds, including metals, such as: arsenic, cadmium and chromium. During cigarette smoking some metals released with the smoke are present both in the main stream, which is inhaled by a smoker, but also in the side stream, which is inhaled by people passively exposed [13]. Bearing in mind that soil and dust can be significant pathways of exposure, particularly in the vicinity of mining or smelting sites [14,15], in the case of some girls and boys habits of hand-to-mouth and biting nails can also constitute an additional source of exposure.

Almost all the study population used tap water to prepare meals. The WHO guideline for drinking water, based on the increasing awareness of toxicity of As, recommended a value of up to 10 μg/L [16]. Tap water in this area is allowed for consumption and, therefore, it can be assumed that there was no risk of exposure to As related to water consumption. Only six people used water from a private well. Additionally, in the study subjects dietary supplements consumed by them, especially those prepared from seafood e.g. fish liver oil, shark cartilage, seaweed, algae, collagen fish, could constitute another source of exposure to arsenic. In the study groups from 25 to 65% of the individuals took different types of supplements. Moreover, the people with a high AsT concentration had more frequent fish and pate with liver consumption than those with a low AsT concentration. Metabolic transformations of arsenic take place in the liver. Therefore, As^{III} and DMA are mainly accumulated in this organ. Studies performed using animals have confirmed this regularity [17]. Also seafood is an important source of As for many populations [18]. AsB is the main compound containing As that is present in most fish and it is non-toxic and not metabolized. Fish and other seafood also contain iAs and significant amounts of other organic compounds such as: arsenosugars and arsenolipids. These organic compounds are metabolized mainly to DMA. Arsenic species concentration in different seafood types varies widely. For instance in fish the range of AsT concentrations is from 0.005 to 5.03 μg/g of wet weight and in seaweed it is from 0.062 to 102 μg/g of wet weight [19]. In our study population, in the groups with a low AsT concentration, AsB accounted for 51–66%, while in a high AsT GH, GH, WH it accounted for about 90% and in the MH for 75%, which is very characteristic. Such a high proportion of AsB in the study groups indicates high consumption of seafood, especially in the groups with a high AsT concentration. This is also confirmed by the results obtained during the questionnaire studies.

Urine concentration of organic and inorganic arsenic forms in the study subjects differed considerably from concentrations observed in other populations. For instance, in a Chinese population in men consuming water with a concentration of As below 10 μg/l, the average urinary content of various forms of As was as follows: iAs - 53.4%, DMA - 28.0%, MMA - 4.4% and AsB - 14.3%. In this population consumption of rice and other As-containing foods was the main contributor to

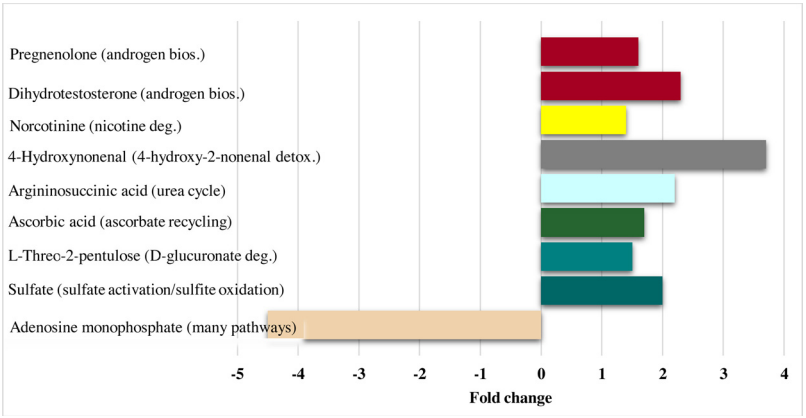


Fig. 6. Mean fold change of intensity signal from the potentially identified compounds belonging to the specific pathways (information in brackets) in the group of men with high urinary concentration of total arsenic (AsT) in comparison with the corresponding group of men with low AsT. bios. - biosynthesis; deg. - degradation; detox - detoxification.

urinary iAs [6]. In the studies carried out in New Hampshire (USA) in individuals with a low to moderate As exposure, mean concentration of iMD was 7.1 μg/L and AsB - 30.2 μg/L [20]. In the GL, BL, WL, ML and BH the average concentration of iMD was at a similar level as the one in New Hampshire population, while in the GH, WH and MH in particular – it was considerably higher. Concentration of AsB in the study groups with a low AsT concentration was about 3 times lower, and in the groups with a high AsT concentration it was 3–5 times higher. The specific profile of various forms of arsenic in the urine and differences in consumption of some products present in our study population may be related also to a different spectrum and intensity of disorders associated with the environmental exposure to As. We wanted to investigate whether in the study population with high and low AsT concentrations there are differences in metabolism without identifying the source of exposure. Therefore, we did not exclude the people who were passive and active smokers, took dietary supplements, had more frequent fish, rice and pate with liver consumption as well as those with habits of hand-to-mouth and biting nails.

4.2. Urinary metabolomics

Almost every organ in the body can be affected by exposure to organic or inorganic arsenic, with health effects, such as: skin lesions [21], cancer of internal organs, e.g. bladder, kidney and liver cancer [22,23] as well as various adverse health problems, such as: weakness, anemia, diarrhoea, hepatomegaly [24], neuropathy, lung and cardiovascular diseases [25,26], conjunctivitis [27], diabetes [28]. Our metabolomics studies of the urine allowed potential identification of many compounds, s.i. of which was significantly different in the compared groups of girls, women, boys and men with low and high AsT concentrations. These results shed new light on the mechanisms of action of arsenic. The compounds that were significantly different in the groups with low and high AsT concentrations were mainly metabolites of neurotransmitters and hormones, and they belonged to the pathways of amino acids and tricarboxylic acids as well as to the pathway of glycolysis. Additionally, significant differences were observed in the s.i. from metabolites of C and B_6 vitamins as well as phenolic compounds.

In the groups of individuals with a high AsT concentration when compared to those with a low AsT concentration, significant differences in the s.i. from serotonin, catecholamines, mineralocorticoids, glucocorticoids and androgens metabolites were observed.

In the GH lower s.i. from the products of serotonin degradation was observed in comparison with the GL but in the case of women, the situation was reversed – the WH had higher s.i. than the WL. Serotonin plays important roles in the gastrointestinal tract where it regulates intestinal movement as well as in the central nervous system where, among others, it affects mood, sleep, memory, and learning [29,30]. The observed changes in the s.i. from serotonin metabolites may be associated with the occurrence of the above-mentioned disorders of varying severity in the groups of girls and women with a high AsT concentration.

Higher s.i. deriving from metabolites of catecholamines was observed in the GH, BH and WH when compared to the GL, BL and WL. Biosynthesis of catecholamines begins with hydroxylation of tyrosine producing L-dopa [31], which is decarboxylated to dopamine [32]. That, in turn, is hydroxylated to noradrenaline [33]. Transformations of noradrenaline involve a sequence of reactions during which, among others, adrenaline, 3.4-dihydroxyphenylglycol and vanylglycol are formed. Adrenaline can markedly increase blood glucose levels via stimulating hepatic glycogenolysis and via decreasing peripheral glucose uptake. Additionally, adrenaline can raise body temperature, increase heart rate and cardiac output [34]. Elevated plasma levels of adrenaline and noradrenaline have been reported in patients with hypertension [35]. Prolonged elevation of plasma catecholamines levels can contribute to cardiac dysfunction by activation of vascular smooth muscle cells resulting in ischemia, functional hypoxia, oxidative damage as a consequence of formation of oxidized catecholamines and oxygen free radicals [34]. Higher s.i. deriving from metabolites of catecholamine in the GH, BH and WH may pose a risk of the development of hypertension and cardiovascular diseases. As it is known, exposure to high concentrations of arsenic causes a number of pathological changes in the organisms of the studied population, manifesting, among others a number of „civilization" diseases [25,26,28]. The results presented in this work prove that arsenic exposure can cause a number of changes at the cellular level, changing the metabolic pathways. Understanding these changes with the metabolomic profiling can be an important element in studying the mechanisms of arsenic action.

In the BH higher s.i. from cortisone, which is a metabolite of glucocorticoids, was observed. Glucocorticoids are essential in maintaining homeostasis under stress conditions and play a key role in biological processes, such as growth, reproduction, metabolism, immune and inflammatory processes, functioning of the central nervous and cardiovascular systems as well as maintenance of water-electrolyte homeostasis [36–38]. It has been also shown that the increase in glucocorticoids concentration affects a decrease in peripheral glucose uptake, which is associated with an increase in insulin secretion and induction of insulin resistance. In the liver glucocorticoids intensify the process of gluconeogenesis, thereby increasing the release of substrates for gluconeogenesis from peripheral tissues, in particular from muscles. Mobilization of substrates for gluconeogenesis increases protein catabolism in non-hepatic tissues, in particular in muscles [39,40]. Glucocorticoids also promote development of visceral fat. Cortisol has been shown to affect preadipocytes, accelerating their differentiation into adipocytes. It also regulates expression of mature fat cell genes, promoting their hypertrophy and lipid accumulation. Chronic exposure to excess of glucocorticoids results in the excess of triglycerides stored in the liver, due to their impaired metabolism by β-oxidation [37,41]. The observed in the study population changes in s.i. from metabolites of neurotransmitters and hormones may be also an important etiologic factor in the development of lipid disorders and liver disease. This has been confirmed by the studies showing that arsenic exposure was associated with liver inflammation and steatosis as well as with a high cholesterol level [42,43]. It has been also stated that arsenic alters lipid metabolism in a pattern that suggests disruption of the tricarboxylic acid (TCA) cycle and increased ketogenesis [44].

In the BH not only higher s.i. from cortisone but also from 18-hydroxycorticosterone -metabolite of mineralocoricoids was observed. It has been shown that a high concentration of cortisol also activates mineralocorticoid receptors [45], which results in sodium and water retention in kidney tubules, increased volume of extracellular fluids and may lead to the development of hypertension [46,47]. The observed in the GH, BH and WH higher s.i. from metabolites of catecholamines, mineralocorticoids and glucocorticoids may be also related to the observed higher s.i. from metabolites of leucine, tryptophan, purine degradation (in the GH, WH), urea cycle (in the WH and MH), glycolysis (in the WH) and with lower s.i. from metabolites of tricarboxylic acid cycle (in the BH) as well as from adenosine monophosphate (in the MH).

In the WH and MH high s.i. from metabolites of androgens biosynthesis was observed. Androgens are synthesized in testes, ovaries, placenta and other tissues and are required for normal sexual development of males and females. In the groups of boys, women and men with a higher AsT concentration in the urine (BH, WH, MH), higher s.i. from the compounds being metabolites of nicotine was observed than in the groups with a lower AsT concentration (the BL, WL, ML). These results may indicate that the study individuals from the BH, WH and MH were exposed in a passive or active way to tobacco smoke. In the studies of other authors it has been indicated that exposure to tobacco smoke is also associated with an increased concentration of testosterone in the urine. In metabolomics studies carried out among 127 men exposed to arsenic, even after excluding individuals exposed to tobacco smoke, increased s.i. coming from testosterone was observed in the

examined urine samples. Authors of those studies believe that exposure to low concentrations of arsenic coming from the environment may be sufficient to induce changes in testosterone concentration [6]. In our studies, in the BH, WH and MH apart from an increase in the amount of metabolites of nicotine transformations in the urine, also higher s.i. coming from the compounds that are androgens and steroid hormones metabolites was observed. Thus, it may be assumed that in those groups exposure to arsenic and tobacco smoke might have had an additive influence on the increase of metabolites of these hormones in the urine. Another factor that might have had an effect on the total and free testosterone concentration is the level of physical activity [48]. However, in the case of the compared study groups with low and high AsT concentrations there were no significant differences with regard to the level of physical activity.

In the GH, WH and MH high s.i. from D-glucuronate degradation and, additionally, in men from sulfate were observed. In humans, glucuronic acid and sulfate are often linked to endogenous neurotransmitters and hormones (e.g. dopamine, noradrenaline, serotonin, testosterone, bilirubin) and exogenous molecules (e.g. nicotine, toxic or poisonous substances). These processes (glucuronidation and sulfonation) inactivate and detoxify molecules by increasing their water solubility, which promotes their removal from the body via the kidneys or gastrointestinal tract. Glucuronides are produced by uridine diphosphate (UDP)-glucuronosyltransferase enzymes in the liver that append glucuronic acid to functional groups. In the gastro-intestinal tract, some strains of microbiota express ß-glucuronidase enzymes that can remove glucuronic acid and effectively reverse actions of inactivation [49]. For example, it has been shown that bile acids that are also conjugated to sulfate can be strongly metabolized by the microbiota and deconjugated [50]. Urinary excretions of L-threo-2-pentulose (L-xylulose) after oral administration of glucuronolactone were measured in normal healthy individuals and in patients with diabetes mellitus, acute hepatitis in a recovery stage, chronic hepatitis and liver cirrhosis. A marked increase in L-xylulose excretion has been observed in cirrhotic, acute and chronic hepatitis patients compared to the normal healthy individuals [51]. The latest metabolomics study has shown that levels in the serum of a variety of metabolites, including glucuronic acid, were reduced in patients with hepatitis B cirrhosis in comparison to healthy individuals [52]. It can be assumed that in our study, higher s.i. from sulfate and glucoronate degradation in the urine may be related to the impaired glucuronidation and sulfonation in the people with a high AsT concentration who had higher s.i. from catecholamine, nicotine and hormones. It may be promoted by a change in the intestinal flora of the digestive tract. Microbiota within the digestive tract plays a very important role in human health [53]. One recent study has demonstrated that mice exposure to arsenic via water (10 µg/L) changed the types of microbes in their guts and their metabolic profile [54].

In the MH, where concentration of iMD in the urine was almost four times higher than in the BH and almost twice as high as in the GH and WH, higher s.i. from metabolite of 4-Hydroxy-2-nonenal was observed. This is a highly reactive neurotoxic product of lipid peroxidation that is also implicated in the pathogenesis and progression of Alzheimer's and Parkinson's diseases This compound reacts and modifies, e.g. proteins, DNA, RNA and lipids, inducing toxic effects that are involved in the pathology of Alzheimer's disease [55–57]. It has been found that in the brain and cerebrospinal fluid of Alzheimer's disease patients the level of free and protein-bound 4-Hydroxy-2-nonenal was significantly higher in comparison to healthy people [58].

Harmful effects of toxic elements on human health may be modulated by some components of a diet. Diet components may interact with those elements or compete for transport mechanisms, which, in turn, may reduce their absorption from the gastrointestinal tract. Protective effect of some nutrients may also result from their antioxidant and anti-inflammatory properties [59]. In the compared study groups with low and high AsT concentrations there were significant differences with regard to signal intensity from the compounds being metabolites of polyphenols, vitamin B_6 and vitamin C.

In the examined girls and boys groups with high and low AsT concentrations significant differences with regard to s.i. from metabolites belonging to 4-hydroxybenzoate biosynthesis pathway were observed, which indicates differences with regard to consumption and/or absorption of phenolic acids. Dietary plant polyphenols can be converted in the colon by microorganisms into phenolic acids and in this form they can be excreted in the urine. In studies on rats fed with diets containing 5% of cranberry, primary phenolic acid excreted in the urine was 4-hydroxycinnamic acid [60]. Epidemiological studies have shown that increased urinary excretion of phenolic acids in humans is associated with decreased overall mortality [61]. In the BH s.i. from the compound belonging to the 4-hydroxybenzoate biosynthesis pathway was higher than in the BL, but in the girls it was the opposite - in the GH s.i. was lower than in the GL. The observed higher s.i. of phenolic acids in the BH compared to BL can be considered a very beneficial effect.

In the BH and WH, higher s.i. from metabolites of serotonin and dopamine than in the BL and WL were observed, also s.i. from products of vitamin B_6 metabolism was higher, whereas in the GH s.i. from vitamin B_6 and serotonin metabolites were significantly lower than in the GL. Vitamin B_6 is an important cofactor in the synthesis of not only serotonin, but also dopamine, adrenaline and melatonine. Even slight vitamin B_6 deficiency may reduce serotonin synthesis [62]. The observed higher s.i. from metabolites of vitamin B_6 indicates higher consumption of products that constitute a good source of this component in the BH and WH and lower in the GH. Frequency of meat and sunflower seed consumption, which are, among others, a good source of this vitamin, was significantly higher in the individuals with a high AsT concentration than in those with a low AsT concentration. Due to the small number of individuals in the groups, the frequency of consumption of products in particular subgroups of the girls, women, boys and men was not analyzed. Perhaps, in the GH consumption of vitamin B_6 was insufficient.

In the GH and BH lower s.i. from vitamin C was observed when compared to the GL and BL. Only in the MH s.i. from vitamin C in the examined urine samples was significantly higher than in the ML. Vitamin C and other food ingredients can counteract toxic effects of metals. Such a protective effect may be a consequence of their ability to directly reduce toxicity through antioxidant and anti-inflammatory activity [59]. Taking into consideration described in the literature beneficial effects of antioxidants, including vitamin C, the lower s.i. from this compound in the groups of young people with a high AsT concentration compared to the groups with a low AsT concentration, may be particularly unfavorable in this case.

The obtained study results revealed occurrence of many differences with regard to the intensity of signals coming from compounds belonging to various transformation pathways in the compared study groups. Bearing in mind the fact, that some part of the disorders associated with environmental exposure to arsenic may be modulated by changes in a diet and lifestyle, it seems that in the study group implementation of a wide-ranging educational program and monitoring its effectiveness is a necessity.

5. Conclusions

As it was emphasized, this is the first report binding exposure to arsenic with changes in metabolic profiles of adults and children living in a copper smelter area. So far published works emphasize the endpoints of exposure to arsenic however, presented data gives great insight into the mechanisms leading to pathological effect. This may be a contribution to further studies on changes in metabolism caused by arsenic and its metabolites. Summing up, in the study population with a low AsT concentration in the urine there were significant differences in signal intensity from many compounds being metabolites of neurotransmitters, nicotine and hormones transformations in comparison with age and sex matched groups with a significantly higher AsT

concentration (serotonin in the girls and women; catecholamines in the girls, boys and women; mineralocorticoids and glucocorticoids in the boys, androgens in the women and men and nicotine in the boys, women and men). Those changes might have been related to higher s.i. from metabolites of leucine, tryptophan, purine degradation (in the GH, WH), urea cycle (in the WH and MH), glycolysis (in the WH), with lower s.i. from metabolites of tricarboxylic acid cycle (in the BH) as well as from adenosine monophosphate (in the MH) in comparison with the matched groups with low AsT. In the MH, in the case of whom iMD concentration in the urine was several times higher than in the other study groups, higher s.i. from metabolite of a highly reactive neurotoxic product of lipid peroxidation (4-hydroxy-2-nonenal) was observed. In addition, significant differences in s.i. from metabolites of nutrients, which may modulate harmful effects of As, were observed (vitamin C in the girls, boys and men, vitamin B_6 in the girls, boys and women as well as phenolic compounds in the boys and girls). We hypothesize that higher s.i. from metabolites of sulfate (in MH) and of glucoronate degradation (in the BH, WH and MH) than in the low AsT groups may be related to the impaired glucuronidation and sulfonation and higher s.i. from catecholamines, nicotine and hormones. Our results indicated that even a low exposure to As is associated with metabolic changes and that urine metabolomics studies could be a good tool to reflect their wide spectrum connected to specific environmental exposure to As.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jtemb.2019.03.009.

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