E.V. Drozdova, K.V. Kaliasniova, V.E. Syakhovich, N.A. Dalhina

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Research article

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POLYMORPHISMS OF XENOBIOTIC METABOLISM ENZYME GENES CYP2E1, GSTM1, GSTT1, EPHX1 AS BIOMARKERS OF SENSITIVITY TO EXPOSURE TO WATER DISINFECTION BYPRODUCTS (USING CHLOROFORM AS AN EXAMPLE)

E.V. Drozdova[1], K.V. Kaliasniova[1], V.E. Syakhovich[2], N.A. Dalhina[1]

[1]Scientific and Practical Center for Hygiene, 8 Akademicheskaya Str., Minsk, 220012, Republic of Belarus
[2]National Anti-Doping Laboratory, 31 ag. Lesnoi, Minsk region, 223040, Republic of Belarus

Chloroform accumulation in the body and the increase in its steady-state concentrations in blood of exposed people have been established to be associated with polymorphisms in enzyme genes involved in metabolism of water disinfection byproducts (A415G of EPHX1 gene, C1091T of CYP2E1 gene, deletions of GSTT1 and GSTM1) (p < 0.000001). These genes polymorphisms correlate with higher chloroform levels in blood of people consuming chlorinated drinking water: by 43.8 % and higher for GSTM1 null genotype; by 68.2 % and higher for GSTT1; by 80.4 % and higher for EPHX1 (p < 0.01). Polymorphism in EPHX1 gene makes chloroform accumulation much more probable (blood levels ≥ P75), which is the most pronounced when combined with GSTT1 gene polymorphism.

The study results allow us to consider hetero- and homozygous polymorphic genotypes AG/GG for the EPHX1 gene, CT/TT for the CYP2E1 gene, and the null allele in the GSTT1 and GSTM1 genes as genetic predisposition factors for chloroform accumulation in the body. This increases the probability of health outcomes associated with chronic exposure to this disinfection by-product.

The A415G polymorphism of the EPHX1 gene and GSTT1 deletion, their combinations including the combination with the GSTM1 null and/or the C1091T polymorphism of the CYP2E1 gene can be used as the most informative biomarkers of sensitivity when assessing risks associated with exposure to trihalomethanes (chloroform) at levels not exceeding MPC in water.

Keywords: CYP2E1, GSTM1, GSTT1, EPHX1 genes, disinfection by-products, drinking water, gene polymorphism, biomonitoring, health risk assessment, biomarkers of susceptibility.

Disinfection is an integral stage in water treatment. It guarantees that population is provided with epidemiologically safe drinking water and this is especially vital for surface water sources as less protected from any external impacts. At the same time, use of reagents for water disinfection creates up to 400 disinfection by-products (DBPs); some of them are able to produce long-term effects. DBPs composition and levels in treated drinking water vary significantly depending on levels of natural organic compounds in water prior to treatment and an applied method of disinfection [1, 2]. Tap water supply systems widely rely on various chlorination methods with trihalomethanes (THMs) as typical indicator DBPs (chloroform, dichloro-bromomethane, chlorodibromomethane and bromoform). Since all of them are volatile organic compounds, they can enter the body not only orally but also by inhalation and dermal absorption. The latter make a substantial contribution to the total daily DBPs load due to intensive household water use (showering, bathing, cleaning, washing, doing the dishes, etc.) [3–5]. Although these compounds occur in water in very low and even trace concentrations, simultaneous exposure to several of them creates public health risks associated with water use, especially under long-term exposure [1–11]. Use of water containing several trihalomethanes creates elevated risks of pregnancy complications (intrauterine growth retardation, low birth weight,

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Alena V. Drozdova – Candidate of Medical Sciences, Associate Professor, Deputy Director for Science (e-mail: drozdovaev@mail.ru; tel.: +7 (017) 370-50-15; ORCID: https://orcid.org/0000-0002-3032-0895).

Ekaterina V. Kolesneva – Senior Researcher at the Laboratory of Industrial Toxicology (e-mail: kolesneva_kate@mail.ru; tel.: +375-17-388-48-22; ORCID: https://orcid.org/0000-0003-1605-4786).

Vitaly E. Syakhovich – Head of the Research Laboratory (e-mail: rlab.head@antidoping.by; tel.: +375-17-265-58-88; ORCID: https://orcid.org/0000-0002-2558-6824).

Natallia A. Dalhina – Researcher (e-mail: dlginan@rambler.ru; tel.: +375-17-351-23-84; ORCID: http://orcid.org/0000-0003-4012-2507).

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premature birth, and congenital anomalies) [1, 6]. Established carcinogenic effects of chloroform, bromoform and dichlorobromomethane allowed the International Agency on Cancer Research (IARC) to rank them as 2B carcinogens (possibly carcinogenic for humans); the evidence was provided by animal experiments [1, 2, 7–9]. Epidemiological studies established a correlation between exposure to trihalomethanes and bladder cancer and colorectal cancer [1, 10, 11]. Considering all the latest data on hazards posed by trihalomethanes, there is a trend to develop stricter standards for these DBPs levels in drinking water in international regulatory documents. However, any revision of national standards should be based on reliable evidence considering water use in the Republic and peculiar sensitivity of the population to exogenous pollutants. This may be achieved by applying certain methodical approaches [12].

It is very difficult to estimate actual effects of trihalomethanes on the human body due to their complex chemical composition, especially when it comes down to a mixture of DBPs, and multiple ways of introduction. More precise and objective quantification of health risks is achieved, among other things, by using data on internal exposure, that is, levels of pollutants (their metabolites) in biological media [13–16]. Chloroform levels in blood have been proven to be reliable biomarkers of exposure to trihalomethanes in water. Chloroform concentration in blood grows just after showering, bathing, doing the dishes by hand, or drinking hot drinks made of tap water (the highest levels are detected after showering / bathing) and then declines rapidly [17–20]. However, low chloroform levels can be detected in blood even 8 hours after water use. Its slower partitioning out of adipose tissues анв relatively high (e.g., daily) frequency of exposure events such as showering / bathing are thought to produce steady-state blood chloroform concentrations. Examining a blood sample independently of water use establishes such levels and allows more precise estimation of internal exposure for subsequent health risk assessment [21].

So, THMs blood levels depend on intensity of exposure and body weight. But they are also affected by some other factors, activity of xenobiotic metabolism being the most significant one. Inter-individual variations in xenobiotic metabolism capacities may be due to polymorphisms of the genes coding for the enzymes themselves or of the genes coding for the receptors or transcription factors which regulate the expression of the enzymes. Also, polymorphisms in several regions of genes may cause altered ligand affinity, transactivation activity or expression levels of the receptor subsequently influencing the expression of the downstream target genes [22–24]. As a result, slower toxicant excretion and / or greater production of toxic metabolites and their accumulation in the body make adverse biological effects much more probable. This, in its turn, leads to greater probability of chronic non-communicable diseases caused by environmental exposures. Therefore, polymorphisms of xenobiotic metabolism genes in a genotype can underlie differences in individual susceptibility to chemical environmental exposures. Abnormal alleles (polymorphisms) of enzyme genes participating in xenobiotic metabolism are valuable predictors of aforementioned diseases and can therefore be used as markers of body susceptibility to effects produced by toxicants [24–27].

Cytochrome P450 2E1 (CYP2E1) is a basic enzyme that catalyzes trihalomethanes metabolism in human and rat bodies. Secondary metabolism of destruction products (phosgene) is also important but its relative significance depends on availability of glutathione, other thiols and other nucleophile compounds (hystidine and cysteine). Reduced glutathione is able to eliminate practically all chloroform metabolites that occur under relatively low chloroform levels. A balance between oxidative and reduced trichloromethane metabolism (chloroform in particular), depends on a substance, tissue, dose and oxidative stress intensity. For the CYP2E1 gene, the focus is most often on closely linked polymorphisms as per PstI/RsaI restriction endonucleases when a mutant allele makes for elevated transcription and enzymatic activity. Frequency of this allele is different in different populations; the homozygous allele occurs in 6 % of Asians and 35 % have the heterozygous one whereas the latter occurs only in 6 % of Europeans.

Polymorphism of the GSTM1 gene localized in the chromosome 1 (glutathione-S-transferase of μ class) is associated with two alleles: functional GSTM(+) and nonfunctional null GSTM(−) with substantial deletion due to which protein product is not synthesized at all. The GSTT1 gene localized on the chromosome 22 (glutathione-S-transferase (GST) teta-1) also has two alleles: functional GSTT1(+) and nonfunctional null GSTT1(−) corresponding to partial or complete deletion that results in lower activity of a protein or its complete absence. L.C. Backer with colleagues (2008) established that research participants with GSTT1(−) had higher chloroform levels in blood after showering than GSTT1(+)-participants [20]. Kenneth P. Cantor and others (2010) confirmed a hypothesis in their study that there was a correlation between bladder cancer and genetic polymorphisms of the GSTM1 and GSTZ1 genes [28]. Some studies provide evidence of intrauterine growth retardation in case a mother has polymorphisms of the following genes: CYP2E1, MTHFR (F.C. Infante-Rivard, 2004) [29], CYP2E1 and GSTZ1 (B. Zhou with colleagues, 2018) [30], CYP2E1 (S.G. Bonou, 2017) [31]. The cross-sectional study performed by P. Yang with colleagues (2016) revealed an association between semen quality in a given population under exposure to THMs in water and polymorphisms of the CYP2E1, GSTZ1 and GSTT1 genes [32]. In Caucasians, prevalence of GSTM1(−) and GSTT1(−) genotypes equaled 40–50 % and 10–20 % accordingly [33].

The microsomal epoxide hydrolase is a vital component in xenobiotic metabolism. The gene that codes for it (EPHX1) has two well-known functionally significant polymorphisms able to change the enzyme properties. The first is in the 3rd exon (T337C or tyrosine being replaced with hystidine in position 113 (Tyr113His)) and in the 4th exon (A415G or hystidine being replaced with arginine in position 139 (His139Arg)). The T337C polymorphism reduces the enzyme activity by 50 % (a 'slowing down' allele) and the A415G polymorphism increases it by approximately 25 % (an 'accelerating' allele). Accelerated transformation of natural epoxides into highly active metabolites damages DNA thereby in-creasing the quantity of chromosome aberrations and causing development of several pathologies.

In the Republic of Belarus, so far there have been no studies investigating THMs levels in blood of people provided with tap water from surface water sources as well as studies addressing influence of genetic variability within a population on these levels. In our study, the working hypothesis was that gene polymorphisms of xenobiotic metabolism genes participating in chloroform metabolism could lead to potentially higher chloroform levels in biological media of exposed people under long-term exposure. Ultimately, this may create elevated health risks associated with harmful effects produced by disinfection byproducts on the human body.

The aim of this study was to investigate effects produced by polymorphisms of the CYP2E1, GSTM1, GSTT1, and EPHX1 enzyme genes on disinfection byproducts metabolism and substantiate biomarkers of individual susceptibility to exposure to trihalomethanes (using chloroform as an example) in an exposed population.

Materials and methods. We created two groups of volunteers who lived in Minsk. The test group included exposed people living in Moskovskii and Frunzenskii districts. They were provided with tap water from a surface water source disinfected by chlorination (150 people overall). The reference group included non-exposed people who lived in Pervomaiskii district and were provided with tap water from underground water sources that did not require chlorination (47 people). Both groups were comparable as per age (18–40 years) and sex.

Chloroform levels in tap water were below MPC on the test territory prior to the study and during it; they varied between 0.49 and 0.52 MPC (MPC ≤ 0.2 mg/dm^3). Chloroform was not identified in water distribution networks on the reference territory within the method's sensitivity (< 0.0125 mg/dm^3). Chloroform was measured in water by gas chromatography (LOD for chloroform equals 0.0125 mg/dm^3).

Peripheral blood samples were collected from all the participants in healthcare institutions (polyclinics) in Minsk in spring; sampling was performed in the morning. All the stages in the study conformed to the ethical standards and all the participants gave their informed con-

sent to it; the study also involved questioning them regarding individual water use.

All blood samples were analyzed for chloroform (as biomarkers of exposure) at National Anti-Doping Laboratory using gas chromatography – low resolution mass spectrometry. The method was developed within this research (SOP LM 174-2020 The method for identifying trihalomethanes (chloroform) in biological media) and relied on using AGILENT 7890 gas chromatographer with AGILENT 7000 triple quadrupole GSC/MS system (Thermo Fisher Scientific, USA) [34].

The genotypes of all the participants were examined to identify the A415G polymorphism of the EPHX1 gene, C1091T of the CYP2E1 gene and null mutations of glutathione-S-transferase genes, μ and θ class, (GSTT1(−) и GSTM1(−)). Candidate genes for this study were selected based on pathways of trihalomethane (chloroform) metabolism in the body. Genotype assay was performed in the Scientific and Practical Center for Hygiene. DNA was extracted from blood samples by using Nukleosorb, a commercial reagent kit for DMA extraction, complete set B, produced by Primetech LLC (Belarus) in accordance with the manufacturer instructions. We established genotypes as per target locuses by using the polymerase chain reaction (PCR) in real time mode with the C1000 Touch PCR thermal cycler (BioRad, USA). The obtained results were then analyzed with relevant software. Table 1 provides profiles of polymorphisms, localization of the analyzed genes and used restriction enzymes.

Statistical analysis. We analyzed differences in distribution of genotype and allele frequencies in the test and reference groups by using χ^2. We estimated whether the distribution of the observed genotype and allele frequencies in the analyzed population corresponded to the Hardy – Weinberg equilibrium. Qualitative indicators were given as absolute values and fractions. We applied several conventional indexes to describe qualitative indicators (chloroform levels in blood): Max or maximum values, Min or minimal values; Me or median, upper and lower quartiles (interquartile range) as [P25; P75], 10, 90 and 95 percentiles (P10, P90, P95), and confidence interval 95 % CI. Quantitative data were statistically analyzed considering specific distribution of data: independent samples were compared as per an analyzed indicator with independent t-test and Mann – Whitney U-test. To compare an indicator in several independent groups, we applied the nonparametric Kruskal – Wallis test (H). Odds ratios were calculated for each polymorphism and their combinations considering 95 % confidence interval of identifying higher chloroform levels ($\geq$ P75) in blood of people with certain polymorphisms against those without them.

In this study, the statistical significance was taken at $p < 0.05$. All the data were analyzed with conventional applied statistical software packages Statistica 12.0 and Microsoft Office Excel.

Table 2 shows how genotypes and alleles of the analyzed genetic polymorphisms were distributed in two groups; the distribution corresponded to the Hardy – Weinberg principle.

T a b l e 1

Primer sequences and restriction enzymes used in PCR analysis of target candidate gene polymorphisms

Gene, localization	Polymorphism	Primer sequence, (5' › 3')	Product length, bp	Restriction enzyme	Alleles, bp
CYP2E1 10q24.3	5`-regulatory area C1091T	F CCAGTCGAGTCTACATTGTCA R TTCATTCTGTCTTCTAACTGG	411	RsaI	C (412) T (351, 61)
GSTM1 1q13.3	Deletion	F GAACTCCCTGAAAAGCTAAAGC R GTTGGGCTCAAATATACGGTGG	219	-	norm (219) deletion (0)
GSTT1 22q11.2	Deletion	F TCACCGGATCATGGCCAGCA R TTCCTTACTGGTCCTCACATCTC	459	-	norm (459) deletion (0)
EPHX1 1g42.1	4th exon A415G (His139Arg)	F ACATCCACTTCATCCACGT R ATGCCTCTGAGAAGCCAT	210	RsaI	A (210) G (163, 47)

The A415G polymorphisms of the EPHX1 gene (His139Arg), the C1091T gene CYP2E1,
GSTT1(−) and GSTM1(−): distribution of alleles and genotypes in the test and reference group

Genotypes/ alleles*	Genotype frequency, %		p (for different groups)
	The test group	The reference group	
C1091T CYP2E1			
CC	90.0	95.7	$p = 0.225$
CT	10.0	4.3	
TT	0.0	0.0	
C	95.0	97.9	$p = 0.393$
T	5.0	2.1	
GSTT1			
n	76.7	83.0	$p = 0.361$
del	23.3	17.0	
GSTM1			
n	63.3	63.8	$p = 0.951$
del	36.7	36.2	
A415G EPHX1			
AA	78.0	83.0	$p = 0.461$
GA	12.7	12.8	$p = 0.986$
GG	9.3	4.2	$p = 0.263$
A	84.3	91.3	$p = 0.228$
G	15.7	8.7	

Note: **AA, CC is homozygous wild-type genotype; AG, CT is heterozygous genotype; GG, TT is homozygous mutant genotype; A, C is wild-type allele; G, T is mutant allele; n is wild-type gene; del is homozygous deletion.

The results obtained by investigating distribution of xenobiotic metabolism gene polymorphisms in the test and reference groups are in line with literature data on distribution of xenobiotic metabolism gene polymorphisms in the European population. We examined frequency of the C1091T polymorphic locus of the CYP2E1 gene and detected the heterozygous CT genotype in 10 % of the people in the test group and in 4.3 % of the people in the reference group. The remaining genotypes identified in the participants as per this locus corresponded to the homozygous wild-type CC genotype. Shares of the null GSTM(−) and GSTT1(−) equaled 36.7 % and 23.3 % accordingly in the test group and 36.2 % and 17.0 % in the reference group. Frequency of the 4th exon A415G polymorphism of the EPHX1 gene was analyzed and as a result the polymorphic allele G was established in 15.7 % of the people in the test group; the mutant homozygotes GG genotype, in 9.3 %; and the heterozygous AG genotype, in 12.7 %. The same indicators equaled 8.7 %, 4.2 % and 12.8 % accordingly in the reference group.

Fifty-eight point seven percent of the people in the test group and 51.1 % of the people in the reference one had a mutant homo- and heterozygous genotype at least as per one of four genes: GSTM1, GSTT1, EPHX1, and CYP2E1. In the reference group, a polymorphism of one of four genes was identified in 32.9 % of the cases; polymorphisms of two genes, in 18.7 %; polymorphisms of three genes, in 7.3 %. Polymorphisms of several genes were identified simultaneously in the test and reference groups in the following combinations: 6.7 % and 8.5 % accordingly for the GSTM1+EPHX1; the GSTM1+GSTT1, 5.3 % and 4.3 %; the GSTT1+EPHX1, 2.7 % and 0.0 %; the GSTT1+CYP2E1, 2.0 % and 2.1 %; the EPHX1+CYP2E2, 2.7 % and 2.1 %; the GSTM1+GSTT1+EPHX1, 2.7 % and 2.1 %; the GSTM1+GSTT1+CYP2E1, 2.0 % and 2.1 %; the GSTT1+EPHX1+CYP2E1, 2.7 % and 0.0 %.

Statistically significant differences in relative frequencies of the mutant homozygous, heterozygous and normal homozygous geno-

type were not identified as per the analyzed genes in both groups and all the compared pairs ($p > 0.05$). This indicates that the samples were comparable.

Results and discussion. Chloroform was identified in 100 % of the samples taken in both groups. Its levels varied between 0.03 and 0.54 ng/ml, P95 0.27 ng/ml, in the test group; and between 0.004 and 0.37 ng/ml, P95 0.13 ng/ml, in the reference group. Differences in chloroform levels in blood of unexposed and exposed (through water) people were statistically significant ($U = 2336$, $p < 0.01$) [34]. Chloroform levels identified in blood were not higher (one sample excluded) than reference concentrations (0.0004 mg/l) proposed by Russian experts and established as per aspartate aminotransferase levels in blood (K.V. Chetverkina with colleagues, 2018) [16].

The next stage involved estimating associations between polymorphisms of the relevant genes encoding for xenobiotic metabolism enzymes and chloroform levels in blood. The research article describes identified associations and regularities only for the test group since we did not establish any statistically significant differences between allele distribution in the test and reference groups, but at the same time we established statistically significant differences for basic associations of the analyzed genes polymorphisms with chloroform blood levels for the test group corresponded to those detected in the reference one.

To analyze the associations, results of chloroform identification in blood of the volunteers from the test group were generalized as per 42 conditional sub-groups (variants) depending on the presence / absence of the analyzed gene polymorphisms: the analyzed gene polymorphisms are absent in a genotype (sub-group No. 2); the analyzed gene polymorphisms are present in a genotype (No. 3); one of the analyzed gene polymorphisms (mono) in a genotype (No. 4–7); a gene polymorphism in a genotype combined with other gene polymorphisms (No. 8–11); polymorphisms of two or three genes in different combinations in a genotype (No. 12–22); polymorphisms of

specific genes (No. 23–26) or their specific combinations (No. 27–42) are absent in a genotype. Since the chloroform concentrations were distributed normally only in some sub-groups, further data analysis relied on non-parametric methods. For each sub-group, we calculated a number of samples and their share of the total number of samples in the test group. Chloroform levels were calculated as Max, Min, Me, P25, P75, P10, P90. We also calculated a number of samples in a sub-group (absolute values and their % of the total number of samples in a sub-group) with a chloroform level ≥ P75 and P90 values, ≤ P10, P25, P75 and P90 values for the test group as a whole.

Table 3 provides the data generalized as per sub-groups and chloroform levels in blood of the volunteers from the test group depending on presence (absence) of the analyzed polymorphisms (26 sub-groups that are the most interesting for investigation); the results are visualized as a range chart covering major 19 sub-groups, Figure 1.
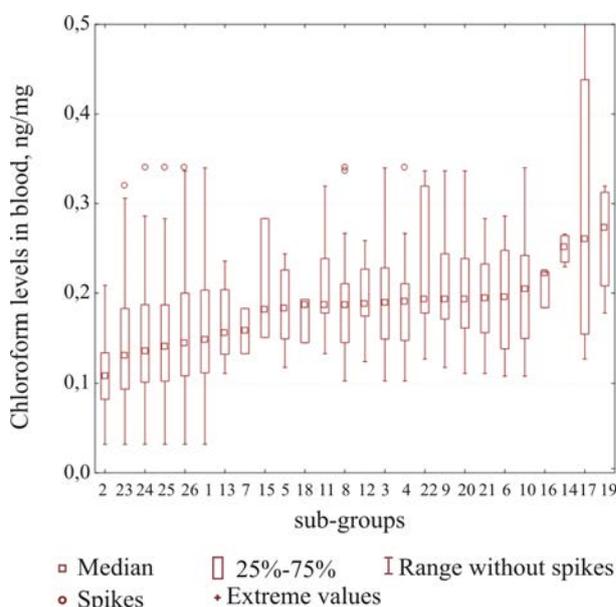

Figure 1. The range chart showing chloroform levels in blood of the volunteers from the test group depending on the presence of polymorphisms of the analyzed xenobiotic metabolism genes CYP2E1, GSTM1, GSTT1, and EPHX1 (the sub-groups are given in the order following the growing median values of chloroform levels)

T a b l e 3

Chloroform levels in blood of the volunteers from the test group, ng/ml

No.	Sub-group as per presence / absence of polymorphisms *(clarification)*	Number of tests	% of the total samples	Me [P25 ÷ P75]	P10	P90	The share of samples (%) with a chloroform levels higher (lower) than the relevant percentile as per the test group as a whole			
							≥ P75	≥ P90	< P10	< P25
1.	The test group as a whole	150	100.0	0.1488 [0.1118 ÷ 0.2035]	0.0819	0.2392	25.3	10.0	10.0	25.3
2.	Polymorphisms are absent	62	41.3	0.1082 [0.0822 ÷ 0.1341]	0.0628	0.1568	4.8	0.0	24.2	56.5
3.	Polymorphisms are present	88	58.7	0.1892 [0.1495 ÷ 0.2281]	0.1310	0.2660	60.2	17.0	0.0	23.9
4.	The GSTM1 polymorphism *(mono)*	30	20.0	0.1903 [0.1480 ÷ 0.2105]	0.1279	0.2351	30.0	6.7	0.0	26.7
5.	The GSTT1 polymorphism *(mono)*	9	6.0	0.1826 [0.1497 ÷ 0.2257]	0.1178	0.2440	44.4	11.1	0.0	22.2
6.	The EPHX1 polymorphism *(mono)*	8	5.3	0.1951 [0.1387 ÷ 0.2478]	0.1081	0.2862	50.0	37.5	0.0	37.5
7.	The CYP2E1 polymorphism *(mono)*	2	1.3	0.1581 [0.1333 ÷ 0.1829]	0.1333	0.1829	0.0	0.0	0.0	50.0
8.	The GSTM1 polymorphism *(not only mono)*	55	36.7	0.1869 [0.1455 ÷ 0.2105]	0.1272	0.2387	30.9	9.1	0.0	27.3
9.	The GSTT1 polymorphism *(not only mono)*	35	23.3	0.1930 [0.1711 ÷ 0.2440]	0.1451	0.3061	48.6	28.6	0.0	14.3
10.	The EPHX1 polymorphism *(not only mono)*	33	22.0	0.2040 [0.1494 ÷ 0.2422]	0.1310	0.3061	54.5	30.3	0.0	24.2
11.	The CYP2E1 polymorphism *(not only mono)*	15	10.0	0.1869 [0.1778 ÷ 0.2385]	0.1455	0.3061	40.0	20.0	0.0	13.3
12.	The GSTM1+GSTT1 polymorphisms	8	5.3	0.1873 [0.1745 ÷ 0.2268]	0.1243	0.2586	37.5	12.5	0.0	12.5
13.	The GSTM1+EPHX1 polymorphisms	10	6.7	0.1556 [0.1326 ÷ 0.2039]	0.1219	0.2230	30.0	0.0	0.0	40.0
14.	The GSTT1+EPHX1 polymorphisms	4	2.7	0.2512 [0.2346 ÷ 0.2643]	0.2294	0.2660	100.0	75.0	0.0	0.0
15.	The GSTT1+CYP2E1 polymorphisms	3	2.0	0.1819 [0.1514 ÷ 0.2832]	0.1514	0.2832	33.3	33.3	0.0	0.0
16.	The EPHX1+CYP2E1 polymorphisms	3	2.0	0.2222 [0.1839 ÷ 0.2237]	0.1839	0.2237	66.7	0.0	0.0	0.0
17.	The GSTM1+GSTT1+EPHX1 polymorphisms	4	2.7	0.2597 [0.1550 ÷ 0.4383]	0.1272	0.5401	50.0	50.0	0.0	25.0
18.	The GSTM1+GSTT1+CYP2E1 polymorphisms	3	2.0	0.1869 [0.1455 ÷ 0.1930]	0.1455	0.1930	0.0	0.0	0.0	33.3
19.	The GSTT1+EPHX1+CYP2E1 polymorphisms	4	2.7	0.2723 [0.2082 ÷ 0.3128]	0.1778	0.3195	75.0	75.0	0.0	0.0
20.	Polymorphisms of more than one gene	39	26.0	0.1930 [0.1617 ÷ 0.2385]	0.1325	0.3061	46.2	23.1	0.0	17.9
21.	Polymorphisms of two genes	28	18.7	0.1944 [0.1565 ÷ 0.2325]	0.1325	0.2626	46.4	17.9	0.0	17.9
22.	Polymorphism of three genes	11	7.3	0.1930 [0.1778 ÷ 0.3195]	0.1455	0.3366	45.5	36.4	0.0	18.2
23.	The GSTM1 polymorphisms are absent	95	63.3	0.1310 [0.0935 ÷ 0.1829]	0.0718	0.2398	22.1	10.5	15.8	63.2
24.	The GSTT1 polymorphism is absent	115	76.7	0.1363 [0.1013 ÷ 0.1872]	0.0773	0.2222	18.3	4.3	13.0	57.4
25.	The EPHX1 polymorphism is absent	117	78.0	0.1414 [0.1025 ÷ 0.1869]	0.0773	0.2225	17.1	4.3	12.8	57.3
26.	The CYP2E1 polymorphism is absent	135	90.0	0.1446 [0.1084 ÷ 0.2000]	0.0812	0.2378	23.7	8.9	11.1	50.4

Chloroform was identified in levels varying between 0.1025 and 0.5401 ng/ml, *Me* = 0.1892 ng/ml [0.1495; 0.2281], P90 = 0.2660 ng/ml, in blood of the exposed volunteers from the test group who had a polymorphism of at least one analyzed gene in their genotype. Chloroform levels were between 0.0321 and 0.2087 ng/ml, *Me* = 0.1082 ng/ml [0.0822; 0.1341], P90 = 0.1568 ng/ml, in blood of the exposed people without the analyzed gene polymorphisms in their genotype. We estimated validity of the established

differences in chloroform levels between the sub-groups with gene polymorphisms and their combinations (No. 3–22) and the sub-group without polymorphisms (No. 2) as per the Mann – Whitney test. The revealed differences were statistically significant ($p < 0.01$) for all the sub-groups, except the sub-group No. 7 'CYP2E1 polymorphism (mono)' (two-sided $p > 0.05$). This might be due to a small number of people in this sub-group. Additionally, we estimated significance of differences between the sub-groups with polymorphisms (No. 3–22) and the sub-groups without relevant polymorphisms or their combinations (the sub-groups No. 23–26 and other 16 sub-groups without any combinations of polymorphisms). The differences turned out to be significant for the sub-groups No. 3–6, 8–11, 14, 19–22 ($p < 0.05$) and to be statistically insignificant for the sub-groups No. 7, 12–13, 15–18 ($p > 0.05$).

We compared differences between 12 basic sub-groups (No. 2, 4–7, 12–19) using the Kruskal – Wallis method and the median test; as a result, the differences turned out to be statistically significant: H (12, N+150) = 80.5, $p < 0.01$, $\chi^2 = 63.5$, cs = 12, $p < 0.01$ (with the Bonferroni correction). Pair comparisons between 16 basic sub-groups (No. 4–19) with the Mann – Whitney test (99 pairs compared overall) established statistically significant differences between chloroform levels in blood of the volunteers in the following sub-groups: No. 4 and No. 14 (U = 10, Z = – 2.66, $p < 0.01$), No. 4 and No. 19 (U = 22, Z = – 2.00, $p < 0.05$), No. 5 and No. 14 (U = 3, Z = – 2.24, $p < 0.05$), No. 12 and No. 14 (U = 3, Z = – 2.12, $p < 0.05$), No. 13 and No. 14 (U = 1, Z = – 2.62, $p < 0.01$), No. 13 and No. 19 (U = 4, Z = – 2.19, $p < 0.05$), No. 13 and No. 9 (U = 100, Z = – 2.03, $p < 0.05$), No. 8 and No. 14 (U = 22, Z = – 2.64, $p < 0.01$), No. 8 and No. 19 (U = 44, Z = – 1.97, $p < 0.05$). Chloroform levels (median ones) were 32 % and 43.1 % lower in the sub-group No. 4 (GSTM1 polymorphism (mono)) against the sub-groups No. 14 (GSTT1+EPHX1 polymorphisms) and No. 19 (GSTT1+EPHX1+CYP2E1 polymorphisms) accordingly. They were also 24.1 %, 61.5 % and 75.0 % lower in the sub-group No. 13 (GSTM1+EPHX1 polymorphisms)

against the sub-groups No. 9 (GSTT1 polymorphism (not only mono)), No. 14 (GSTT1+EPHX1 polymorphisms) and No. 19 (GSTT1+EPHX1+CYP2E1 polymorphisms) accordingly; chloroform levels were also 34.4 % and 45.7 % lower in the sub-group No. 8 (GSTM1 polymorphism (not only mono)) against the sub-groups No. 14 and No. 19. Chloroform levels were higher in blood of the exposed people in the sub-group No. 14 against the sub-groups No. 4, 5, 12, 13 and 8 with differences varying between 23.2 % and 38.1 %, as well as in the sub-group No. 19 against the sub-groups 4, 8 13 with differences varying between 30.2 % and 42.9 % (Figure 2).

We analyzed differences between chloroform levels in blood depending on the presence / absence of a specific gene polymorphism (the results are visualized in Figure 3). Present polymorphisms of the GSTT1 and EPHX1 genes as well as their simultaneous presence leads to a growth in chloroform levels (the sub-groups No. 10, 14, 17, and 19).

We compared how samples with different chloroform levels in blood (< P10, < P25, ≥ P75, ≥ P90 of the total test group) were distributed in the sub-groups. We established that all the samples with chloroform levels < P10 (0.0819 ng/ml) (24.2 % of the total number of samples in the sub-group) and 92.1 % of the samples with chloroform levels < P25 (0.1118 ng/ml) (56.5 % of the sub-group) belonged to the sub-group No. 2 'Polymorphisms are absent' whereas the share of the samples with chloroform levels < P25 was two times lower (23.9 %) in the sub-group 3 'Polymorphisms are present'.

In the test group, 44.7 % of the samples with chloroform levels ≥ than the upper quartile (P75) (0.2035 ng/ml) were taken from people with the GSTT1 or GSTM1 gene polymorphisms; 47.4 %, with the EPHX1 gene polymorphisms; 15.8 %, the CYP2E1 gene polymorphisms; the analyzed polymorphisms were absent only in 7.9 % of such cases. The highest share of the samples with chloroform levels ≥ P75 was identified in the sub-group No. 5 'GSTT1 polymorphism (mono)' (44.4 %), No. 9 'GSTT1 polymorphism (not only mono)' (48.6 %), No. 6 'EPHX1 polymorphism (mono)' and No.17 'GSTM1+GSTT1+EPHX1 polymor-

phisms' (50 %), No. 10 'EPHX1 polymorphism (not only mono)' (54.5 %), No. 3 'Polymorphisms are present' (60.2 %), No. 16 'EPHX1+ CYP2E1 polymorphisms' (66.7 %), No. 19 'GSTT1+ EPHX1+CYP2E1 polymorphisms' (75 %),

No. 14 'GSTT1+EPHX1 polymorphisms' (100 %). The EPHX1 gene polymorphisms were present in genotypes in all the sub-groups with the share of the samples with chloroform levels being ≥ P75 equal to 50 % and higher.
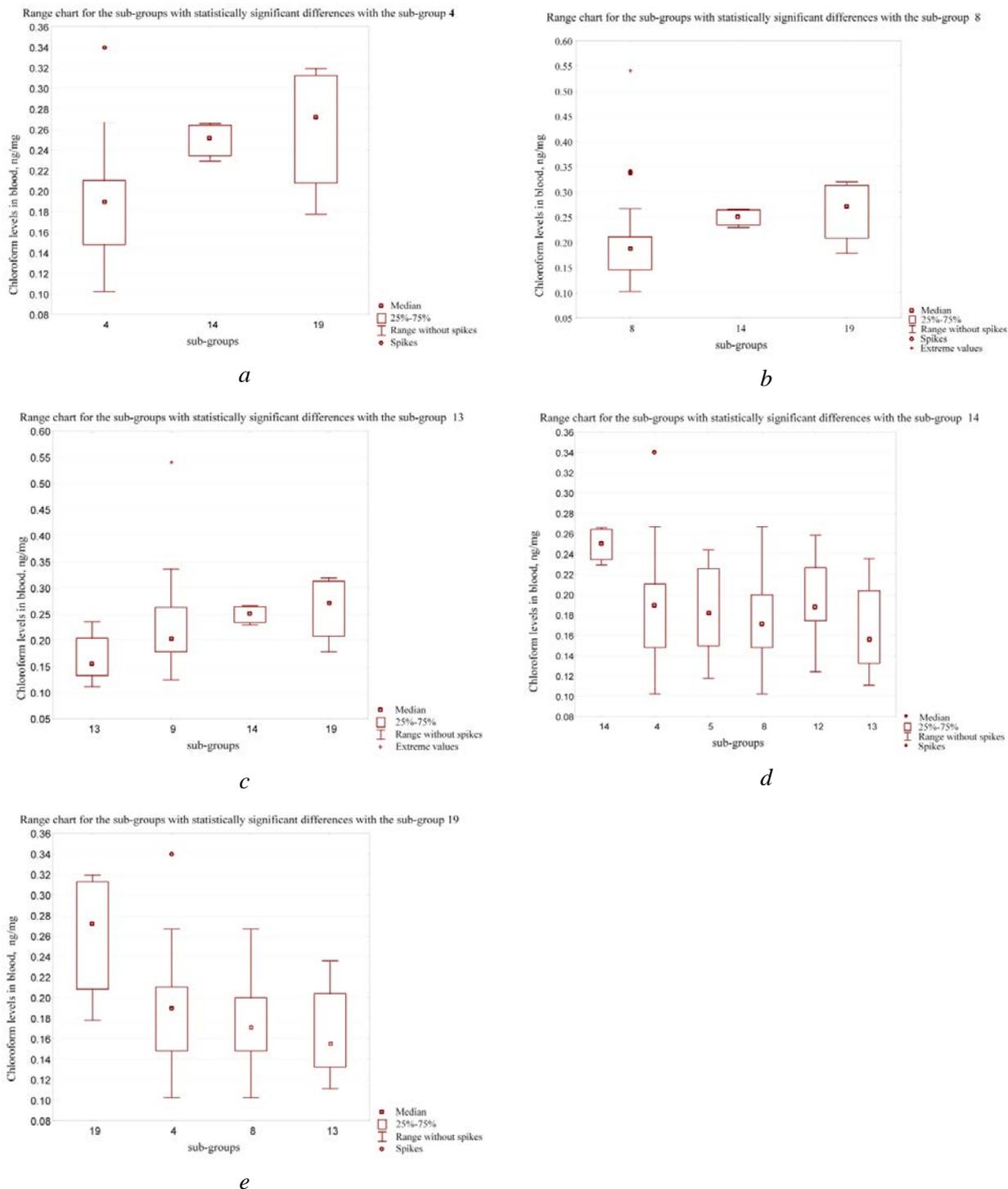

Figure 2. Chloroform levels in blood of the volunteers from the test group: range charts for the sub-groups with statistically significant differences with the sub-groups: *a,* with the sub-group No. 4; *b,* with the sub-group No. 8; *c,* with the sub-group No. 13; *d,* with the sub-group No. 14; *e,* with the sub-group No. 19
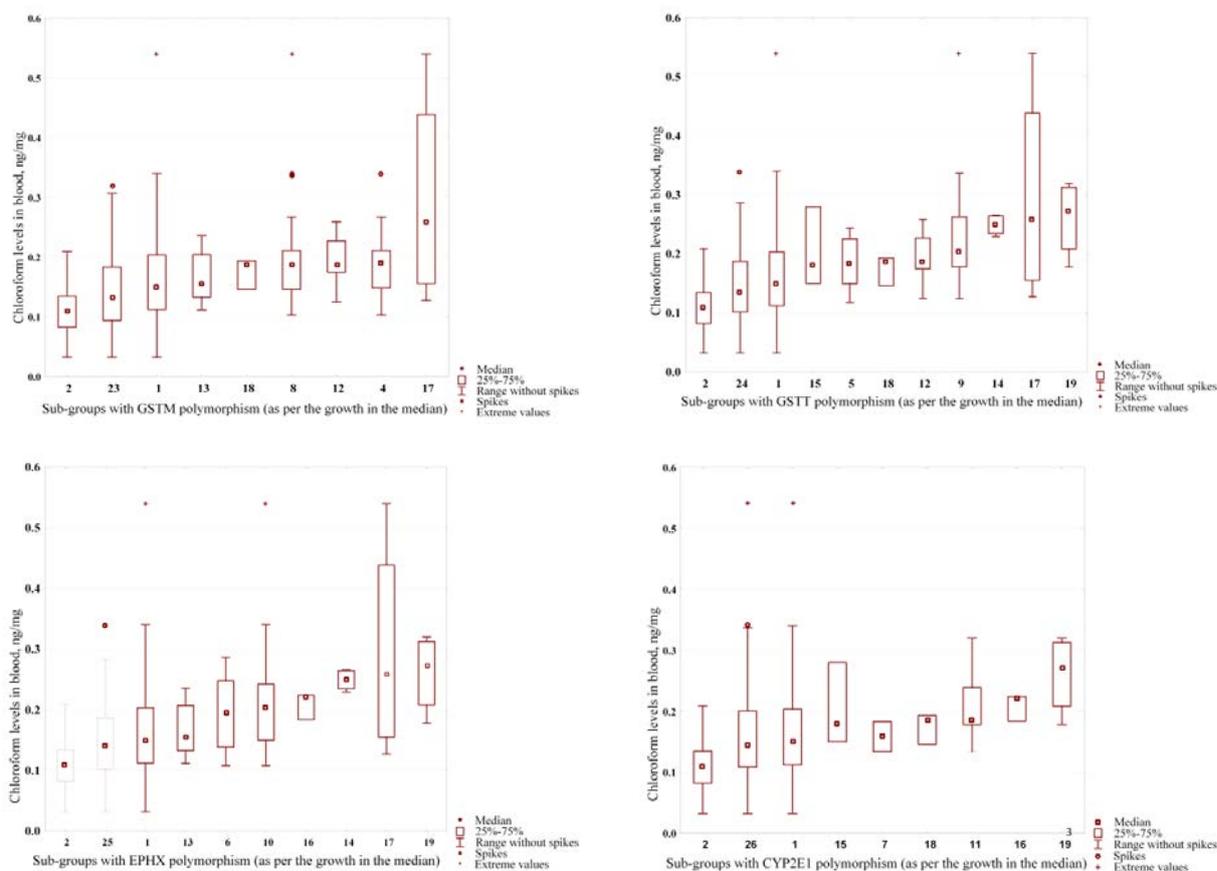
Figure 3. Chloroform levels in blood of the volunteers from the test group: range charts for the sub-groups with present / absent polymorphisms of the CYP2E1, GSTM1, GSTT1, and EPHX1 genes
(the sub-groups are given in a sequence following the growth in the median level)

All the samples with chloroform levels in blood $\geq$ P90 (0.2392 ng/ml) belonged to the volunteers with gene polymorphisms in their genotype; 66.7 % of them had the GSTM1 or EPHX1 gene polymorphisms; 33.3 %, the GSTT1 gene polymorphisms; 20.0 %, the CYP2E1 gene polymorphisms (accordingly 75 % of the samples in the sub-groups No. 13 'GSTT1+EPHX1' and No. 19 'GSTT1+ EPHX1+CYP2E1', 50 % of the samples in the sub-group No. 17 'GSTM1+GSTT1+EPHX1', 37.5 % of the samples in the sub-group No. 6 'EPHX1 polymorphism (mono)'.

We calculated odds ratios (OR) for the analyzed population; the results are given in Table 4. We established that odds ratios (OR) of chloroform levels in blood being $\geq$ P75 were significantly higher for the exposed people with the polymorphisms of the analyzed genes (or their combinations) in their genotype than for their counterparts without any poly-

morphisms (OR = 29.8, 95 % CI: 8.7–102.5). Depending on a specific polymorphism, the differences in the calculated odds ratios varied between 8.4 times (the sub-group No. 4 'GSTM1 polymorphism (mono)') and 59 times (the sub-group No. 19 'GSTT1+ EPHX1+CYP2E1') against the sub-group where the analyzed polymorphisms were absent. The sub-group No. 7 'CYP2E1 polymorphism (mono)' and No. 18 'GSTM1+GSTT1+CYP2E1 polymorphisms' were the only exceptions (chloroform levels were < P75). The detected differences were statistically significant ($p < 0.05$) except the sub-group No. 15. The highest probability of a relatively higher chloroform level in blood ($\geq$ P75) was identified for the cases when the EPHX1 gene polymorphism was present in a genotype, especially in a combination with the GSTT1 gene polymorphism (the differences were statistically significant for all the combinations, $p < 0.05$) (Table 4, Figure 1).

T a b l e 4

Odds ratios (OR) for polymorphic genes and their combinations for chloroform levels in blood being ≥ P75

Sub-group as per presence / absence of polymorphisms *(clarification)*	OR (95 % CI) (for the samples with chloroform levels ≥ P75 with polymorphisms and without them)	Relative risk (RR)
3. Polymorphisms are present	29.8 (8.7–102.5)	12.4
4. GSTM1 polymorphism *(mono)*	8.4 (21–34.1)	6.2
5. GSTT1 polymorphism *(mono)*	15.7 (2.7–90.8)	9.2
6. EPHX1 polymorphism *(mono)*	19.7 (3.2–119.9)	10.3
7. CYP2E1 polymorphism *(mono)*	0*	0.0
8. GSTM1 polymorphism *(not only mono)*	8.8 (2.4–32.1)	6.4
9. GSTT1 polymorphism *(not only mono)*	18.6 (4.9–70.7)	10.0
10. EPHX1 polymorphism *(not only mono)*	23.6 (6.1–90.8)	11.3
11. CYP2E1 polymorphism *(not only mono)*	13.1 (2.8–62.0)	8.3
12. GSTM1+GSTT1 polymorphisms	11.8 (1.9–74.5)	7.8
13. GSTM1+EPHX1 polymorphisms	8.4 (1.4–50.1)	6.2
14. GSTT1+EPHX1 polymorphisms	–**	20.7
15. GSTT1+CYP2E1 polymorphisms	9.8 (0.7–141.4)***	6.9
16. EPHX1+CYP2E1 polymorphisms	39.3 (2.7–565.8)	13.8
17. GSTM1+GSTT1+EPHX1 polymorphisms	19.7 (2.0–191.8)	10.3
18. GSTM1+GSTT1+CYP2E1 polymorphisms	0*	0.0
19. GSTT1+EPHX1+CYP2E1 polymorphisms	59.0 (4.6–750.5)	15.5
20. Polymorphisms of more than one gene	16.9 (4.5–63.1)	9.5
21. Polymorphisms of two genes	51.1 (10.8–241.5)	14.9
22. Polymorphisms of three genes	24.6 (4.3–141.9)	11.5

Note: * all the samples in the sub-groups with these polymorphisms contained chloroform in a concentration below P75, ** means it was impossible to calculate OR since all the samples had chloroform levels > P75 in this sub-group, *** means the established differences are not valid, $p > 0.05$.

Conclusions. In the test group, the polymorphic locus C1091T of the CYP2E1 gene was identified in 10 %, of the cases; null genotypes of the GSTM1 and GSTT1 genes, 36.7 % and 23.3 % accordingly; the 4th exon polymorphism A415G of the EPHX1 gene, 15.7 % (the mutant homozygotes GG accounted for 9.3 % and the heterozygous genotype AG accounted for 12.7 %).

The analyzed polymorphisms (A415G of the EPHX1 gene, C1091T of the CYP2E1, null mutations of the glutathione transferase genes GSTT1 and GSTM1) in a genotype are associated with slower chloroform excretion and metabolism, its accumulation in the body and elevated steady-state chloroform levels in blood of people under long-term exposure to disinfection byproducts in water. Chloroform concentrations in water within MPC (0.2 mg/dm^3) created the following chloroform levels in exposed people's blood:

Me = 0.1892 ng/ml [0.1495; 0.2281] in people with a polymorphism / polymorphisms and Me = 0.1082 ng/ml [0.0822; 0.1341] in people with normal genotypes of the analyzed genes (U = 492, Z = 8.53, $p < 0.0000001$). Chloroform levels (as per the median value) were 80.4 % (and more) higher in blood of people with the A415G polymorphism of the EPHX1 gene; 68.2 % (and more) higher in people with the null allele of the GSTT1 gene; 43.8 % (and more) higher in people with the null allele of the GSTM1 gene (the differences were statistically significant at $p < 0.01$).

Odds ratios (OR) for chloroform levels in blood being ≥ P75 were significantly higher (29.8 times) for exposed people with the analyzed gene polymorphisms (their combinations) than for people without them in their genotypes. The EPHX1 gene polymorphism makes even higher chloroform levels in blood (≥ P75, ≥ P90) much more probable especially

when it is combined with the GSTT1 gene polymorphism.

These established peculiarities make it possible to consider hetero- and homozygous polymorphic AG/GG genotypes as per the EPHX1 gene, CT/TT as per the CYP2E1 gene as well as a null allele in the GSTT1 and GSTM1 genes to be factors of genetic predisposition to chloroform accumulation in the body. The latter stimulates negative health outcomes due to risks associated with chronic exposure to this disinfection byproduct. Given all the above stated, the aforementioned polymorphisms can be used as biomarkers of individual sensitivity when estimating susceptibility to exposure to chloroform that penetrates the body with tap water. They can be also eligible for developing more effective preventive measures.

We suggest using the A415G polymorphism of the EPHX1 gene and deletion of the GSTT1 glutathione transferase gene as well as their combinations, including those with the GSTM1 gene deletion and / or the C1091T polymorphism of the CYP2E1 gene as the most informative biomarkers of sensitivity when assessing health risks associated with exposure to chloroform in tap water when its levels do not exceed maximum permissible ones.

A promising trend in further research would be to investigate associations between exposure to trihalomethanes and diseases with pathogenetic causation considering genetic susceptibility in a given population.

Competing interests. The authors declare no competing interests.

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