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Benchmark dose approach in investigating the relationship between blood metal levels and reproductive hormones: Data set from human study

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ABSTRACT

The main objective of this research was to conduct a dose–response modeling between the internal dose of measured blood Cd, As, Hg, Ni, and Cr and hormonal response of serum testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). The study included 207 male participants from subjects of 5 different cohorts (patients with prostate, testicular, and pancreatic cancer, patients suffering from various thyroid and metabolic disorders, as well as healthy volunteers), enrolled from January 2019 to May 2021 at the Clinical Centre of Serbia in Belgrade, Serbia. Benchmark dose–response modeling analysis was performed with the PROAST software version 70.1, showing the hormone levels as quantal data. The averaging technique was applied to compute the Benchmark dose (BMD) interval (BMDI), with benchmark response set at 10%. Dose-response relationships between metal/metalloid blood concentration and serum hormone levels were confirmed for all the investigated metals/metalloid and hormones. The narrowest BMDI was found for Cd-testosterone and Hg-LH pairs, indicative of high confidence in these estimates. Although further research is needed, the observed findings demonstrate that the BMD approach may prove to be significant in the dose–response modeling of human data.

1. Introduction

Toxic and essential metals and metalloids are naturally present in the Earth's crust. However, they are prevalent in the air, soils, and water due to natural and anthropogenic processes, while pollution increases the quantities of these elements in environmental settings even to hazardous levels (Rahman and Singh, 2019; Vareda et al., 2019). Hence, some metals can be considered noteworthy pollutants, especially in locations subjected to a great deal of anthropogenic pressure (Martí-Cid et al., 2008). Metals may be detected in various items, including food, water, air, cigarette smoke, and alcoholic drinks (Pizent et al., 2012). They can readily leach, spread to new media, and become more bioavailable, allowing them to be absorbed by living organisms (Vareda et al., 2019). Although these elements enter the human body primarily by inhalation and ingestion food is regarded as the primary intake source for most non-occupationally exposed people (Ejaz et al., 2007; Martí-Cid et al., 2008; Satarug et al., 2019). Because these substances are not biodegradable, they accumulate at all stages of the food chain, producing various harmful effects to both the environment and humans (Vareda et al., 2019). Accordingly, it is important to mention that both non-essential and essential elements at specific quantities can be hazardous to live organisms (Vareda et al., 2019). As a result, the link between environmental exposure to arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb), together with nickel (Ni), chromium (Cr), cobalt (Co), and antimony (Sb), and various human illnesses has been widely studied (Buha et al., 2018; Leff et al., 2018; Djordjevic et al., 2019; Huat et al. 2019; Kumar and Sharma, 2019; Sevim et al., 2020; Wallace et al., 2020; Wallace and Buha Djordjevic, 2020; Živančević et al., 2021). Furthermore, the International Agency for Research on Cancer (IARC) has classified several of these metals (As, Cd, Cr, and Ni) as category 1 carcinogens, indicating that they are harmful to humans and/or experimental animals (IARC, 2018a, 2018b, 2012). While Cr(VI) is classified as a human carcinogen (EPA, 1998a), EPA has classified Cr(III) as a Group D, not classifiable as to carcinogenicity in humans (EPA, 1998b).

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Several of these metals have also been classified as endocrine-disrupting chemicals (EDCs), compounds that may potentially impair the activity of the endocrine system (Nascimento et al., 2018). By definition provided by the World Health Organization (WHO), endocrine-disrupting chemicals are 'an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse effects in an intact organism, or its progeny, or (sub)populations' (Bergman et al., 2012). Currently, many challenges in this field of work are being addressed, including improving techniques for the determination of EDCs, controlling low-dose effects of EDCs, the so-called non-monotonic dose–response phenomena (NMDR), together with the relevance of using the threshold concept (Goumenou et al., 2021; Roig et al., 2013). Furthermore, these metals/metalloids represent a significant hazard to human health, especially given that, in day-to-day life, people are exposed to mixtures of many chemicals rather than single substances through various routes of intake (Tsatsakis et al., 2016). EDCs imitate hormones or block their receptors, influencing the entire body and possibly causing numerous chronic illnesses such as diabetes, obesity, thyroid disorders, and different homeostatic imbalances, as well as reproductive toxicity (Buha Djordjevic et al., 2021; Erkekoglu et al., 2021; Nascimento et al., 2018; Sevim and Kara, 2021).

Infertility is considered a worldwide public health issue, while 15% of all reproductive-age couples are affected (Anyanwu and Orisakwe, 2020). Among them, male factors (e.g., poor sperm quality) are responsible for 40% of all reported infertility cases (Anyanwu and Orisakwe, 2020). While men's reproductive health may be compromised due to a combination of genetic, lifestyle, and environmental factors, it has been demonstrated that environmentally relevant metals/metalloids may negatively impact male reproductive function (Pizent et al., 2012; Tariba Lovaković, 2020). Metals/metalloids have been posited to directly interfere with gametogenic cells, Leydig cells, or spermatozoa. These consequences may result in decreased fertility and congenital malformations linked to genetic disorders (Chowdhury, 2009). Various studies in experimental animals have indicated the ability of toxic metals/metalloids to cause an imbalance in reproductive hormones (testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH)), eventually leading to the impairment of male reproductive function. The hypothalamic-pituitary–gonadal axis is essential for the secretion of FSH and LH. In turn, LH stimulates the Leydig cells to produce testosterone, while both FSH and testosterone stimulate the Sertoli cells to generate sperm, later deposited in the epididymis until reaching its maturation (Adedara et al., 2019). Cd has been identified as a reprotoxic element that directly impacts Sertoli and Leydig cells, causing cytotoxic and functional impairment and generating oxidative stress in both somatic and germ cells (Ilieva et al., 2020). Cd has also been shown to diminish sperm count, sperm motility, gene expression of testicular 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase, and serum testosterone level, likely as a result of perturbated redox status parameters, as well as inflammation, and activation of apoptosis in testis (Habib et al., 2019). A substantial drop in enzymatic antioxidant activities has also been demonstrated after the Cd exposure (Alharthi et al., 2020), while even a single dose of this metal was found to lower testicular and epididymal weights in rats, as well as FSH, LH, and testosterone serum concentrations (Onoja et al., 2021). Similarly, As, Hg, Cr, and Ni were shown to cause reproductive impairment and alterations in testosterone, LH, and FSH levels, mainly due to their ability to trigger oxidative stress and dampen antioxidative mechanisms (Abarikwu et al., 2017; Adedara et al., 2019; Albasher et al., 2020; Bashandy et al., 2021; Biswas and Kumar Mukhopadhyay, 2020). An *in vitro* study has demonstrated that Ni can trigger ROS generation in Leydigs cells, directly leading to a decrement in testosterone synthetase expression and testosterone production (Han et al., 2018). Given that humans are constantly exposed to various toxic metals/metalloids at once (Živančević et al., 2021), animal studies have been conducted to explore the reproductive toxicity of metal mixtures. For example, Cd, Hg, and Pb mixture applied orally to the rats for 9 days (20 mg/kg; Cd, 1.61 mg/kg; Hg, 0.40 mg/kg Pb) reduced redox status parameters such as superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH), and increased malondialdehyde (MDA), one of the main biomarkers of lipid peroxidation, as well as Pb, Cd, and Hg testes concentrations. The mixture enhanced irregular sperm morphology and plasma prolactin levels while decreasing epididymal sperm count, viability, FSH, LH, and testosterone levels (Anyanwu et al., 2020). However, animal studies usually test a single chemical, through a single route of administration, in a limited dosing regimen (or even only one high-dose that lacks the physiological relevance to normal human exposure circumstances) for quite a short period, which makes assessing the risk of endocrine disruptors extremely difficult (Goumenou et al., 2021; Hernandez et al., 2019; Pizent et al., 2012). The benefit of utilizing human data in the risk assessment process is that animal-to-human extrapolation is bypassed (Edler et al., 2014; Grandjean and Budtz-Jørgensen, 2013). The benchmark dose (BMD) modeling was created as a manner of fine-tuning the estimate of the point of departure (POD, also known as the reference point (RP)) (Crump, 1984; Haber et al., 2018). BMD is defined as the dosage that corresponds to a particular change in an adverse reaction related to the response in unexposed individuals, while the lower 95 percent confidence limit is considered the BMD level (Dakeishi et al., 2006; Filipsson et al., 2003). Nowadays, BMD modeling is widely accepted in toxicological health risk assessment as the primary method for calculating PODs/RPs and creating the so-called "safe dose" estimates, including reference doses (RfDs), acceptable daily intake values (ADIs), and other comparable values, referred to as "risk values" (Haber et al, 2018). Both the US Environmental Protection Agency (US EPA) and the European Food Safety Authority (EFSA) have suggested the BMD approach as a viable tool for analysing data from epidemiological investigations (Haber et al., 2018).

Hence, this research aimed to analyse the dose–response relationship between the measured concentrations of toxic metals (Cd, Hg, Ni, Cr)/metalloid (As) and serum hormone levels (testosterone, FSH, and LH) in blood samples obtained from a case-control study in male population from Belgrade, Serbia, using the BMD concept.

2. Material and methods

2.1. Study population

The sample collection was conducted at the Clinical Centre of Serbia, Belgrade, Serbia in Belgrade, Serbia, from January 2019 to May 2021. A total of 207 samples were collected from subjects of 5 different cohorts (patients with prostate, testicular, and pancreatic cancer, patients suffering from various thyroid and metabolic disorders, as well as healthy volunteers). The inclusion criterion was age over 18 years. The median age of the participants, who were not occupationally exposed to the investigated toxic metals/metalloid, was 46 (min − 20; max − 94). All participants signed informed consent. The study was designed and conducted according to the Ethical guidelines defined by the Declaration of Helsinki and approved by the Ethical Committee of the Clinical Centre of Serbia (license number 526/9, 31/8 and 579/19), School of Medicine University of Belgrade (license number 1322/XII-5), and the Ethics Committee for Biomedical Research, University of Belgrade – Faculty of Pharmacy (license number 650/2 and 288/2).

2.2. Sample collection and preparation

Blood samples were collected by venepuncture after a 12-h fasting period. Oncology patients had their blood sampled before the surgery and before the start of chemotherapy. Each sample was labelled with an identifying number, the participant's name, surname, and the date and time of venepuncture. For toxic metal determination, an aliquot of blood in Vacutainer tubes containing K2EDTA (BD Vacutainer® system) was used. The K_2EDTA tubes were tested for the presence of toxic metals by

aspirating water type 1 (OmniaTap 6, ASTM Typ l, Stakpure, Niederahr, Germany) using the BD Vacutainer system. They were determined to be Cd, As, Hg, Cr and Ni free. For serum preparation for hormone analyses, blood was obtained in vacutainers without any additives. After the blood coagulation (20 min at ambient temperature), serum separation was conducted by centrifugation at 3000*g* for 30 min. Both EDTA-blood and serum were stored frozen (−20 °C) until the analysis.

2.3. Toxic metal analysis

Blood samples (1 ml of EDTA blood) were used to determine the toxic metals/metalloid (Cd, As, Hg, Cr, Ni). The samples were prepared by the microwave digestion (program: (i) heating for 15 min to 180°C, (ii) maintaining the temperature at 180°C for 15 min, and (iii) ventilation for 15 min) in Teflon containers with 7 ml 65% HNO_3 and 1 ml 30% H_2O_2 (Milestone START D, SK-10 T, Milestone Srl, Sorisole, Italy). After the cooling, samples were quantitatively transferred into a 10 ml volumetric flask. A blank containing 7 ml of 65% HNO_3 and 1 ml of % H_2O_2 was analysed alongside the samples. Graphite furnace atomic absorption spectrophotometry (AAS GTA 120 graphite tube atomizer, 200 series AA, Agilent technologies, Santa Clara, CA, USA) was used for Cd analyses. The ICP-MS method (ICP-MS 7700, Agilent Technologies, Santa Clara, CA, USA) was used to determine As, Hg, Cr, and Ni. An external standard technique (multielement standard solution 1 g/L in diluted nitric acid (Merck, Darmstadt, Germany)) was applied for calibration. The accuracy of both AAS and ICP-MS was validated with standard reference material (SRM) whole blood Level 2 (Seronorm TM, Sero, Billingstad, Norway). The same procedure was applied for SRM preparation and analysis as for the EDTA-blood samples. The trueness of the method was determined by recovery approach using spiked samples (middle range concentration). The trueness ranged, for Cd 89.5% to 103.2%, for As from 97.3 to 114.7%, for Hg from 96.5 to 107.2%, for Cr from 91.6% to 112.7%, and for Ni from 91.9% to 106.2%.

2.4. Hormone analyses

FSH, LH, and testosterone were analysed in serum samples. For hormone assay analysis, chemiluminescent immunoassay (CLIA) and electrochemiluminescent immunoassay (ECLIA) techniques were used on the Liason (DiaSorin Inc, USA) and Cobas e411 analyser families (Roche Ltd., Switzerland), with commercial reagents, according to good laboratory practices. The quantitative determination of FSH and LH is a sandwich chemiluminescent immunoassay, while for testosterone direct, competitive chemiluminescent immunoassay. All tests have been calibrated using appropriate International standards and Reference materials.

2.5. Benchmark dose–response modeling

PROASTweb 70.1 software (https://proastweb.rivm.nl/; Dutch National Institute for Public Health and the Environment, RIVM) was used for BMD modeling, carried out following the software specifications and the EFSA recommendations (Hardy et al., 2017). Metal/metalloid concentrations (Cd, As, Hg, Ni, Cr) were analysed for all the hormonal parameters measured in the study. Hormone levels were analysed as quantal data (0 – hormone level within the reference range, 1 – hormone level outside the reference range). All stated reference ranges are given by the test manufacturers (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim) and based on literature data: Testosterone 2.49–8.36 for men aged 20–49 and 1.93–7.40 ng/ml for men over 50 years of age (Marshall and Tanner, 1970). For FSH, 1.5–12.4 mIU/ml was used as a reference range (Gudeloglu and Parekattil, 2013, Beastall et al. 1987), while for LH 1.7–8.6 mIU/ml was used (Beastall et al. 1987, Ciccone and Kaiser, 2009, Franchimont, 1983). The averaging technique was applied to compute the Benchmark dose (BMD) interval. The BMD method estimates the dosage that will increase incidence based on a statistically significant change in effect previously established, i.e., benchmark response (BMR). BMD denotes the dosing interval corresponding to a set BMR, often 5% or 10%, whereas BMDL denotes the lower confidence limit for the calculated BMD (Hardy et al., 2017). It was suggested that the BMD interval, rather than individual results, be displayed as the outcome of each BMD analysis. A BMDL value is required as a reference point to get a level of substance intake that is unlikely to have adverse effects on human health. To create a BMDU/BMDL connection, a BMDU (upper limit of BMD reliability) value is also needed (Edler et al., 2014). The Benchmark response (BMR) was set at 10% in the modeling procedure (recommended by the software). In the case of quantal response, the size of the effect (i.e. 10%, as applied in the current study) is expressed as additional risk - an absolute change in frequency of response (additional risk in %) divided by the non-affected fraction in the control population (100 minus the background response in %) (Hardy et al., 2017). The software uses a model averaging approach to fit the data into approved models and produce the BMD interval (BMDI) (Hardy et al., 2017). Rather than performing single model studies, we utilized the model averaging technique since it compensates for model and data uncertainties and is highly recommended by the EFSA scientific committee (Hardy et al., 2017). When dose–response was apparent, the model averaging technique combined the data obtained by fitting all accessible models in the program (two-stage, log-logistic, Weibull, log-probabilistic, gamma, exponential, and Hill), delivering the BMDI, as well as the graphical output for each tested parameter (EFSA, 2017). The number of bootstrap runs was set at 200 for the model averaging technique by the default.

3. Results

In Table 1, parameters of descriptive statistics (median with range and geometric mean value) are presented for each of the 5 measured metals/metalloid (Cd, As, Hg, Cr, Ni). The table shows that the highest median and geometric mean was calculated for Ni, followed by Hg. On the other hand, the lowest median was calculated for As and the lowest geometric mean for Cr. Table 2 represents parameters of descriptive statistics for hormones and metals/metalloid (Cd, As, Hg, Cr, Ni) measured in blood samples of the male population from Serbia for each of the cohorts. The highest median for Testosterone was obtained for healthy volunteers. On the other hand, the highest LH and FSH were measured in the prostate and testicular carcinoma cohorts, respectively. The lowest testosterone level was measured in patients with pancreatic carcinoma, while the lowest LH and FSH levels were measured in patients with thyroid and metabolic disorders and healthy volunteers, respectively. The highest As values were measured in the pancreatic carcinoma cohort, while the highest Cd and Hg were measured in the thyroid and metabolic disorders cohort. The highest Cr median value was obtained in the testicular carcinoma cohort, while the highest Ni was measured in healthy subjects. On the other hand, the lowest Cd, Hg, and Cr values were measured in healthy volunteers, while the lowest As and Hg and Ni medians were calculated for prostate carcinoma, pancreatic carcinoma, and thyroid and metabolic disorders cohorts, respectively.

The quantal dose–response relationship between the concentration of five selected metals/metalloid (Cd, As, Hg, Cr, Ni) in blood and serum

Table 1
Descriptive statistics for the selected metals (Cd, As, Hg, Cr, Ni) measured in blood samples of the male population from Serbia.

Metal/metalloid	Median (Range 5% − 95%)	Geometric mean
Cd (μg/L)	1.476 (0.032–4.280)	1.25
As (μg/L)	0.971 (0.004–17.491)	0.747
Hg (μg/L)	5.219 (0.022–48.892)	2.66
Cr (μg/L)	1.324 (0.004–11.522)	0.647
Ni (μg/L)	7.609 (0.005–48.035)	3.32

Table 2
Descriptive statistics for hormones and metals/metalloid (Cd, As, Hg, Cr, Ni) measured in blood samples of the male population from Serbia for each of the cohorts.

	Cohort (number of subjects) Parameter	Healthy volunteers (66)	Prostatic carcinoma (46)	Testicular carcinoma (58)	Pancreatic carcinoma (12)	Thyroid and metabolic disorders (25)
Years	25% Percentile	33.00	62.50	29.00	35.50	45.00
	Median	40.00	68.00	38.00	66.00	60.00
	75% Percentile	56.50	71.00	43.50	70.00	65.50
	Geometric mean	42.01	66.05	36.08	52.38	55.80
Testosterone	25% Percentile	3.963	3.833	2.520	2.920	12.96
(ng/ml)	Median	**5.140**[h]	5.075	4.120	**3.650**[l]	18.97
	75% Percentile	6.638	6.640	5.605	5.050	31.93
	Geometric mean	4.891	4.822	3.577	3.171	19.54
LH	25% Percentile	4.078	4.988	1.710	4.755	2.460
(mIU/ml)	Median	5.490	**6.945**[h]	5.730	6.205	**3.790**[l]
	75% Percentile	8.740	10.16	10.59	14.86	6.300
	Geometric mean	5.924	7.021	3.795	7.722	3.802
FSH	25% Percentile	3.428	5.553	3.450	2.473	3.375
(mIU/ml)	Median	**4.590**[l]	9.025	**10.09**[h]	5.735	4.700
	75% Percentile	7.545	13.78	27.06	9.303	10.35
	Geometric mean	5.461	9.221	6.757	5.616	5.455
Cd	25% Percentile	0.7858	0.9903	2.575	19.35	94.62
(μg/L)	Median	**1.102**[l]	1.322	3.000	20.95	**112.7**[h]
	75% Percentile	1.542	1.880	3.300	31.30	125.8
	Geometric mean	1.404	1.322	2.88	23.26	107.7
As	25% Percentile	0.7085	0.2688	0.4791	13.21	11.49
(μg/L)	Median	1.389	**0.4925**[l]	0.8951	**14.29**[h]	13.10
	75% Percentile	6.528	0.9612	1.133	15.06	15.20
	Geometric mean	1.804	0.5486	0.6343	13.71	13.55
Hg	25% Percentile	1.801	8.393	0.8270	4.293	3.181
(μg/L)	Median	**3.663**[l]	13.92	7.883	7.511	**15.34**[h]
	75% Percentile	6.470	23.44	36.46	11.93	27.94
	Geometric mean	3.201	13.25	5.614	4.865	10.79
Ni	25% Percentile	5.405	6.039	1.539	10.43	1.864
(μg/L)	Median	**14.18**[h]	9.498	3.776	12.05	**3.143**[l]
	75% Percentile	37.24	16.91	10.74	13.72	3.544
	Geometric mean	11.84	10.48	3.834	11.55	2.258
Cr	25% Percentile	0.8461	1.591	0.9488	1.025	0.8523
(μg/L)	Median	**1.139**[l]	2.565	**2.621**[h]	1.418	1.421
	75% Percentile	3.332	4.515	5.187	3.816	2.403
	Geometric mean	1.448	2.577	2.434	1.799	1.388

[h] – highest value
[l] – lowest value.

concentrations of testosterone, LH and FSH was assessed using the BMD-modeling. dose–response relationship was confirmed for all the tested metal-hormone pairs. In a total of 200 iterations, model averaging was conducted using the bootstrap approach, while models were weighed by the Akaike information criterion (AIC) (Jensen et al., 2019). In this approach, the results of various models are integrated and assessed according to model fit, such that models that are more closely related to the data provide more to the assessment. The model weights, which determine the quality of fit of several models to a dose–response data set, are presented in the Table 3 for all the investigated parameters.

For quantal data, the BMR is defined as a specified increase in incidence over the background, while the confidence interval for the estimated background indicates how well it could be determined, given the data provided (EFSA, 2014). The model averaging iteration results were presented as BMDL and BMDU, which reflect the bottom and upper bounds of the 95 percent confidence interval, respectively. The obtained results are presented in Table 4. The lowest BMD was obtained for LH in the case of Cd (BMDL = 0.000112), As (BMDL = 8.77e−05), Cr (BMDL = 1.53e−05) and Ni (BMDL = 2.93e−05). The lowest BMDL was obtained for testosterone in the case of Hg (BMDL = 0,0122), while, in the case of As, the lowest BMDL was obtained for FSH (BMDL = 3.14e−05). However, it should be noted that, in the case of some of these parameters, the estimated BMDI was quite wide (i.e., BMDI for Cr-LH and Ni-LH pairs). The resulting BMDI intervals might be attributed to statistical uncertainties of the models and/or limited data (Hardy et al., 2017). On the other hand, some of the confidence intervals were narrow (less than a factor of 10 intervals), indicating a high certainty in the estimates (Vieira Silva et al., 2021). This was the case with Cd-testosterone and Hg-LH.

Table 3
The models used in model averaging and model weights for all of the investigated hormone-metal pairs.

	Model	Cd	As	Hg	Cr	Ni
Testosterone	two.stage	0.0068	0.0574	0.1693	0.1215	0.1429
	log.logist	0.2102	0.1775	0.0598	0.0279	0.1429
	Weibull	0.1496	0.1498	0.0604	0.0285	0.1429
	log.prob	0.2593	0.1848	0.1798	0.2106	0.1429
	gamma	0.0963	0.1369	0.171	0.2106	0.1429
	EXP	0.1142	0.1454	0.1798	0.2085	0.1429
	HILL	0.1637	0.1483	0.1798	0.1925	0.1429
LH	two.stage	0.0708	0.1431	0.0015	1e-04	0.0753
	log.logist	0.153	0.1431	0.0015	0.0922	0.1727
	Weibull	0.153	0.1431	0.2675	0.0922	0.1693
	log.prob	0.1657	0.1431	0.2675	0.0922	0.1834
	gamma	0.153	0.1416	0.0015	0.2854	0.1611
	EXP	0.15	0.1431	0.2702	0.1971	0.1101
	HILL	0.1545	0.1431	0.1904	0.2408	0.128
FSH	two.stage	0.0373	0.1433	9e-04	0.0093	0.0452
	log.logist	0.1741	0.1433	9e-04	0.1903	0.1888
	Weibull	0.1575	0.1433	0.0011	0.1866	0.1796
	log.prob	0.1905	0.1433	0.4582	0.1903	0.1965
	gamma	0.1454	0.1404	0.2724	0.1722	0.1708
	EXP	0.1439	0.1433	0.1326	0.1045	0.0976
	HILL	0.1513	0.1433	0.1339	0.1468	0.1216

The PROAST program generated bootstrap curves (number of runs: 200), based on model averaging technique, shown in Fig. 1 for testosterone, Fig. 2 for LH, and Fig. 3 for FSH. According to both EFSA and

Table 4
Benchmark dose (BMD) confidence intervals BMDI (BMDL - BMDU) for the selected metals (Cd, As, Hg, Cr, Ni) and hormone concentration measured in blood samples of the male population from Serbia (PROASTweb 70.1 software, https://proastweb.rivm.nl; model averaging method based on quantal BMR of 10%).

	Testosterone (ng/mL)		LH (mIU/mL)		FSH (mIU/mL)	
Metal	BMDL	BMDU	BMDL	BMDU	BMDL	BMDU
Cd (μg/L)	**0.273**	**3.62**	0.000112	9.61	0.00118	4.99
As (μg/L)	0.0209	8.58	8.77e-05	51,900	0.000613	44,200
Hg (μg/L)	0.0122	9270	**4.08**	**55.6**	0.971	41.2
Cr (μg/L)	0.0434	108	1.53e-05	121,000	3.14e-05	45.7
Ni (μg/L)	0.00234	745,000	2.93e-05	43,600	7.06e-05	41,500

BMDL: lower 95% confidence limit of the Benchmark dose; BMDU: upper 95% confidence limit of the Benchmark dose; LH: luteinizing hormone; FSH: follicle-stimulating hormone. Narrow confidence intervals (less than a factor 10) are bolded (high certainty in the estimates).

RIVM, the Monte Carlo error in the confidence interval is roughly 10% after 200 bootstrap iterations, which can be considered acceptable for the purpose of risk assessment (Slob, 2018).

4. Discussion

In the present study, toxic metals/metalloid (Cd, As, Hg, Cr, Ni) were measured in blood samples of the male population from Serbia which consisted of 5 different cohorts (patients with prostate, testicular, and pancreatic cancer, patients suffering from various thyroid and metabolic disorders, as well as healthy volunteers). Indeed, the presence of the cancer itself may influence the level of reproductive hormones. For example, prostate cancer is frequently linked to low testosterone concentrations, while its connection with reduced serum LH and increased FSH has also been suggested (Mearini et al., 2008). This is partially following our study results, which meant slightly lower testosterone and FSH values than the healthy volunteers, and the highest LH level was measured in patients with this type of carcinoma. Furthermore, testicular cancer has also been connected to a decrease in LH and an increase in FSH values (Petersen et al., 1999). This is following our study, where the highest FSH values were noted in patients with this kind of carcinoma. It should also be noted that, in oncology patients, in some cases, chemotherapy and radiotherapy might lower testosterone levels, which would return to normal after the completion of the therapy (Sarfraz et al., 2015). On the contrary, the association between chemotherapy and a rise in the FSH level has been suggested due to Sertoli cell injury and loss of FSH's negative feedback regulation (Delessard et al., 2020). Similarly, LH serum level has been used in clinical practice to evaluate Leydig cell impairment after chemotherapy (Delessard et al., 2020). However, in our study, all oncology patients had their blood sampled before the surgery and before the start of chemotherapy. Therefore, chemotherapy and/or radiation could not affect the level of hormones in their body. The goal of our study was to assess the possible dose–response relationship between metal(loids) levels in blood and serum hormone in the entire investigated population. Hence, in the further steps of our investigation, all cohorts were combined. Although aware of the other factors which might alter hormone level (such as various types of carcinoma, chemotherapy, genetic factors, lifestyle, etc.), by using BMD approach, we aimed to investigate whether low internal levels/doses of toxic metals/metalloids contribute to a higher risk of alterations in hormonal levels, including testosterone, LH and FSH. These changes, in turn, might lead to a wide range of adverse outcomes. No

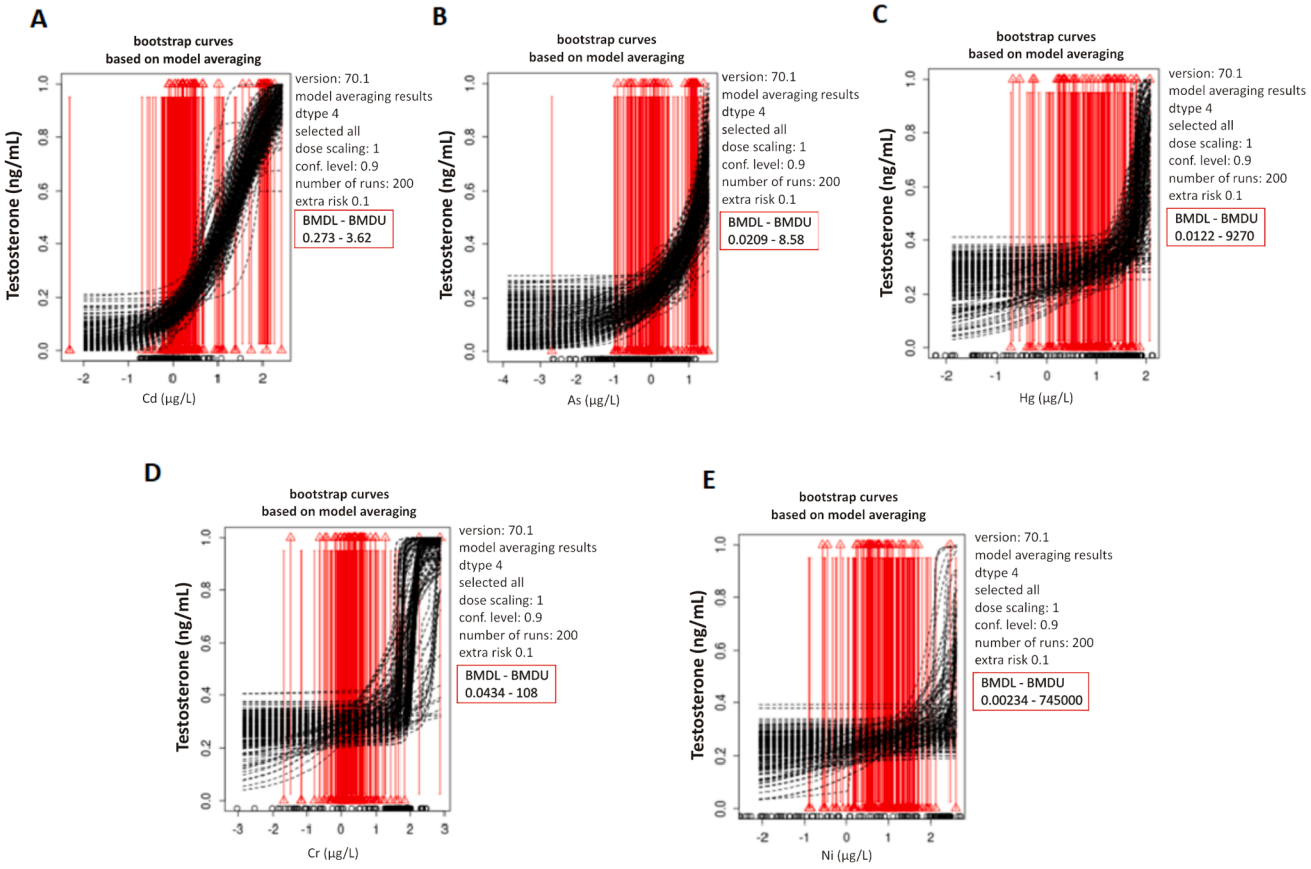

Fig. 1. The dependence of the testosterone serum level (ng/ml) on the concentration of (A) Cd; (B) As; (C) Hg; (D) Cr; and (E) Ni measured in blood samples of the male population from Serbia as variables based on Model averaging. The number of runs: 200.

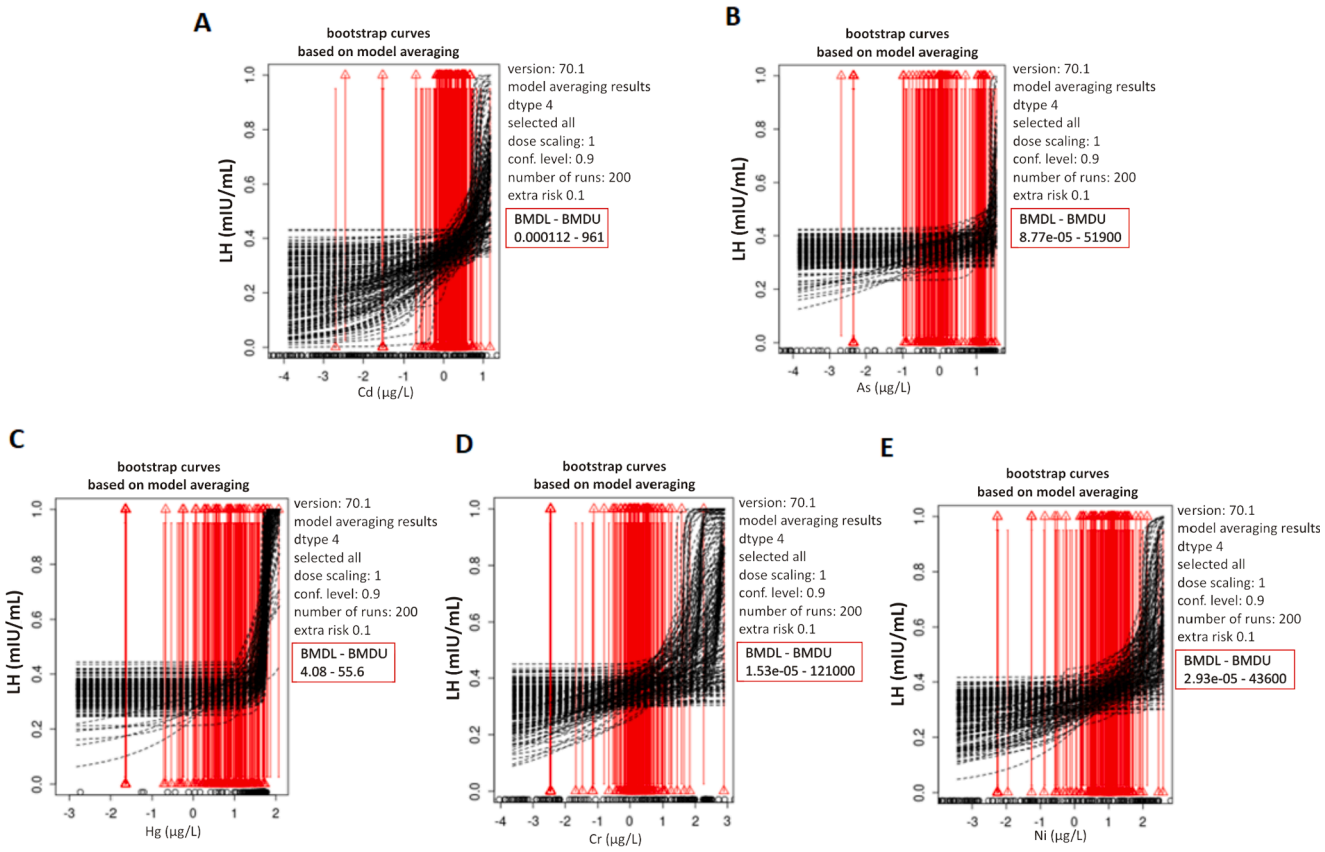

Fig. 2. The dependence of the LH serum level (mIU/mL) on the concentration of (A) Cd; (B) As; (C) Hg; (D) Cr; and (E) Ni measured in blood samples of the male population from Serbia as variables based on Model averaging. The number of runs: 200.

conventional toxicological test which is strictly controlled can correctly reflect a real-life scenario of endocrine disruptor exposure. As a result, sound and validated judgments on the limits of exposure to specific chemicals cannot always be drawn simply on the findings of such test results. An answer might be found in designing long-term experiments in which very low doses are applied. This is especially important in EDCs research because even minimal doses of these substances have been shown to influence endocrine function, while these chemicals can sometimes even be characterized by a non-monotonic dose–response (Lagarde et al., 2015; Renieri et al., 2017; Vandenberg, 2014). Non-monotonic dose–response curves (NMDRCs) are defined as a shift in the direction of the slope of a dose–response relationship over a dose range (Kohn and Melnick, 2002; Lagarde et al., 2015); i.e. maximum reactions can take place at extremely low and very high doses, as well as at midrange dose levels. This demonstrates that data collected from high-dose studies cannot always be utilized to anticipate what sort of biological response would be produced by low-dose research.

The linkage between toxic metals and reproductive hormones has also been drawn from epidemiological data. In a study using National Health and Nutritional Examination Survey (NHANES) data from 1999 to 2004, blood Cd and serum testosterone showed significant positive crude trends in males (Kresovich et al., 2015), as well in the NHANES data from 2011 to 2012 (Lewis and Meeker, 2015). On the other hand, Chen et al. (2016) identified an inverse relationship between this metal and serum testosterone level (Chen et al., 2016). Meeker et al. (2008) found inverse associations between Cd and semen volume. In the same study, As levels in the blood above 5.8 g/L were found to be connected to low sperm motility after adjusting for smoking and age, despite the dose–response pattern in all the groups is not linear (Meeker et al., 2008). Another study conducted by Meeker et al. (2010) demonstrated that in a model adjusted for other metals, As was marked as a notable risk factor for reduced sperm volume. Additionally, As increased the risk of low LH level after adjusting for age, BMI, and current smoking (Meeker et al., 2010). Cr, Ni, and Hg impact on reproductive health mainly was explored through studies covering occupational exposure. Workers exposed to Cr (VI) exhibited substantially greater serum FSH levels and reduced semen concentration and motility (Li et al., 2001). A study of workers exposed to Cr (VI) found a 67% reduction in sperm content and an inverse association between this parameter and Cr levels in the blood. In the same study, workers exposed to Cr and Ni had lower linear sperm motility, while sperm abnormalities had a strong positive connection with blood Ni content (Danadevi et al., 2003). Similarly, in a study that explored endocrine function in Hg exposed workers, a positive correlation was found between the serum total testosterone levels and cumulative Hg exposure (Barregard et al., 1994).

Nevertheless, multivariable linear regression was most commonly used to test the relationship between toxic metals and testosterone/LH/FSH levels in these investigations, which varies from the technique applied in the present study. The proven link between the hazardous agent's internal dosage and the observed reaction of the quantal type suggests that PROAST software can be used to process data from epidemiological studies to achieve appropriate findings.

Although the BMD concept has been applied in various animal research, there is currently limited information on its usage to interpret data from human investigations (Mayfield and Skall, 2018). As an outcome, EFSA recommended investigating the viability of using this method to analyze data from epidemiological studies (Hardy et al., 2017). The benchmark dose (BMD) concept is a method for identifying a potential link between the dose of the test substance and the examined effect. This approach is considered more advanced in comparison with its alternative, No Observed Adverse Effect Level (NOAEL), which has been most widely utilized (Hardy et al., 2017). The benchmark dose

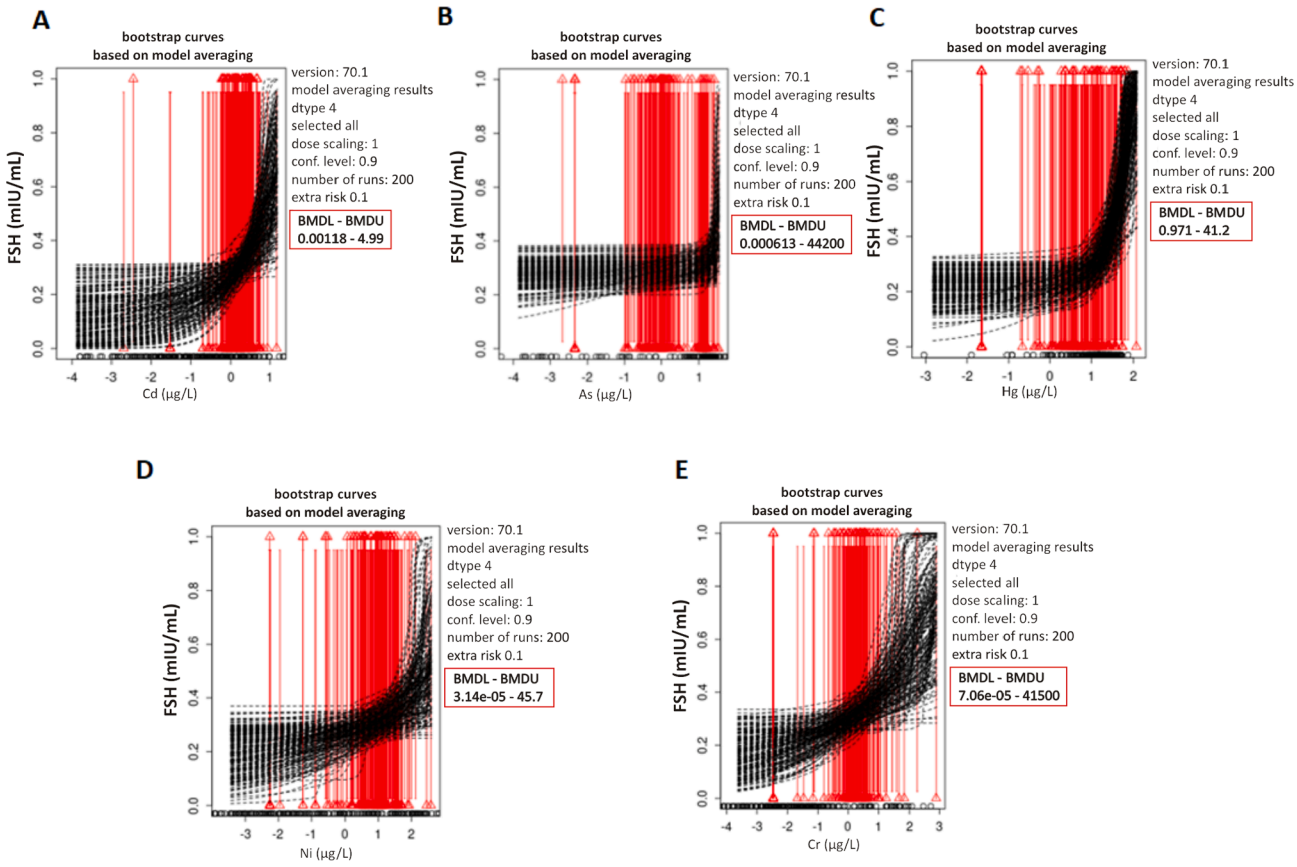

Fig. 3. The dependence of the FSH serum level (mIU/mL) on the concentration of (A) Cd; (B) As; (C) Hg; (D) Cr; and (E) Ni measured in blood samples of the male population from Serbia as variables based on Model averaging. The number of runs: 200.

(BMD) technique, in contrast to the NOAEL approach, has the ability of modeling the whole data set to compute a specific endpoint rather of reflecting a single data point (Hardy et al., 2017). While NOAEL approach produces the highest tested dose that has no adverse effects in the certain experiment, BMD is a statistically calculated concentration that predicts variations in the rate of reaction to an adverse effect (Pink et al., 2020). Additionally, BMD has been recommended by both the U.S. Environmental Protection Agency (US EPA) and European Food Safety Authority (EFSA) as a promising tool in analysing data from epidemiological studies (Haber et al., 2018). Furthermore, EFSA has recommended using BMD approach for epidemiological studies and stated the need for creating a separate guidance document covering the analysis of human data (Hardy et al., 2017). Applying this approach, our previous investigation has attempted to determine the dose–response relationship between Cd measured in breast tissue and serum estradiol levels in 96 women. Having in mind that the number of studies assessing human data using the BMD technique is limited, these results might prove important for future dose–response analysis of the data acquired from such studies (Buha Djordjevic et al., 2021). One of the few studies which applied the BMD concept in the light of human exposure to toxic metals focused on renal failure and sought to determine the linkage with Cd exposure. N-acetyl-beta-D-glucaminidase, beta (2)-microglobulin, retinol-binding protein, and albumin in urine were measured as markers of Cd-induced renal impairment. In contrast, BMDL value of Cd in urine was determined for each of these effect indicators (Jin et al., 2004). Using BMD methodology, Shi et al. (2021) inferred a strong positive association between urinary Cd and typed 2 diabetes mellitus in analysis based on the 1999–2006 NHANES data. In this study, for each population, BMD and BMDL values for 5% BMR were 0.297 (0.198) and 0.190 (0.178) mg/g creatinine, respectively (Shi et al., 2021). Lin et al. (2008) used data from a study on patients who had been occupationally exposed to Pb to calculate the dosage responsible for Pb-induced kidney impairment. Total proteins, beta (2)-microglobulin, and N-acetyl-beta-D-glucaminidase levels in urine were used as indicators of exposure, whereas biomarkers of effect were Pb levels in the blood. BMR was set to 10%, and data were analysed in BMDS software as dichotomized (Lin et al., 2008). However, studies applying the BMD concept to epidemiological data remain limited.

In the current study, it was possible to compute BMD from a human study and obtain the relationship between concentrations of different measured metals and quantal-type response connected to testosterone, LH, and FSH blood levels in the male population. For example, relatively low calculated BMD values indicate even levels lower than 0.273, 0.0209, 0.0122, 0.0434, and 0.00234 µg/L for Cd, As, Hg, Cr, and Ni, respectively, could lead to a 10% increased risk for alterations in testosterone serum levels. The lowest BMDL was observed for Ni, while the narrowest BMDI was observed for Cd (0,273–3,62 µg/L). Another 10-fold BMDI was observed for Hg and LH (4.08–55.6 µg/L), while the narrowest BMDI for FSH was also observed in the case of Hg. In the light of this, it is essential to mention that, in the current investigation, the second-highest median (5.219 µg/L) and geometric mean (2.66 µg/L) values were also obtained for Hg. In comparison, the highest value of these parameters was obtained for Ni (median of 7.609 µg/L and the geometric mean of 3.32 µg/L). Furthermore, for almost all the measured metals/metalloids, median and geometric mean values were higher than the obtained BMDL values. Having in mind that people are daily exposed to toxic metals/metalloids through various sources, such as contaminated air, food, drinking water, and tobacco smoke (Stojsavljević et al., 2019; Vardhan et al., 2019; Živančević et al., 2021), environmental exposure certainly contributes to these internal doses in the general population. However, it is worth noting that several of the generated BMD intervals were quite broad, implying a significant level of

uncertainty when interpreting computed results, especially in the case of Ni and Cr with LH and FSH. Furthermore, it is unclear if investigated 10% increase in risk for alterations of the levels of measured reproductive hormones, which could be considered non-disease-specific markers, is biologically significant, especially when considering that a variety of variables influences their blood levels. These include environmental and genetic and maternal factors, smoking habits, alcohol consumption, and other chemical exposure (Kempenaers et al., 2008). The recent version of PROAST software has included the possibility of dose–response assessment of mixtures. To accomplish this, doses related to various chemicals in a mixture experiment are inserted into the software, along with the doses of the singular compounds. By incorporating a compound as a covariate in the dose–response model, the model is fitted to all compounds in one fit (Van der Ven et al., 2022). However, this type of analysis is currently possible only for controlled toxicity testing and not for epidemiological studies. Nevertheless, the results of the quantal BMD analysis conducted in this study represent a 10% increase in risk for alterations of the levels of measured reproductive hormones in the presence of measured metals/metalloids, meaning that they might contribute to the development of disturbances of the reproductive hormone level, and have to be taken into account, among all the other factors. Finally, despite its limitations, this study implicates EDCs have dose–response behaviour and suggests the BMD method for epidemiological data analysis. Further research on this topic is necessary so that the extrapolation of data from animals to humans can be, at least partly, replaced by the BMD approach in processing data obtained from human studies, thus facilitating risk assessment, especially for EDCs. Moreover, by exploring various potential mechanisms of toxicity (oxidative stress, inflammation, interaction with bioelements, etc.), our future animal research will investigate whether low metal/metalloid doses (extrapolated from obtained human data) may cause adverse effects under the controlled conditions.

5. Conclusion

In this paper, four metals (Cd, Hg, Cr, Ni) and metalloid As have been linked to a higher risk for substantial alterations of reproductive hormone levels (testosterone, LH, and FSH) measured in the blood samples of the male population from Serbia. Dose-response relationship between all the measured metals and hormones was confirmed, highlighting the requirement for more research on the relationship between these metals and their impact on the human body's hormonal balance. The narrowest BMDI was found for Cd-testosterone and Hg-LH pairs, indicating a high certainty in these estimates. Furthermore, medians for these two metals measured in Serbian population were within the range of the corresponding BMD intervals (higher than the BMDL, but lower than the BMLU). These findings are sufficient to warrant further studies on metal-induced reproductive toxicity and highlight the potential of analysing human data by using the suggested BMD technique.

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CRediT authorship contribution statement

Katarina Baralić: Conceptualization, Formal analysis, Investigation, Writing – original draft. **Dragana Javorac:** Formal analysis, Investigation, Data curation. **Đurđica Marić:** Formal analysis, Investigation. **Danijela Đukić-Ćosić:** Formal analysis, Investigation, Supervision. **Zorica Bulat:** Formal analysis, Investigation, Supervision. **Evica Antonijević Miljaković:** Methodology, Investigation. **Milena Anđelković:** Conceptualization, Supervision. **Biljana Antonijević:** Conceptualization, Supervision. **Michael Aschner:** Writing – review & editing, Supervision. **Aleksandra Buha Đorđević:** Conceptualization, Visualization, Data curation, Writing – review & editing, Supervision, Funding acquisition, Project administration.

Declaration of Competing Interest

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

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Further reading

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