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ALAD and *APOE* polymorphisms are associated with lead and mercury levels in Italian pregnant women and their newborns with adequate nutritional status of zinc and selenium

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ABSTRACT

The impacts of single-nucleotide polymorphisms (SNPs) in *ALAD* and *VDR* genes on Pb health effects and/or kinetics are inconclusive at low exposure levels, while studies including *APOE* SNPs are rare. In this study, we examined the associations of *ALAD*, *VDR* and *APOE* SNPs with exposure biomarkers of Pb and other trace elements (TEs) in Italian pregnant women (N = 873, aged 18–44 years) and their newborns (N = 619) with low-level mixed-element exposure through diet, the environment or endogenously. DNA from maternal peripheral venous blood (mB), sampled during the second and third trimesters, was genotyped for *ALAD* (rs1800435, rs1805313, rs1139488, rs818708), VDR (rs2228570, rs1544410, rs7975232, rs731236) and *APOE* (rs429358, rs7421) using TaqMan SNP assays. Personal and lifestyle data and TE levels (mB, maternal plasma, hair and mixed umbilical cord blood [CB]) from the PHIME project were used. Multiple linear regression models, controlling for confounding variables, were performed to test the associations between SNPs and TEs. The geometric means of mB-Pb, mB-Hg, mB-As and mB-Cd (11.0 ng/g, 2.16 ng/g, 1.38 ng/g and 0.31 ng/g, respectively) indicated low exposure levels, whereas maternal plasma Zn and Se (0.72 μg/mL and 78.6 ng/g, respectively) indicated adequate micronutritional status. Variant alleles of *ALA*D rs1800435 and rs1805313 were negatively associated with mB-Pb levels, whereas a positive association was observed for rs1139488. None of the VDR SNPs or their haplotypes had any association with Pb levels. Regarding *APOE*, the ϵ4 allele was associated with lower mB-Hg and CB-Hg, while a positive association was found with the ϵ2 allele and CB-Pb when the model included only newborn girls. The observed associations indicate possible modification effects of *ALAD* and *APOE* SNPs on Pb or Hg kinetics in women and their newborns with low exposure to non-essential TEs, as well as an adequate nutritional status of Zn and Se.

1. Introduction

It is well known that genetic variability within or between populations may play a role in susceptibility and adaptability to metal(loid) toxicity, as genetic background can influence the uptake, accumulation, distribution and retention of a toxicant within the body, as well as any potential toxic effects (Skerfving and Bergdahl, 2015). These gene–environment interactions are especially important for vulnerable populations, including pregnant women, because metal(loid)s, such as lead (Pb), mercury (Hg), arsenic (As) and cadmium (Cd), can be particularly damaging; they can affect not only the mother but also her unborn child. For pregnant women, Pb is especially challenging, as studies suggest that a lifelong accumulation of Pb in the bones can be triggered to release in blood during pregnancy (particularly during the second half of gestation) and passed through the placenta, which may result in cumulative prenatal Pb exposure (Skerfving and Bergdahl,

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2015; ATSDR, 2020). Genetic variability in the aminolevulinate dehydratase (*ALAD*), vitamin D receptor (*VDR*) and apolipoprotein E (*APOE*) genes has been previously reported to affect Pb toxic effects and/or kinetics (Broberg et al., 2015; Ding et al., 2016; Mani et al., 2019). Furthermore, *ALAD* and *VDR* single-nucleotide polymorphisms (SNPs) are both well-established susceptibility biomarkers for Pb toxicity at higher levels of exposure yet with controversial results when exposure levels are low (Broberg et al., 2015; Mani et al., 2019; ATSDR, 2020). Moreover, studies conducted on pregnant populations subjected to low environmental exposure are very scarce.

ALAD is a gene that codes the ALAD enzyme. The latter is also known as porphobilinogen synthase (PBGS), a multimer metalloenzyme that is involved in the haem biosynthetic pathway and contains zinc (Zn)-binding sites essential for its activity (Jaffe, 2020). Compared to Zn, Pb has a significantly higher affinity for this enzyme, making ALAD an important Pb-binding enzyme; when Pb binds to the enzyme, it inhibits it by displacing Zn from its active site (Skerfving and Bergdahl, 2015; ATSDR, 2020). As polymorphisms of the *ALAD* gene can influence the binding affinity of Pb towards ALAD or alter its expression, changes in the amount of bioavailable Pb within the body, as well as the severity of its effects, may result. Several studies have been carried out to assess the influence of *ALAD* polymorphisms on blood Pb levels, but the majority have been conducted on only one, namely, rs1800435 (also known as *ALAD1* for the common allele and *ALAD2* for the variant). At higher exposure levels the variant allele *ALAD2* is associated with higher blood and bone Pb levels representing protective role against toxic effects in other tissues (Kelada et al., 2001; Broberg et al., 2015; Skerfving and Bergdahl, 2015; ATSDR, 2020), however at low exposure levels *ALAD2* was as well found associated with lower blood Pb levels (Tasmin et al., 2015; Stajnko et al., 2020). Recently, other characterised SNPs that could potentially influence Pb concentrations have been identified, including rs1805313, rs1139488 and rs818708 (Szymańska-Chabowska et al., 2015; Warrington et al., 2015; Li et al., 2017; Stajnko et al., 2020). However, their functional significance is less clear at lower as well as at higher Pb exposure levels. The variant alleles of rs1805313 and rs818708 may lower *ALAD* expression levels, the first in blood and liver cells (Warrington et al., 2015) and the second through miRNA (Li et al., 2017). Moreover, variant allele of rs1805313 was associated with lower blood Pb levels in general populations (Warrington et al., 2015; Stajnko et al., 2020). It should also be noted that Pb is not the only element that can substitute Zn within the binding site of the ALAD enzyme. Hg, Cd, As, tin (Sn) and selenium (Se) show similar characteristics, although they have lower affinities and various effects (Bernard and Lauwerys, 1987; Rocha et al., 2012; Braga et al., 2012). Therefore, at low exposure levels, assessing the associations of *ALAD* polymorphisms with a wider set of trace elements (TEs) is important (Baierle et al., 2014).

Vitamin D is a crucial compound in the human body, as it is involved in various biological processes. One notable interaction includes its binding to VDR, which then promotes the expression of various calcium (Ca)-binding proteins. Consequently, VDR plays a significant role in maintaining Ca homeostasis (Onalaja and Claudio, 2000; da Silva Lopes and Abe, 2021). As Pb has similar physico-chemical properties as Ca, it has the ability to bind to the same binding proteins. This results in high Pb concentrations within calcifying tissues, particularly in cases with high Pb exposure and/or nutritional Ca deficiency (Mani et al., 2019). Furthermore, when Ca resources are scarce, the synthesis of Ca-binding proteins is increased, which can modify Pb kinetics (Broberg et al., 2015; Onalaja and Claudio, 2000). Therefore, besides vitamin D and Ca dietary intake, the genetic differences influencing Ca absorption and excretion (da Silva Lopes and Abe, 2021), such as *VDR* polymorphisms, may affect Pb levels. Concerning the *VDR* gene, multiple SNPs have been identified, such as *FokI* (rs2228570), *BsmI* (rs1544410), *ApaI* (rs7975232) and *TaqI* (rs731236). Some studies have demonstrated the influence of the *FokI* and *BsmI* variant alleles on lower VDR expression and consequently on diminished Ca absorption, bone mineral density and blood Pb levels (ATSDR 2020; Broberg et al., 2015; Mani et al., 2019). However, the effects of *BsmI*, *ApaI* and *TaqI* polymorphisms on the toxicokinetics of Pb studied at lower exposure levels gave inconsistent results (Broberg et al., 2015).

ApoE is a multifunctional glycosylated lipid-binding protein that plays a crucial role in lipid metabolism and as a signalling molecule (Getz and Reardon, 2009), depending on the location of apoE protein synthesis. It occurs in three distinct protein isoforms (apoE2, apoE3 and apoE4) encoded by alleles ϵ2, ϵ3 and ϵ4, respectively. Protein isoforms differ from one another by single Cys-Arg interchanges at residues 112 and 158, with E2 harbouring Cys at both sites, E3 having Cys-112 and Arg-158, and E4 having Arg at both sites. These structural differences are related to presumably different impacts on lipid-binding, receptor-binding, antioxidative and metal-binding characteristics (Egert et al., 2012; Kara et al., 2017; Mahley, 1988; Miyata and Smith, 1996; Tudorache et al., 2017; Baierle et al., 2014). Consequently, these polymorphisms can have varying influences on human health across different life stages and conditions, making apoE4 an example of antagonistic pleiotropy in Western-style living populations. For apoE4, it is believed to be associated with better innate immune function (Vitek et al., 2009), higher levels of vitamin D (Huebbe et al., 2011; Soares et al., 2021) and Ca (Huebbe et al., 2011), higher progesterone levels and higher fertility in women of childbearing age (Jasienska et al., 2015). However, among the elderly population, carriers of ϵ4 alleles were associated with a higher risk of developing cardiovascular events and neurodegenerative diseases, such as late-onset sporadic Alzheimer's disease (Egert et al., 2012; Kara et al., 2017; Tudorache et al., 2017). ApoE2 was suggested to be a protective factor for these diseases, a risk factor for type III hyperlipoproteinemia and also as potential risk factor for a high bone turnover (Tudorache et al., 2017; Dieckmann et al., 2013). The potential direct or indirect influence of ApoE isoforms on TE kinetics, sometimes in combination with specific health endpoints, has been observed in various vulnerable population groups, including pregnant women (Trdin et al., 2020), their newborns (Wright et al., 2003; Ng et al., 2013, 2015; Snoj Tratnik et al., 2017; Trdin et al., 2020) and cardiovascular patients (Ding et al., 2016).

In the present study, our main aim was to assess the influence of four *ALAD*, four *VDR* and two *APOE* SNPs on the biomarkers of Pb exposure in Italian pregnant women and their newborns (from the coastal province of Trieste) who participated in the Public Health Impact of Long-Term, Low-Level Mixed Element Exposure in Susceptibility Population Strata (PHIME) study (Valent et al., 2013a). Possible associations with other TEs, particularly Hg because of expected seafood consumption, were also tested. The research is an extension of our previous work (Trdin et al., 2020), where the influence of *APOE* polymorphisms on TEs was tested on a much smaller population (Croatian PHIME cohort) with the different microelement nutritional status compared to present Italian population.

2. Material and methods

2.1. Study population and sampling

During the period 2006–2009, pregnant women were recruited in Italy, Slovenia, Croatia and Greece as part of the PHIME project, which was originally designed to assess exposure to various metals, with a primary interest in seafood consumption and potential Hg exposure. In Italy, the project was approved by the Ethics Committees of the University of Udine and the Institute for Maternal and Child Health, IRCCS Burlo Garofolo in Trieste (CE/V-70-05/02/2007; CE/V-109-12/04/2010) and conducted in accordance with the Declaration of Helsinki. Signed informed consent was obtained from each participant. For the present study, archived maternal peripheral venous blood (mB) samples (N = 873) recruited in the province of Trieste, Italy, between April 2007 and March 2009 were utilised for DNA isolations and genotyping. Additionally, we used questionnaire data and TE concentrations for samples of mB, maternal plasma (mP), maternal hair (mH) and mixed

umbilical cord blood (CB) of their newborns (N = 619). The PHIME project has been described in detail elsewhere, including the recruitment process, exclusion criteria and detailed protocol (Valent et al., 2013a, 2013b; Miklavčič et al., 2013). In brief, pregnant women were enrolled in the study during their second trimester at the Institute for Maternal and Child Health, IRCCS 'Burlo Garofolo'. During recruitment, they gave their informed consent, filled out a short questionnaire and donated a hair sample cut close to the scalp. A blood sample was collected during weeks 20–22, whenever possible. Because of time constraints or other personal reasons, the final time sampling window was wider. The majority (78%) of the pregnant women were sampled for peripheral venous blood during their second trimester (75% between 20 and 21 weeks of gestation, 1% between 18 and 19 weeks, and 2% between 22 and 26 weeks), while 22% gave blood during their third trimester (19% between 31 and 32 weeks of gestation, 2% between 27 and 30 weeks, and 1% between 33 and 36 weeks). Trained research personnel collected mixed CB during delivery. Both were collected in Vacutainer Blue Cup NaH tubes, which were specific for the determination of TEs.

Questionnaires were designed to collect personal details. A brief questionnaire was first completed during pregnancy at enrolment, and then a long questionnaire was given to the participants to be completed independently during the first few weeks following birth. Parity was characterised as the number of pregnancies the women had prior to the current pregnancy, which had surpassed 24 weeks of gestation. Estimation gestation week (EGW) at blood sampling and estimation gestation age (EGA) at delivery were estimated by menstrual history. The frequency of daily seafood intake was estimated from questions determining how often the participants consumed 150 g of different seafood (never, <1 × /month, 1–3 × /month, 1 × /week, 2–4 × /week, 5–6 × /week, 1 × /day, 2–3 × /day or > 3 × /day) (Miklavčič et al., 2013; Valent et al., 2013a).

2.2. Determination of trace elements

TE measurements in mB, CB and mH were performed at the Jožef Stefan Institute, Ljubljana, Slovenia, and in mP at the University Medical Centre Ljubljana, Institute of Clinical Chemistry and Biochemistry, Ljubljana, Slovenia.

Hg levels in mH (N = 867), mB (N = 872) and CB (N = 616), were determined with atomic absorption spectrometry using a direct mercury analyser (Milestone, USA) following thermal combustion (650 °C) and amalgamation (Miklavčič et al., 2013). The limit of detection (LOD), calculated at three times the standard deviation (SD) of blank samples, was determined as 0.02 ng/g when measuring Hg in blood and 0.2 ng/g when measuring Hg in hair.

To determine Pb, Cd, As, Se, Zn, Cu and Mn concentrations in mB (N = 824) and CB (N = 577), the biological samples were prepared according to the method described by Barany et al. (1997) and Jagodic et al. (2017). Briefly, the samples were diluted 10 times with alkaline solution containing Triton X-100 and disodium ethylenediaminetetraacetic acid (EDTA) and then analysed with inductively coupled plasma mass spectrometry (ICP MS) (7500ce, Agilent, Tokyo, Japan), equipped with an octopole reaction system (ORS). The LODs were 1.30, 0.12, 0.13, 5.00, 20.0, 11.0 and 2.00 ng/g for Pb, Cd, As, Se, Zn, Cu and Mn, respectively, in mB and CB.

Zeeman electrothermal atomic absorption spectroscopy (ET-AAS) (Varian SpektrAA-800 ETAA spectrometer) was used to measure Se concentration in mP (N = 841) (Kobal et al., 2004), while flame AAS (Varian SpekrtAA-250 Plus FAAS) was used to measure Zn concentration in mP (N = 838) (Tsalov and Zaprianov, 1983).

For all measurements, strict quality control procedures were followed. On a daily basis, blank samples, control samples and reference materials were measured together with the samples.

2.3. SNP genotyping for ALAD, VDR and APOE

Following the manufacturer's instructions, DNA was extracted from 0.5 mL of mB (N = 873) using the FlexiGene® DNA kit (Qiagen, Hilden, Germany). The quantity and quality of the isolated DNA were determined with an ultraviolet–visible (UV-VIS) spectrophotometer NanoDrop 2000c (Thermo Fisher Scientific, USA). Until genotyping, genomic DNA was stored at −80 °C.

SNP genotyping for *ALAD* (rs1800435, rs1805313, rs1139488 and rs818708), VDR (rs2228570, rs1544410, rs795232 and rs731236) and APOE (rs7412 and rs429358) was performed using pre-designed TaqMan SNP Genotyping Assays (Applied Biosystems, USA). Polymerase chain reaction (PCR) was performed in reaction volumes of 5 μL, which contained 2.5 μL of FastStart Essential DNA Probes Master (Roche, Germany), 1.875 μL of ultrapure nuclease-free water (Life Technologies, USA), 0.125 μL of 44X TaqMan probe/primer mix and 0.5 μL of genomic DNA. The PCR thermal cycling conditions were as follows: one cycle of pre-PCR (50 °C, 2 min), one cycle of activation step (95 °C, 10 min), 50 cycles of annealing and amplification (95 °C, 15 s following 61 °C, 1 min) and one cycle of post-PCR (40 °C, 30 s). For amplification and fluorescence detection, LightCycler® 480 Instrument II and LightCycler480® version 1.5.1 (Roche, Germany) were used. Approximately 10% of the randomly selected samples were repeated as controls within each SNP genotyping. Basic SNP characteristics are given in Table 1.

2.4. Statistics

2.4.1. Population data

Descriptive statistics were used to assess the study cohort's characteristics obtained from the questionnaires (mothers' age, pre-pregnancy body mass index [BMI], parity, education, daily seafood intake, smoking habits, EGW at venous blood sampling, country of birth, employment, marital status, home size, alcohol consumption, intake of supplements, number of amalgam fillings, number of miscarriages, newborn EGA at delivery, newborn sex, length, and weight, and type of delivery) and were expressed in terms of arithmetic means (AM), along with standard deviation (SD) and minimum and maximum values for continuous variables, and in terms of frequency and percentage distribution for categorical variables. The distribution of TEs in different samples was presented with box plots including the median, 25th and 75th percentiles, and outliers, as well as in a table with the AM, SD, range, geometric mean (GM) and 95% confidence interval (CI). When the determined TE values were under the LOD, they were substituted with LOD/2.

2.4.2. Gene SNPs

The frequency of each genotype was presented in the form of a number and a percentage, and it was tested with Pearson's chi squared test for deviation from the Hardy–Weinberg equilibrium (HWE) ($p > 0.05$). Linkage disequilibrium (LD) and the construction of haplotypes between four *ALAD* SNPs and between four *VDR* SNPs were analysed using Haploview (version 4.2, Day Lab at the Broad Institute, Cambridge, USA) (Broad Institute 2018, Accessed January 25, 2022).

2.4.3. Comparisons between TEs and gene SNPs

A comparison of TE levels between carriers and non-carriers of SNP variant alleles, their combination and haplotypes was performed using the Mann-Whitney *U* test and between genotypes using the Kruskal-Wallis test.

2.4.4. Multiple linear regression models

The associations between TE concentrations and gene SNPs were estimated using multiple linear regression models, adjusting for different explanatory variables; all applicable TEs (Pb, Hg, Zn, Se, Cu and Mn for mB; Zn and Se for mP; Hg for mH; Pb, Hg, Zn and Mn for CB) were tested for *ALAD* and *APOE SNPs*, and only Pb in the case of *VDR* SNPs. Several available variables were preliminarily added to the

Table 1
The SNPs studied and their basic characteristics.

GENE	SNP ID	Alternative Name	Chr/Location	Nucleotide Change	Amino Acid Change	TaqMan Assay ID
ALAD	rs1800435[a]	*ALAD1/2, MspI*	9/exon	C > G	Lys > Asn	C_11495146_10
	rs1805313		9/intron	A > G		C_11495186_1_
	rs1139488	*RsaI*	9/exon	A > G	Tyr > Ter	C_3045785_10
	rs818708		9/intron	G > A		C_1632155_20
VDR	rs2228570 (aka rs10735810)	*FokI*	12/exon	A > G (F>f)	Thr > Met	C_12060045_20
	rs1544410	*BsmI*	12/intron	C > T (B>b)		C_8716062_20
	rs7975232	*ApaI*	12/intron	C > A (A>a)		C_28977635_10
	rs731236	*TaqI*	12/exon	A > G (T>t)	Ile > Ile	C_2404008_10
APOE	rs7412		19/exon	C > T	Arg > Cys	C_904973_10
	rs429358		19/exon	T > C	Cys > Arg	C_3084793_20

Chr – chromosome.
[a] In the literature the variant allele (ALAD2) is commonly referred as C allele. However, SNP alleles could be reported regarding the choice of DNA strand (Nelson et al., 2012). In the present study, ALAD2 is given as G allele accordingly to dbSNP, NCBI (NCBI, 2022).

models, including adjustment for accompanying essential and co-exposure TEs. The final selected variables were chosen based on the obtained preliminary modelling results, data quality, known physiological processes during pregnancy, literature data (Bocca et al., 2020; Liang et al., 2019; Liang et al., 2019) and avoidance of possible collinearity. The mB-Zn or CB-Zn level was included in the model as a rough substitution for missing haemoglobin and iron values (Gibson et al., 2008; Houghton et al., 2016). All TEs were log (ln) transformed to approximate a normal distribution.

The associations between each gene SNP and the concentration of each applicable TE in maternal blood were tested in separate models, adjusted for the mother's age, pre-pregnancy BMI, parity (*parous/nulliparous*), education (high school or lower/university or higher), EGW, seafood frequency intake, smoking (yes/no), mB-Zn levels and newborn sex (girls/boys) (Model 1a). To exclude the influence of sampling during two time points in pregnancy (second and third trimesters), which can have a significant impact on TE levels because of physiological changes in the progression of pregnancy (Hertz-Picciotto et al., 2000; ATSDR, 2020), similar multiple linear regressions were performed by including only pregnant women sampled during their second trimester (Model 1b).

The effect of each gene SNP on the concentration of each applicable TE in CB was investigated after controlling for the mother's age, pre-pregnancy BMI, parity (*parous/nulliparous*), education (high school or lower/university or higher), seafood frequency intake, smoking (yes/no), mixed CB-Zn levels and newborn sex (girl/boy), length, weight and EGA (Model 2a). To exclude the possible influence of sex, the associations were tested separately for boys (Models 2b) and girls (Model 2c).

Multiple regression models were performed for allele and genotype categorisations (stratifications) for all *ALAD*, *VDR* and *APOE* SNPs, except for the *ALA*D rs1800435 polymorphism, in which only allele categories were applied because of an insufficient number of participants with the *ALAD*2/2 genotype. For *APOE* categories, the genotype ϵ2/ϵ4 was excluded, as its function can resemble that of ϵ3/ϵ3. Because of the low number, the homozygous ϵ2 and ϵ4 genotypes were combined with the heterozygous ϵ2/ϵ3 and ϵ3/ϵ4 genotypes, respectively.

In cases in which only the dependent/response variable (TE) was log transformed, the confounding variables' estimation coefficients (b) were exponentiated (exp(b)) for easier interpretation. The subtraction of exp (b) from 1 and its multiplication with 100 result in a percentage change in the TE level for a one-unit change in the independent/explanatory variable. When both the dependent and explanatory variables were log transformed, the estimation coefficient (b) displayed can be interpreted as the percentage of change in the dependent variable for every 1% increase in the independent variable (Kelada et al., 2019). The possible multicollinearity of the independent variables was tested using the variance inflation factor.

Models 1a and 1b were also performed on the concentrations of TEs measured in maternal hair and plasma.

All statistical analyses were performed using STATA12/SE (and/or R) statistical software, while OriginPro®version 2020b software (OriginLab Corporation, USA) was used for visualising the results. The level of statistical significance (p-value) was set to ≤ 0.05, and the level of marginal significance was set to > 0.05 and ≤ 0.1.

3. Results

3.1. Study population

All the general characteristics of the study population are presented in Table 2. The average age of the women at recruitment was 32.7 years, and the majority of them were born in Italy (92.6%). The majority did not smoke during pregnancy (88.9%), and, on average, they consumed 0.35 portions of seafood per day. The majority were employed (84.6%) and were married or living together with a partner (90.0%). 57.7% were *nulliparous*, which meant that their current pregnancy was their first beyond 24 weeks of gestation. 33.8% were *primiparous*, and the remainder were *multiparous*. None of the women were *grand multipara* ($n \geq 5$) with increased risks of any obstetric complication, neonatal morbidity or perinatal death. On average, the women gave birth on week 39.4 ± 1.4 of their pregnancy ($N = 743$), with 2.56% of the births being pre-term, 97.3% being full term and no pregnancies being overdue.

3.2. TE levels

Fig. 1 presents the distribution of TEs (Pb, Hg, Cd, As, Se, Zn, Cu and Mn) in the samples of mB, mP, mH and CB in the form of box plots; a table including AMs, SDs, ranges, GMs and 95% CIs and number of samples can be found in the supplementary data (Table A1). Table A1a presents the data for all maternal samples, stratified by sampling time (second vs third trimesters), while Table A1b presents the CB data for all newborns, stratified by gender. In general, the concentrations of potentially toxic non-essential TEs (Pb, Hg, Cd and As) were low.

The GMs (95% CI) for Pb in mB and CB were 11.0 ng/g (10.7–11.3 ng/g) and 10.4 ng/g (10.0–10.9 ng/g), respectively. Only two women exceeded the recommended mB-Pb value for pregnant women, which was set at 50 ng/mL (CDCCenters for Disease Control and Prevention, 2010; Taylor et al., 2014), while 8% had a value greater than 20 ng/mL, which was recently discussed as a possible new recommendation value (ATSDR, 2020) because of the unknown threshold of subclinical toxicity for Pb.

The GMs (95% CI) for Hg in mB and CB were 2.16 ng/g (2.04–2.30 ng/g) and 3.88 ng/g (3.62–4.16 ng/g), respectively. Ten percent of the women exceeded the EPA's recommended mB-Hg level for the maximum oral reference dose (0.1 μg/kg per day), which is at 5.8 ng/mL (Taylor et al., 2014). Hg was mostly present in the form of methyl Hg (MeHg), as reported by Miklavčič et al. (2013), according to a speciation

Table 2
General characteristics of the participants.

Participants	AM ± SD (min-max)	%	N
MOTHERS (m)			873
mAge (years)	32.7 ± 4.6 (18–44)		873
mPre-pregnancy BMI (kg/m^2)	22.5 ± 0.1 (15.6–46.7)	100	873
Underweight (<18.5)		7.67	67
Normal (18.5 – < 25)		72.3	631
Overweigh (25 – < 30)		14.5	127
Obesity (30 – < 40)		5.27	46
Severe obesity (≥40)		0.2	2
mParity		100	872
0		57.7	503
1		33.8	295
2		6.77	59
3		1.15	10
4		0.34	3
mEducation		100	764
Elem. or high school/University or higher		65.7/34.3	502/262
mYears of education		100	873
<5		0	0
5		0.23	2
6–8		7.90	69
9–13		46.8	409
14–19		38.3	334
20+		6.76	59
mSeafood intake (150 g portion/day)	0.35 ± 0.25 (0–1.57)		762
mSmoking during pregnancy		100	873
Yes/No		11.1/88.9	97/776
mNumber of cigarettes/day during pregnancy		100	873
0 – < 5		92.2	805
5 – < 10		4.81	42
10–20		2.98	26
mEGW (weeks)		100	871
2nd trimester (14–26)/3rd trimester (27–40)	20.6 ± 0.6 /31 ± 1.1 (18–26)/(27–36)	78.0/22.0	679/192
mCountry of birth		100	869
Italy/Other		92.6/7.36	805/64
mEmployment		100	733
Yes/No		84.6/15.4	620/113
mMarital status		100	737
Married or living together/Single or separated		90.0/10.0	663/74
mHome size		100	735
<50 m^2		6.94	51
50–100 m^2		68.6	504
> 100 m^2		24.5	180
mAlcohol consumption during pregnancy (drinks per week)		100	870
<4/>4		96.0/4.02	835/35
mSupplements intake		100	873
Yes/No		70.3/29.7	614/259
mNumber of amalgam fillings		100	582
<3		20.5	119
3–5		32.0	186
6–9		32.5	189
> 9		15.1	88
mNumber of miscarriages		100	873
0		80.9	706
1		15.6	136
2		2.63	23
3+		0.92	8
NEWBORNS (c)			619
cEGA (weeks)	39.5 ± 1.4 (30–42)	100	557
Pre-term (<37)		1.97	11
Boys/Girls		1.26/0.72	7/4
Full term (37–42)		98.0	546
Boys/Girls		51.5/46.5	287/259
Post-term (> 42)		0	0
cSex		100	571
Boys/Girls		52.3/47.6	299/272
cLength (cm)	50.1 ± 2.1 (30–42)		567
Boys	50.4 ± 2.2		296

(*continued on next page*)

Table 2 (*continued*)

Participants		AM ± SD (min-max)	%	N
		(30–42)		
	Girls	49.7 ± 2.1		271
		(42–54)		
cWeight (g)		3403 ± 469		570
		(1.450–5140)		
	Boys	3462 ± 456		299
		(1.980–4930)		
	Girls	3339 ± 476		271
		(1450–5140)		
cType of delivery			100	564
Vaginal			80.9	456
	Boys/Girls		41.3/39.5	233/233
Caesarean			19.1	108
	Boys/Girls		11.0/8.16	62/46

AM – arithmetic mean; SD – standard deviation; min – minimum; max – maximum; EGW – estimated gestation week of pregnancy at maternal blood sampling; EGA – estimated gestational age at delivery; Elem. - elementary; cLength – newborn length at birth; cWeight – newborn weight at birth.

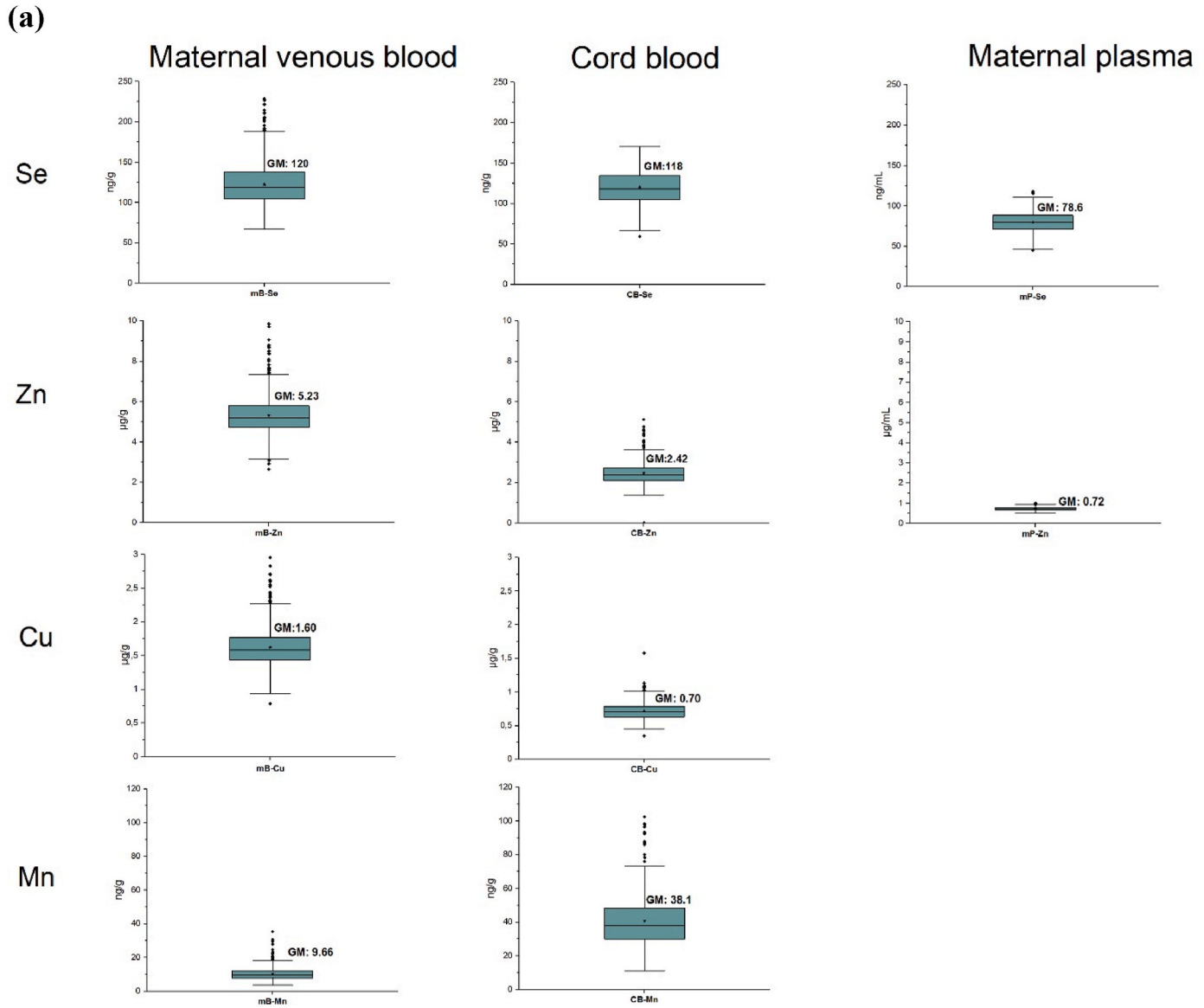
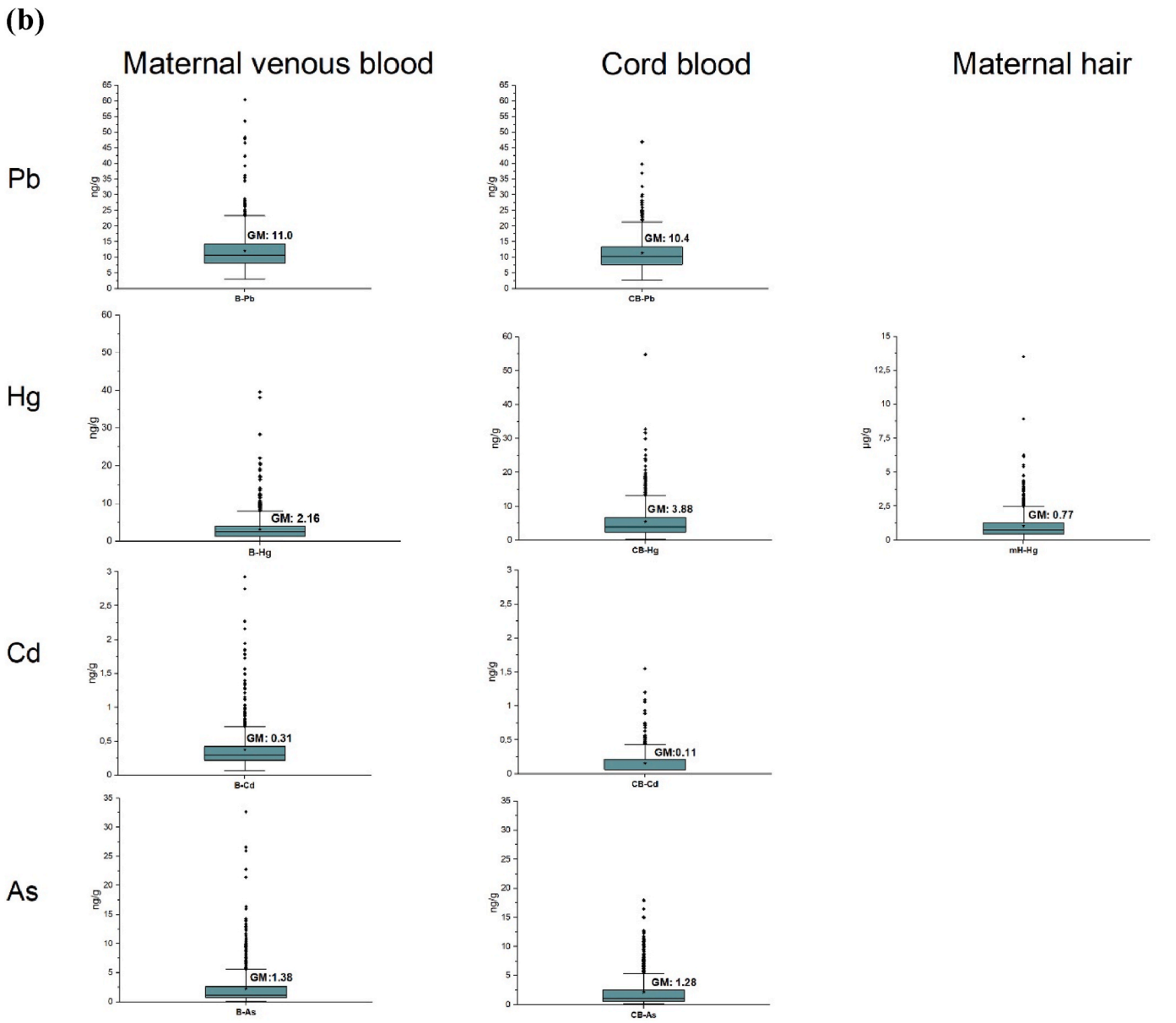


Fig. 1. Distribution of essential (a) and non-essential (b) TEs in maternal blood, plasma, hair and mixed umbilical CB presenting the median, AMs, 25th and 75th percentiles, outliers and GMs.



mB – maternal peripheral venous blood; CB – mixed umbilical cord blood; GM – geometrical mean; mH – maternal hair: mP – maternal plasma; TE trace elements.

Fig. 1. (*continued*).

analysis performed in a subset of samples. The reported MeHg percentages in the samples of maternal blood, newborns' mixed umbilical CB and maternal hair were 91% (N = 223), 99% (N = 330) and 100% (N = 323), respectively.

Cd concentrations in mB and mixed CB were even below the LOD in some cases. The GM (95% CI) for mB-Cd was 0.31 ng/g (0.29–0.32 ng/g), and that for CB-Cd was 0.11 ng/g (0.10–0.11 ng/g). Four percent of the mB-Cd samples and 62% of the CB-Cd samples were under the LOD. Both means were well below the blood reference value for non-smoking adults, which is 1 ng/g (Taylor et al., 2014).

The GM (95% CI) of mB-As was 1.38 ng/g (1.29–1.47 ng/g), and that of mixed CB-As was 1.28 ng/g (1.18–1.39 ng/g). Because of the fast clearance of As in blood, blood levels are not reliable indicators of chronic exposure to low levels of As (ATSDR, 2007). The only credible indicator is As species measured in urine which was not performed for the Italian PHIME study population. Data exists for 136 Croatian PHIME study participants living in the region of Rijeka, geographically close to the region of Trieste, who had similar access to seafood from the North Adriatic Sea, similar daily seafood intake frequency (GM 0.31, range 0–2.21; number of 150 g servings per day) and slightly higher mB-As during the third trimester (GM 2.59, 95% CI: 2.12–3.15 ng/g) (Stajnko et al., 2019). The majority of As in urine was present in the form of non-toxic, biologically inactive arsenobetaine, the major form of As in seafood (Fowler et al., 2015). Evidently, the exposure to toxic inorganic As was negligible in the case of the Croatian and, in all likelihood also, the Italian PHIME study populations.

Regarding essential TEs, Zn and Se were of particular importance, primarily because of their close connection with ALAD and secondarily because of their known protective (mutually antagonistic) interactions with several non-essential TEs, including Pb (Skerfving and Bergdahl, 2015; Bi et al., 2019). Both are also constituents of several enzymes that regulate cellular redox status and take part in cellular antioxidative processes. The GM (95% CI) for mB-Se was 120 ng/g (118–122 ng/g), that for mP-Se was 78.6 ng/g (77.7–79.5 ng/mL) ng/mL, and that for

Table 3
Maternal genotype and minor allele frequencies (MAFs) of the studied SNPs compared with the MAFs reported for populations with European ancestry.

Gene	SNP ID	Genotype	N (%)	MAF %	MAF EU %	HWE p-value
ALAD	rs1800435		872 (100)	10	8	0.531
		CC	703 (80.6)			
		CG	158 (18.1)			
		GG	11 (1.26)			
	rs1805313		871 (100)	35	31	0.580
		AA	367 (42.1)			
		AG	391 (44.9)			
		GG	113 (13.0)			
	rs1139488		870 (100)	35	37	0.638
		AA	369 (42.4)			
		AG	400 (46.0)			
		GG	101 (11.6)			
	rs818708		872 (100)	48	45	0.146
		GG	245 (28.1)			
		GA	414 (47.5)			
		AA	213 (24.4)			
VDR	rs2228570 (*FokI*)		867 (100)	35	38	0.875
		GG	366 (42.2)			
		GA	393 (45.3)			
		AA	108 (12.5)			
	rs1544410 (*BsmI*)		867 (100)	39	40	0.543
		CC	324 (37.4)			
		CT	405 (46.7)			
		TT	138 (15.9)			
	rs7975232 (*ApaI*)		863 (100)	47	40	0.315
		AA	252 (29.2)			
		AC	415 (48.1)			
		CC	196 (22.7)			
	rs731236 (*TaqI*)		867 (100)	38	40	0.413
		AA	342 (39.5)			
		AG	396 (45.7)			
		GG	129 (14.9)			
APOE	rs7412		863 (100)	6	6	0.394
		CC	758 (87.8)			
		CT	100 (11.6)			
		TT	5 (0.58)			
	rs429358		870 (100)	8	10*	0.772
		TT	735 (84.5)			
		TC	130 (14.9)			
		CC	5 (0.57)			

MAF – minor allele frequency for the studied population; MAF EU – minor allele frequency in populations with European ancestry from the 1000 Genome Project (SNP Database, NCBI, 2021) for ALAD and VDR; *given the high variation in the geographic distribution of *APOE*, mostly as a result of *APOE* rs429358 SNP variation (Graeser et al., 2012), we obtained this SNP MAF for Italian population from Tuscany included in the HapMap project (HapMap, CEU, TSI) (NCBINational Center for Biotechnology Information, 2022); HWE – p-values for the Hardy–Weinberg equilibrium for the studied population; N – number of observations.

Table 4
Maternal genotype and allele frequencies of *APOE* in the studied population and comparable literature values from the Italian population.

APOE	Genotype or Allele	Frequencies of Studied Population N (%)	Frequencies of Comparable Italian Population (Simonelli et al., 2001) N (%)
	Genotype	863 (100)	1235 (100)
	ϵ2/ϵ2	5 (0.6)	(1.0)
	ϵ2/ϵ3	92 (10.7)	(8.8)
	ϵ2/ϵ4	8 (0.9)	(1.4)
	ϵ3/ϵ3	632 (73.2)	(69.8)
	ϵ3/ϵ4	121 (14.0)	(18.4)
	ϵ4/ϵ4	5 (0.6)	(0.6)
	Allele*	1726 (100)	2470 (100)
	ϵ2 (rs7412-T, rs429358-T)	110 (6.4)	(6.1)
	ϵ3 (rs7412-C, rs429358-T)	1477 (85.6)	(83.4)
	ϵ4 (rs7412-C, rs429359-C)	139 (8.1)	(10.5)

N – number of observations; * - so-called ε alleles are in fact haplotypes which are in literature almost regularly presented as alleles.

CB-Se was 118 ng/g (117–120 ng/g). The mP-Se GMs (95% CI) were within the European mean levels for pregnant women, and they exceeded the level believed to be sufficient for normal plasma glutathione peroxidase (GPx3) activity (2/3 of maximal activity, 62 ng/mL) (Thomson, 2004). According to Thomson (2004), the observed Se GMs could be sufficient for the normal functioning of several blood GPxs (GPx1, GPx3 and GPx4), selenoprotein P and probably other selenoproteins as well. The GM (95% CI) for mB-Zn was 5.23 μg/g (5.17–5.29 μg/g), and that for P–Zn was 0.72 μg/mL (0.72–0.73 μg/mL), which indicate a good micronutrient status, as not even one woman had plasma levels below the normal reference range reported by Abbassi-Ghanavati et al. (2009) for pregnant women: 0.51–0.80 and 0.50–0.77 μg/mL for the second and third trimesters of pregnancy, respectively. In our population, the GM (95% CI) for mP-Zn was 0.72 μg/mL (0.72–0.73 μg/mL) in the second trimester and 0.71 μg/mL (0.70–0.72 μg/mL) in the third trimester.

3.3. Maternal genotype and allele frequencies of selected SNPs for ALAD, VDR and APOE

Maternal blood DNA was genotyped for the selected SNPs in three different genes: *ALAD*, *VDR* and *APOE*. For all the selected SNPs, the distribution of genotypes was consistent with the HWE and can be found

Table 5
ALAD combinations.

ALAD combination	*ALAD* rs1800435	*ALAD* rs1805313	*ALAD* rs1139488	N (%)	mB-Pb GM (ng/g)
ALADcomb0	CC	AA	AG, GG	181 (79.6)	11.8
ALADcomb1	CG, GG	AG, GG	AA	47 (20.6)	9.4

N – number of observations; mB – maternal venous whole blood.

in Table 3, together with the variant allele frequencies (MAFs) of the studied population and, for comparison, the existing values for the populations with European ancestry (NCBINational Center for Biotechnology Information, 2022).

We found no LD between the analysed *ALAD* SNPs (Figure A1a). However, we could construct four *VDR* haplotypes (VDR-H1, VDR-H2, VDR-H3 and VDR-H4), including three SNPs out of four (*TaqI*, *ApaI* and *BsmI*, Figure A1b). VDR haplotypes frequencies are given in Table A2. In the TEs statistical analysis, the homozygous carriers of the common allele for *TaqI*, *ApaI* and *BsmI* (VDR-H1) were compared to the homozygous carriers of the variant allele, which is actually exactly the same sequence as in the second formed haplotype (VDR-H2).

Two different SNPs located within the *APOE* gene form six genotypes (ϵ2/ϵ2, ϵ2/ϵ3, ϵ2/ϵ4, ϵ3/ϵ3, ϵ3/ϵ4 and ϵ4/ϵ4), and their frequencies (%) and for comparison the existing values for Italian population (Simonelli et al., 2001) are listed in Table 4.

3.4. ALAD, VDR and APOE SNPs and TE levels

3.4.1. Comparison of TE levels according to maternal alleles and/or genotypes (bivariate analysis)

A simple comparison of all measured TE levels (GM, 95% CI, AM, SD, P50, min-max) between the carriers and non-carriers of variant alleles and/or between the genotypes for each specific SNP was conducted, except As as an appropriate indicator of inorganic As exposure. The results are presented as supplements in Tables A3a (maternal samples) and A3b (CB samples). Statistically significant differences in TE distribution among gene alleles and/or genotypes were observed between *ALA*D rs1800435 SNP and mB-Pb (p 0.048), ALAD rs818708 and mB-Cu (p 0.023), and *APOE* SNPs and Hg in mB and CB (p 0.011 and p 0.02, respectively). By contrast, no such statistically significant differences were observed for almost any of the TEs in terms of the four *VDR* SNP alleles and two haplotypes analysed (VDR-H1 and VDR-H2), except for CB-Se and *BsmI*, CB-Hg and *ApaI*, and CB-Se and *TaqI*. Nevertheless, in all cases, the GM differences were very small and, therefore, unreliable without evaluation using multiple linear regression models.

No statistically significant difference in mB-Pb levels was observed for *ALAD* SNPs rs1805313, rs1139488 and rs818708. Nevertheless, a lower mB-Pb was observed for the variant carriers of rs1800435 and rs1805313 and for the homozygous common allele carriers of rs113988b (Table A3a). Basing on these results, we formed *ALAD* combination groups. *ALAD* alleles associated with lower Pb levels were assigned to group ALADcomb1: carriers of the ALAD2 allele, variant allele for ALAD rs1805313 and homozygous carriers of the common allele ALAD rs1139488. ALAD alleles associated with higher Pb levels were assigned to group ALADcomb0: homozygous for ALAD1, homozygous common for ALAD rs1805313 and variant allele ALAD rs1139488 carriers. The ALADcomb1 group had significantly lower mB-Pb concentrations (GM: 9.4 ng/g) than the ALADcomb0 group (GM: 11.8 ng/g) (Table 5 and A3a).

3.4.2. Multiple linear regression models

To determine the associations between the selected SNPs and measured TE levels, multiple linear regression models were conducted for Pb, Hg, Se, Zn, Cu and Mn measured in mB, Zn and Se in mP, Hg in mH and Pb, Hg, Zn and Mn in mixed CB. Cd was excluded due to low concentrations (97% of the women had mB-Cd under 1 ng/g and 62% of the newborns had mixed CB-Cd under the LOD) and As because of the aforementioned reasons. Additionally, we excluded Cu and Se measured in mixed CB. Their proportion in plasma is substantial and potential arterial-venous concentration differences cannot be ignored. Significant associations between the selected SNPs (alleles and/or genotypes) and TE concentrations were observed in case of *ALAD* and *APOE* and are presented in Tables 6–8. Specifically, Table 6 shows the summary of results for mB, while Tables 7 and 8 present the results for all newborns' umbilical CB (Model 2a) and for boys (Model 2b) and girls (Model 2c) separately. The models explained 12%–31% of the variation (R^2) in TE levels. In their full form, including all explanatory variables, are added in the supplements (Tables A4, A5 and A6).

TEs in maternal blood samples (Table 6)

The models presented in Table 6 confirmed the associations between three *ALAD* SNPs (or their combinations) and mB-Pb. Carriers of the *ALAD2* allele were associated with 8% (95% CI 15-1%) or 9% (95% CI 16-1%) lower mean concentrations of mB-Pb (Model 1a or Model 1b, respectively). *ALAD* SNP rs1805313 homozygous variant allele carriers showed an 11 (95% CI 20-1%) decrease in mean mB-Pb levels compared to homozygous carriers of the common allele when Model 1b was applied. A positive association between ALAD SNP rs1139488 and mB-Pb levels was confirmed with both types of Model 1 when the homozygous variant group was compared to the homozygous common, which was at 11% (95% CI 1–23%) and 13% (95% CI 1–25%), respectively, and only with Model 1b when variant carriers were compared to non-carriers, which was at 7% (95% CI 0–15%). As expected, the strongest association was identified between the ALAD combination and mB-Pb concentrations. ALADcomb1 showed a 21% (95% CI 32-8%) decrease in mean mB-Pb levels compared with ALADcomb0 when Model 1a was applied, and 25% (95% CI 35-12%) when tested with Model 1b.

Regarding *APOE* SNPs, the associations were confirmed for mB-Hg (Models 1a and 1b). A marginally significant difference in mB-Hg levels was observed between carriers of the *APOE* ϵ4 allele and non-carriers and between *APOE* genotypes when the ϵ3/ϵ3 group was compared to *APOE* ϵ4 allele carriers (genotypes ϵ3/ϵ4 and ϵ4/ϵ4) when Model 1a was applied. Statistically significant differences were confirmed with Model 1b. Carriers of ϵ4 showed 20% (95% CI 35-1%) lower levels of mean mB-Hg when compared to all noncarriers or only to noncarriers with the genotype ϵ3/ϵ3.

As mentioned above, Model 1b includes only women sampled during their second trimester to achieve a *genetically clearer* or *genetically highlighted* situation by minimising the influence of progressing pregnancy on TE levels, as well as to have a relatively high number of observations. To obtain an even clearer situation, which can help emphasise the influence of SNPs on TE levels, we have also tested influence of the *APOE* genotype on mB-Hg levels including only nulliparous women sampled during their second trimester (N = 322) (supplements, Table A7, Model 1c). In this situation, presented in details in supplements, the ϵ3/ϵ4 and ϵ4ϵ4 genotypes had 33 (95% CI 50, 10) % lower mean mB-Hg levels compared to the ϵ3/ϵ3 genotype, whereas a model including all women sampled during their second trimester had only 20% (95% CI 35-1%) lower mB-Hg levels (Table 6, Model 1b). Obviously, the protective effect of *ϵ*4 against Hg levels was more evident in the *nulliparous* group. The women in this group were more vulnerable due to less effective placental detoxification function related to first pregnancy and consequently had higher Hg levels as a group. Furthermore, from Model 1b, it was also evident that parity was associated with lower Hg (Table A4).

A model including only women sampled during their third trimester (N = 130) was without statistically significant changes, possibly because

Table 6
Multiple linear regression models with statistically significant associations between maternal *ALAD* and *APOE* SNPs (genotypes and/or alleles) and maternal blood Pb and Hg, including their (GM) group comparison.

	TE (ng/g)			Model 1a				TE (ng/g)			Model 1b			
	Second + Third Trimester			Second + Third Trimester				Second Trimester			Second Trimester			
	GM (95% CI)	**N**	**p**	**Exp(b) (95% CI)**	**P**	**R^2**	**N**	**GM (95% CI)**	**N**	**P**	**Exp(b) (95% CI)**	**p**	**R^2**	**N**
***ALAD* rs1800435**	**mB-Pb**							**mB-Pb**						
G-	11.3 (10.9, 11.7)	571		1.00				11.4 (11.0, 11.9)	455		1.00			
G+	10.2 (9.5, 10.9)	141	0.046	0.92 (0.85, 0.99)	**0.026**	0.12	712	10.1 (9.4, 10.9)	115	0.026	0.91 (0.84, 0.99)	**0.024**	0.14	570
***ALAD* rs1805313**	**mB-Pb**							**mB-Pb**						
AA	11.3 (10.8, 11.9)	299		1.00				11.5 (10.9, 12.1)	240	0.143	1.00			
AG	11.0 (10.5, 11.5)	312	0.368	0.97 (0.91, 1.03)	0.300	0.12	711	11.1 (10.5, 11.7)	255		0.96 (0.90, 1.03)	0.310	0.14	568
GG	10.7 (9.9, 11.5)	100		0.95 (0.86, 1.04)	0.241			10.3 (9.5, 11.2)	73		0.89 (0.80, 0.99)	0.031		
***ALAD* rs1139488**	**mB-Pb**							**mB-Pb**						
G-	10.7 (10.2, 11.2)	310		1.00				10.6 (10.1, 11.2)	249		1.00			
G+	11.4 (10.9, 11.9)	400	0.019	1.04 (0.98, 1.11)	0.161	0.12	710	11.6 (11.1, 12.2)	320	0.007	1.07 (1.00, 1.15)	**0.038**	0.14	569
AA	10.7 (10.2, 11.2)	310		1.00				10.6 (10.1, 11.2)	249		1.00			
AG	11.2 (10.7, 11.8)	318	0.025	1.03 (0.96, 1.09)	0.398	0.12	710	11.4 (10.9, 12.0)	253	0.013	1.06 (0.99, 1.13)	0.107	0.14	569
GG	12.1 (11.0, 13.4)	82		1.11 (1.01, 1.23)	**0.037**			12.3 (11.0, 13.7)	67		1.13 (1.01, 1.25)	**0.030**		
***ALAD* comb.**	**mB-Pb**							**mB-Pb**						
ALADcomb0	11.9 (11.0, 12.7)	146		1.00				12.0 (11.1, 13.0)	120		1.00			
ALADcomb1	9.2 (8.0, 10.5)	39	0.001	0.79 (0.68, 0.92)	**0.003**	0.24	185	8.7 (7.6, 10.0)	33	0.001	0.75 (0.65, 0.88)	**0.000**	0.31	153
***APOE* rs7412, rs429358**	**mB-Hg**							**mB-Hg**						
ε4-	2.21 (2.06, 2.38)	596		1.00				2.08 (1.91, 2.25)	482		1.00			
ε4+	1.95 (1.62, 2.34)	102	0.176	0.86 (0.72, 1.02)	***0.091***	0.16	698	1.71 (1.39, 2.09)	80	0.075	0.80 (0.65, 0.99)	**0.037**	0.15	562
ε3/ε3	2.22 (2.06, 2.40)	517		1.00				2.08 (1.90, 2.27)	415		1.00			
ε2/ε2, ε2/ε3	2.17 (1.76, 2.68)	79	0.376	0.99 (0.81, 1.20)	0.912	0.16	698	2.05 (1.62, 2.59)	67	0.200	0.98 (0.78, 1.22)	0.831	0.15	562
ε3/ε4, ε4/ε4	1.95 (1.62, 2.34)	102		0.86 (0.72, 1.03)	***0.092***			1.71 (1.39, 2.09)	80		0.80 (0.65, 0.99)	**0.036**		

mB – maternal venous whole blood; exp(b) – exponentiation of the coefficient (b); conc. – concentration; TE – trace element; GM – geometric mean; N – number of observations; p – statistical significance; R^2 - percentage of variability of TE level explained by the model. Models were adjusted for age, pre-pregnancy BMI, parity, education, seafood frequency intake, smoking, EGW at blood sampling, mB-Zn levels and newborn sex, Significant results ($p \leq 0.05$) are in bold, and marginally significant results ($p > 0.5$ and <0.10) are in bold italics.

Table 7
Multiple linear regression models with statistically significant associations between maternal *APOE* SNPs (genotypes and alleles) and Hg in mixed umbilical CB, including Hg (GM) group comparison.

	TE (ng/g)			Model 2a			
	Boys + Girls			Boys + Girls			
APOE rs7412, rs429358	**GM (95% CI)**	**N**	**P**	**Exp(b) (95%CI)**	**p**	**R^2**	**N**
	CB-Hg			**CB-Hg**			
ε4-	3.88 (3.55, 4.24)	418		1.00			
ε4+	3.35 (2.83, 3.96)	75	***0.052***	0.84 (0.69, 1.02)	***0.086***	0.24	494
ε3/ε3	3.92 (3.56, 4.32)	363		1.00			
ε2/ε2, ε2/ε3	3.64 (2.99, 4.43)	56	***0.091***	0.89 (0.71, 1.11)	0.304	0.25	494
ε3/ε4, ε4/ε4	3.35 (2.83, 3.96)	75		0.83 (0.68, 1.01)	***0.064***		

CB – umbilical cord blood; exp(b) –exponentiation of the coefficient (b); conc. – concentration; TE – trace element; GM – geometric mean; N – number of observations; p – statistical significance; R^2 - percentage of variability of TE level explained by the model. Models were adjusted for age, pre-pregnancy BMI, parity, education, seafood frequency intake, smoking, CB-Zn levels and newborn sex, length, weight and EGA at delivery, Significant results ($p \leq 0.05$) are in bold, and marginally significant results ($p > 0.5$ and <0.1) are in bold italics.

of a much smaller number of observations.

TEs in mixed CB samples (Tables 4 and 5)

When analysing maternal *APOE* genotypes in relation to CB-TEs, we observed that maternal genotype influenced CB-Hg levels (Table 7). The presence of the maternal ϵ4 allele was associated with 16% (95% CI 31-minus 2%) reduced mean CB-Hg levels compared to non-presence, although this had marginal statistical significance only (p 0.086). This difference was similar when analysing the *APOE* genotypes. The ϵ3/ϵ4 and ϵ4/ϵ4 groups showed 17% (95% CI 29- minus1%) lower mean CB-Hg levels compared with the ϵ3/ϵ3 group (p 0.064).

Because of the observed statistically significant influence of child sex on mB-Hg levels in Hg-*APOE* Model 1b (Table A4), *APOE* models for CB were re-run for girls and boys separately for all TEs. The results for Hg and Pb are presented in Table 8, regardless of the *APOE* genotype significance on their concentrations. By sex stratification, the marginally statistically significant CB-Hg associations observed for all newborns (Table 7) were lost for the boys (Table 8, Model 2b) but became statistically significant for the girls (Table 8, Model 2c). For girls the presence of maternal ϵ4 allele was associated with 26% (95% CI 44-3%) reduced mean CB-Hg level and with 27% (95% CI 45-4%) if we compared genotypes (ϵ3/ϵ4, ϵ4/ϵ4 vs ϵ3/ϵ3).

The results in Table 8 also showed that girls whose mothers were carrying the ϵ2 allele had 26% (95% CI 5–53%) higher levels of mean CB-Pb compared to ϵ3/ϵ3 carriers and 27% (95% CI 6–53%) compared

Table 8
Multiple linear regression models with associations between maternal *APOE* SNPs (genotypes and alleles) and Pb and Hg in mixed umbilical CB, including TEs (GMs) group comparison, conducted separately for boys and girls.

	TE (ng/g)			Model 2b				TE (ng/g)			Model 2c			
	Boys			Boys				Girls			Girls			
APOE rs7412, rs429358	**GM (95% CI)**	**N**	**p**	**Exp(b) (95% CI)**	**p**	**R^2**	**N**	**GM (95% CI)**	**N**	**p**	**Exp(b) (95% CI)**	**p**	**R^2**	**N**
	CB-Pb							**CB-Pb**						
ε4-	10.0 (9.5, 10.6)	224		1.00				10.9 (10.2, 11.6)	198		1.00			
ε4+	10.6 (9.5, 11.9)	38	0.267	1.04 (0.91, 1.20)	0.526	0.15	262	10.1 (8.6, 11.7)	37	0.222	0.92 (0.79, 1.09)	0.341	0.14	235
ε3/ε3	10.1 (9.5, 10.8)	194		1.00				10.5 (9.8, 11.3)	172		1.00			
ε2/ε2, ε2/ε3	9.2 (8.1, 10.5)	30	0.203	0.89 (0.77, 1.04)	0.133	0.16	262	13.9 (12.1, 15.9)	26	**0.002**	1.26 (1.05, 1.53)	**0.015**	0.17	235
ε3/ε4, ε4/ε4	10.6 (9.5, 11.9)	38		1.03 (0.90, 1.18)	0.681			10.1 (8.6, 11.7)	37		0.95 (0.81, 1.12)	0.530		
	CB-Hg							**CB-Hg**						
ε4-	3.79 (3.34, 4.31)	221		1.00				3.98 (3.53, 4.49)	198		1.00			
ε4+	3.52 (2.90, 4.27)	38	0.294	0.92 (0.69, 1.22)	0.557	0.26	259	3.18 (2.39, 4.23)	37	***0.089***	0.74 (0.56, 0.97)	**0.028**	0.26	235
ε3/ε3	3.83 (3.32, 4.42)	191		1.00				4.02 (3.53, 4.58)	172		1.00			
ε2/ε2, ε2/ε3	3.57 (2.78, 4.60)	30	0.661	0.86 (0.62, 1.18)	0.356	0.26	259	3.72 (2.68, 5.15)	26	0.339	0.91 (0.66, 1.26)	0.568	0.26	235
ε3/ε4, ε4/ε4	3.52 (2.90, 4.27)	38		0.90 (0.68, 1.20)	0.474			3.18 (2.39, 4.23)	37		0.73 (0.55, 0.96)	**0.025**		

CB – umbilical cord blood; exp(b) – exponentiation of the coefficient (b); conc. – concentration; TE – trace element; GM – geometric mean; N – number of observations; p – statistical significance; R^2 - percentage of variability of TE level explained by the model. Models were adjusted for age, pre-pregnancy BMI, parity, education, seafood frequency intake, smoking, CB-Zn levels and newborn length, weight and EGA at delivery, Significant results ($p \leq 0.05$) are presented in bold, and marginally significant results ($p > 0.5$ and <0.1) are presented in bold italic.

to all ϵ2 non-carriers (Table A8, Model 2c).

Observed associations indicate the potential protective role of the maternal ϵ4 allele against Hg levels, as well as the protective role of the maternal ϵ3 and ϵ4 alleles against Pb levels.

4. Discussion

In this study, our aim was to assess the associations between Pb biomarkers of exposure and the gene variants of *ALAD*, *VDR* and *APOE* in a population of pregnant women and their newborns with long-term low-level mixed exposure living in the province of Trieste, Italy (PHIME study participants). Because of possible mixed effects, some other TEs were also followed (Fig. 1, Table A1; Table A3). The GMs (95% CI) in ng/g for non-essential mB-Pb, mB-Hg, mB-Cd and mB-As in our study population were observed at 11.0 (10.7–11.3), 2.16 (2.04–2.30), 0.31 (0.29–0.32) and 1.38 (1.20–1.47), respectively, indicating a low exposure level. Their levels in CB were even lower, except for Hg. Nevertheless, a few statistically significant associations between SNPs and Pb or Hg levels were identified in the multivariate linear regression analyses.

4.1. ALAD polymorphisms

The first three of the four studied *ALAD* SNPs (rs1800435, rs1805313, rs1139488 and rs818708) showed statistically significant associations with mB-Pb by linear regression analyses, particularly in samples obtained in second trimester (Table 6). A negative association was observed for the variant allele of SNPs rs1800435, variant allele rs1805313 and common allele of rs1139488. No associations between any of four ALAD polymorphisms and mixed CB-Pb levels were found.

4.1.1. ALAD rs1800435

In agreement with the proposed hypothesis, the majority of studies conducted on individuals with high Pb exposure displayed associations between the *ALAD2* allele and elevated blood Pb levels. However, the results are fairly inconclusive at low levels of exposure (Broberg et al., 2015; Skerfving and Bergdahl, 2015; ATSDR, 2020). Moreover, some studies have revealed an inverse association between the *ALAD* SNPs rs1800435 and blood Pb, suggesting that the *ALAD2* allele is associated with lower blood Pb in populations with low exposure to Pb (Hu et al., 2001; Krieg et al., 2010; Stajnko et al., 2020). The same was observed in our population of pregnant women (Table 6). This could be attributed to differences in Pb distribution at various exposure levels. At lower levels, the majority of Pb that binds to ALAD2 may occur in other tissues, such as those in the liver, kidneys and placenta, in which ALAD is also highly expressed (Kelada et al., 2001). Although it has been shown that during pregnancy, the release of Pb from the maternal bone can be triggered, although mostly during the third trimester and particularly with simultaneous Ca deficiency (Skerfving and Bergdahl, 2015; ATSDR, 2020), we presume that the majority of Pb in maternal blood in our population came from current exposure, entering the body via ingestion and passing through the liver, part of which can bind to ALAD before entering the central bloodstream. The majority of the women were sampled during the first half of pregnancy (around 20th EGW) and had presumably good nutritional status, which can protect or delay the mobilisation of maternal bone Pb (ATSDR, 2020). Furthermore, as they lived in a sun-rich environment, we predict that they had a sufficient vitamin D status by endogenous synthesis of vitamin D using UV light. Ca was not measured in maternal blood, but other TEs measured (Se, Zn, Cu and Mn) indicated adequate micronutritional status. Release from the bone should increase during the third trimester (ATSDR, 2020), but we did not observe any increase when comparing GM (95% CI) for mB-Pb concentrations between women sampled in the middle of the second trimester or at the beginning/middle of the third trimester: 11.1 ng/g (10.7–11.5 ng/g) (N = 651) and 10.8 ng/g (10.1–11.5 ng/g) (N = 173), respectively (Table A1a).

In the literature, only a few studies have investigated the relationship between *ALAD1/2* polymorphisms and Pb levels in pregnant women, and all included women with higher Pb exposure levels compared to our study. Akyuzlu et al. (2014) reported higher median maternal blood Pb and cord blood Pb levels for carriers of the *ALAD*2 allele, and a similar finding was reported by Yun et al. (2015), in which pregnant women carrying at least one *ALAD*2 allele had higher concentrations of blood Pb. In both studies, they reported an inverse association with that found in our study. However, the concentrations of blood Pb and cord blood Pb in their research significantly surpassed ours, with a maternal blood Pb arithmetic mean of 72 ng/mL (N = 198) in Yun et al. (2015) and approximately 38 ng/mL (N = 97) in Akyuzlu et al. (2014). Furthermore, Akyuzlu et al. (2014) and Yun et al. (2015) did not use statistical models to account for possible confounding factors, making a direct comparison of results less certain.

4.1.2. ALAD rs1805313

Only a handful of studies have investigated the influence of the three remaining *ALAD* SNPs (rs1805313, rs1139488 and rs818708) on Pb levels, and none of these include pregnant women. In our study (Table 6), we confirmed previously reported negative associations between the *ALAD* rs1805313 variant allele and blood Pb levels found in the general population using a genome-wide association study (Warrington et al., 2015) or multivariant regression analyses (Stajnko et al., 2020). According to cellular experimental studies, it is presumed that *ALAD* rs1805313 has an effect on *ALAD* expression in blood cells (Warrington et al., 2015).

4.1.3. ALAD rs1139488

When investigating ALAD rs1139488 SNPs, we observed an association between carriers of at least one variant allele and higher mB-Pb concentrations. When homozygous variants were compared to common homozygous individuals, the observed statistically significant association became even stronger. Our results are also consistent with published research on active occupationally exposed individuals (Szymańska-Chabowska et al., 2015), in which significantly higher levels of blood Pb were associated with the presence of at least one variant allele. Furthermore, other studies observed a similar trend when analysing Pb levels in children (GM of blood Pb: 39.1 mg/L) or occupationally exposed adults, although the difference in blood Pb levels between *ALAD* rs1139488 SNPs genotypes was not statistically significant (Pawlas et al., 2012; Shaik et al., 2018).

4.1.4. ALAD combination

Throughout the present research, the differences in Pb GMs between alleles and genotypes were extremely small, except for *ALAD* combination (GM: 11.8 ng/g and 9.4 ng/g for ALADcomb0 and ALADcomb1, respectively, Table 5). Furthermore, the best R^2 of any linear regression models was achieved when analysing *ALAD* combination (R^2: 0.24–0.31, Table 6). This points to the importance of researching haplotypes or combinations at low exposure levels, which allow us to better see the effects of genetics.

4.2. VDR polymorphisms

The individual *VDR* SNPs and/or their haplotypes have been extensively studied with regard to different diseases, including osteoporosis, cancers, neurodegenerative diseases, Ca absorption and Pb toxicity (Broberg et al., 2015; Köstner et al., 2009; Thakkinstian et al., 2004). However, none of our four studied *VDR* SNPs (*FokI*, *BsmI*, *ApaI* and *TaqI*) or their haplotypes appeared to have any influence on Pb concentrations in mB or in CB when tested with models (data not shown). This could be due to particularly low Pb levels or/and to the above-mentioned assumption that the mothers had good micronutritional status. In such cases, genetic variability is less expressed on the phenotype level. Anyway, the absence of haemoglobin or haematocrit, Ca, Fe and vitamin

D measurements could have also resulted in residual confounding.

4.3. APOE polymorphisms

SNPs within the *APOE* gene appear to have associations with vitamin D levels (Huebbe et al., 2011; Soares et al., 2021), bone Ca metabolism (Huebbe et al., 2011) and Se levels (Trdin et al., 2020). Huebbe et al. (2011) found evidence that *APOE* ϵ4 is linked to higher Ca and vitamin D levels in targeted replacement mice and in humans. They demonstrated that *APOE* ϵ4 has higher intestinal absorption and renal retention of vitamin D, resulting in more efficient intestinal Ca absorption, which is known to be vitamin D dependent. For instance, in *APOE* ϵ4 mice, they observed a higher expression of genes responsible for renal vitamin D binding and for transport and uptake from primary urine (endocytic receptor megalin, Lrp2). Lrp2 is a multi-ligand receptor that is also responsible for selenoprotein P renal uptake. This *APOE* link with multi-ligand Lrp2 and with common multi-ligand *APOE* receptor-2 (Lrp 8) was source of explanation when higher plasma Se levels were associated with the ϵ4 allele in Croatian pregnant women (PHIME study subgroup) (Trdin et al., 2020). Selenoprotein P is a major plasma selenoprotein responsible for Se storage and distribution to tissues, but it also functions as an antioxidant in blood vessels and as a metal-binding protein (detoxification) (Baclaocas and Mackrill, 2020). Experimentally or *in vivo*, it was found to bind metals, such as Ag, Cd, (Me)Hg and Pb (Sasakura and Suzuki, 1998; Chen et al., 2006; Bi et al., 2019; Baclaocas and Mackrill, 2020). Additionally, further experimental data on APOE interaction with selenoprotein P (Jin et al., 2020) and low-molecular metal-binding protective proteins, metallothioneins (Augsten et al., 2011; Graeser et al., 2012), suggest its wider direct or indirect impact on TE metabolism. Our research supports the beneficial effects of *APOE* ϵ4, as the sampled pregnant women carrying this allele had lower levels of mB-Hg and CB-Hg compared with non-carriers (Tables 6–8), which was most evident in mB sampled during the second trimester (Table 6, Model 1b), particularly in nulliparous women (Table A7, Model 1c), and in newborn girls' umbilical CB (Table A8, model 2c). When the associations between the maternal *APOE* genotype and CB-Pb were tested separately for girls and boys, a clear positive association was found for ϵ2 allele carriers when the models included only girls (Table 8, Model 2c; Table A8, Model 2c). However, at very low Pb exposure levels and loosely defined Pb sources (internal bone sources due to past exposures and external sources from current exposure), the observed differences between newborn girls and boys could also be triggered by other factors and should therefore be interpreted with caution. Nevertheless, there are studies that, although inconsistently, point to sex-related differences in Pb toxicokinetics or effects (ATSDR, 2020).

These findings suggest that maternal ϵ4 may have the highest protective function during pregnancy, whereas ϵ2 which was reported to be associated with a higher bone turnover (Dieckmann et al 2013) shows the opposite. The modifying interactions of different apoE isoforms with various metals have rarely been studied, although they can be important, particularly regarding the observed interactions of apoE protein with metal-binding metallothioneins (Augsten et al., 2011; Graeser et al., 2012) and/or selenoprotein P (Jin et al., 2020) in experimental studies. Furthermore, research involving pregnant women, their *APOE* polymorphisms, and blood and cord blood TE levels is limited. Trdin et al. (2020) reported statistically significant higher plasma Se levels in Croatian pregnant women (PHIME participants) carrying at least one ϵ4 allele compared with non-carriers. This finding supports the antagonistic pleiotropy theory and the proposed beneficial effects of *APOE* ϵ4 during early life, in contrast to age-related disadvantages during elderly stages in certain populations (Han and Tuminello, 2011; Smith et al., 2019). In our study, we could not observe any difference in the Se levels of mB and mP between carriers and non-carriers of *APOE* ϵ4. This is presumably because of the much higher levels of Se in our study population, with a GM (95% CI) of P–Se 78.6 ng/g (77.7–79.5 ng/) in contrast to a GM of 55 ng/g in the Croatian population. In differently focused studies, Wright et al. (2003), Ng et al. (2013, 2015) and Snoj Tratnik et al. (2017) have attempted to link the association between Pb or Hg prenatal exposure and child *APOE* genotype to neurodevelopment, but some shortcomings are involved. The absence of maternal genotype, measured metal concentrations in mixed umbilical cord blood instead of arterial or venous cord blood, and the absence of measured metal concentrations in the blood of children at the time of neurodevelopment testing make the results less reliable, particularly at low levels of exposure.

4.4. Study limitations

The main limitations of our study are as follows: possible biases because of missing information on possible maternal stress during pregnancy (Tamayo et al., 2017); self-reported data with questionable self-recall; uncertainty of smoking or drinking frequencies because these could be stigmatising questions during pregnancy; lack of measurements of some variables with possible influences on Pb kinetics, such as haemoglobin or haematocrit, vitamin D, Ca and iron or ferritin; and sampling of mixed CB instead of arterial or venous CB. As obtaining arterial or venous CB is extremely difficult, mixed CB was used with the proposition that it could be accepted as an approximation, particularly for TEs that mostly accumulate within the red blood cells (e.g. Pb, Hg, Cd and, conditionally, Zn). The missing adjustment for haemoglobin or haematocrit levels was partially corrected in the statistical models by incorporating mB-Zn and CB-Zn concentrations. Although the Ca and vitamin D concentrations are likely to be a key confounding variable in relation to *VDR* and *APOE* SNPs, we presumed that in the study population, who seemed to be nutritionally uncompromised and lived in a sunny area, they should not have had a major impact.

Although maternal genotype is believed to have a leading role in foetal development during a healthy pregnancy, the results for CB should be complemented by newborns' genotype in the next step.

It is also important to stress that in the literature, we can observe contradictory results for *ALAD* or *VDR* SNPs and blood Pb levels, especially at low exposure levels. There might be several reasons for this, including the polymorphisms of other genes. Therefore, we must consider that the mB-Pb and CB-Pb levels in our population might also be affected by other polymorphisms, such as a polymorphic variation in the *SLC4A7* gene (Whitfield et al., 2007). The latter is an ion transporter gene that is highly expressed in erythrocytes, whose product could affect Pb transport into the cell (Whitfield et al., 2007). At low Pb exposure levels, various transporter proteins might play important roles, but they often remain underestimated. Furthermore, how the new data on the dynamic coexistence of octamer and hexamer ALAD forms (Jaffe et al., 2020) affect ALAD affinity towards different metals *in vivo* remains unknown, which, again, might influence Pb levels (Jaffe Eileen, personal communication 2022), especially when the exposure levels are low.

5. Conclusions

1. Three *ALAD* SNPs (rs1800435 variant allele aka *ALAD2*, rs1805313 variant allele and rs1139488 common allele) and their combination were negatively associated with mB-Pb levels in Italian pregnant women. Results for each individual variant allele are confirming observations of Stajnko et al (2020), Warrington et al (2015) and Tasmin et al (2015). They might represent alleles that could be protective against Pb effects at low exposure levels; particularly variant allele rs1800435. Nevertheless, the observed individual Pb levels in our population were mostly too low to inhibit ALAD catalytic activity; according to a recent study the possible threshold of B–Pb for affecting ALAD was 50 ng/mL (Huang et al 2020).
2. The estimated absence of associations between mB-Pb or CB-Pb level and the four analysed *VDR* SNPs or their haplotypes was of limited value because we were unable to perform the adjustment by

corresponding Ca and vitamin D levels and/or because the measured levels of Pb could be too low to interact with Ca metabolic pathways.
3. For the first time a negative association was observed between maternal *APOE* ϵ4 allele carriers and Hg levels in mB and mixed CB compared to ϵ4 non-carriers. The negative association was even stronger when ϵ4 allele carriers were compared to ϵ3/ϵ3 carriers. Additionally, APOE modification was also observed for Pb levels in newborn girls' mixed CB. Girls born to mothers carrying the ϵ2 allele had higher CB-Pb levels than girls born to ϵ2 non-carrying mothers. Results point to the possible metal-detoxifying impact of ϵ4 allele, most probably through indirect metabolic interferences.
4. The observed associations indicate the possible modification effects of *ALAD* SNPs on Pb and *APOE* SNPs on Pb and Hg kinetics in nutritionally uncompromised pregnant women (and their newborns). However, the obtained associations and their functional significance should be interpreted with caution due to absence of the additional effect and/or exposure biomarkers (e.g. urinary aminolevulinic acid and placental Pb levels) and because of possible (unavoidable) masking effects of coexisting background variables, which are of higher importance at lower levels of Pb exposure.

Credit author statement

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Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2023.115226.

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