GENETIC VARIABILITY AND LEAD EXPOSURE BIOMARKERS

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Doctoral Dissertation
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GENETSKA VARIABILNOST IN BIOLOŠKI OZNAČEVALCI IZPOSTAVLJENOSTI SVINCU

Doktorska disertacija

Supervisor: Asst. Prof. Ingrid Falnoga

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"The dose makes the poison."

Paracelsus

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Abstract

The thesis investigates associations between the biomarkers of lead (Pb) exposure (and mercury (Hg), where applicable) and genetic variability in in vulnerable populations, including pregnant women and their newborns from Italy, Slovenia, and Croatia, as well as Slovenian men of reproductive age. The research utilizes data from the Public Health Impact of Long-term, Low-level Mixed Element Exposure in Susceptible Population Strata (PHIME) project and the Slovenian Human Biomonitoring (HBM) project, both characterized by low to moderate levels of potentially toxic non-essential Pb and Hg exposure with adequate levels of essential zinc and selenium. We examined how genetic factors influence susceptibility or resilience to low- to moderate-level Pb (and Hg) exposure, with the central focus on key single nucleotide polymorphisms (SNPs) in the *ALAD* (δ-aminolevulinic acid dehydratase), *VDR* (vitamin D receptor), and *APOE* (apolipoprotein E) genes. Associations were estimated and tested using multiple linear regression models, with the adjustment for selected available cofounders.

In both populations, the presence of the *ALAD2* allele was associated with lower blood Pb concentrations. Furthermore, SNP *ALAD* rs1800435 *(ALAD1/2)* was shown to impact blood Pb levels in varying ways depending on the level of exposure by comparing Slovenian men living in historically Pb polluted environment vs non-polluted environment. Our results also confirm that other *ALAD* SNPs—namely rs1805312, rs1805313 and rs1139488—can as well significantly influence Pb levels. Additionally, combinations of *ALAD* SNPs, were found to account for a substantially higher degree of variability in blood Pb levels compared to individual SNPs alone, underscoring the importance of examining multiple genetic variants together, if possible biases are negligible.

The research also addressed the role of *VDR* SNPs in Pb toxicokinetics, finding no significant effect of selected SNPs or their haplotypes on blood Pb levels in either population. This lack of association may stem from the complex physiological role of vitamin D, particularly in pregnant women, where calcitriol production occurs independently of sun exposure or dietary intake. While *VDR* polymorphisms are known to influence the formation of Ca-binding proteins and affect bone density, the intricate interactions between calcitriol, calcium, and Pb—combined with the polymorphic nature of the *VDR* gene and the co-occurrence of polymorphisms in other genes—add layers of complexity that contribute to inconsistent findings also in the literature.

In the investigated population of pregnant women, maternal *APOE* ε2 allele carriers exhibited higher (cord)blood Pb levels and blood Hg levels, but only when the fetus/newborn was female. Conversely, the *APOE* ε4 allele was linked to lower levels of Pb regardless of the fetal sex. These findings align with the possible theory that the ε2 has the least protective effect on maintaining bone mass, which could influence bone mineral changes during pregnancy, while the ε4 allele demonstrates antagonistic pleiotropy, providing protective benefits during reproductive years but potentially having adverse effects later in life.

An insight from this research highlights the importance of considering sex-based differences in future studies on Pb and Hg toxicokinetics. Also, we have shown that parity

should be taken into considerations in these types of studies, as genetic influences were more pronounced in *nulliparous* women.

The observed associations indicate the possible modification effects of *ALAD* SNPs on Pb and *APOE* SNPs on Pb and Hg toxicokinetics even at low exposure levels, while *VDR* SNPs appear to be overwhelmed by other factors at these low exposure levels.

Povzetek

Disertacija preučuje asociacije med biomarkerji izpostavljenosti svincu (Pb) ter, kjer je primerno, tudi živemu srebru (Hg), in genetsko variabilnostjo v ranljivih populacijah, kot so nosečnice in njihovi novorojenčki iz Italije, Slovenije in Hrvaške ter slovenski moški v reproduktivnem obdobju. Raziskava temelji na podatkih projektov »Public Health Impact of Long-term, Low-level Mixed Element Exposure in Susceptible Population Strata" (PHIME) in »Humani biomonitoring« (HBM), ki vključujeta populacije z nizkimi do zmernimi ravnmi potencialno toksičnega, neesencialnega svinca in živega srebra ter ustreznimi koncentracijami esencialnega cinka in selena. Namen disertacije je bil preučiti, kako ključni polimorfizmi posameznih nukleotidov (SNP) v genih *ALAD* (δ-aminolevulinic acid dehydratase), *VDR* (vitamin D receptor) in *APOE* (apolipoprotein E) vplivajo na občutljivost oziroma dovzetnost na nizke in zmerne ravni izpostavljenosti svincu (in živemu srebru). Povezave so bile ocenjene in testirane z večparametričnimi linearno regresijskimi modeli.

Pri obeh populacijah smo potrdili, da prisotnost alela *ALAD2* znižuje koncentracije svinca v krvi. Opazili smo tudi v literaturi večkrat opisan trend, da *ALAD* rs1800435 (*ALAD1/2*) vpliva na raven svinca v krvi glede na stopnjo izpostavljenosti. To smo potrdili na slovenskih moških, in sicer s primerjavo posameznikov z območij, zgodovinsko onesnaženih s svincem, in tistih z neonesnaženih območij. Naši rezultati tudi potrjujejo, da lahko tudi drugi SNP-ji gena ALAD—in sicer rs1805312**,** rs1805313 in rs1139488—vplivajo na ravni svinca. Prav tako smo ugotovili, da kombinacije SNP-jev *ALAD*-a pojasnjujejo bistveno večjo variabilnost ravni svinca v krvi kot posamezni SNP-ji, kar poudarja pomen hkratnega preučevanja več SNP-jev, če predvidevamo, da so možne pristranskosti zanemarljive.

Preučili smo tudi vlogo *VDR* SNP-jev v toksikokinetiki svinca, vendar nismo našli pomembnega učinka izbranih SNP-jev ali njihovih haplotipov na raven svinca v krvi, urinu ali popkovni krvi, in sicer v nobeni od populacij. Razlog za to bi lahko bila zapletena fiziološka vloga vitamina D, zlasti pri nosečnicah, kjer naj bi se proizvodnja kalcitriola (aktivne oblike vitamina D) odvijala neodvisno od izpostavljenosti sončni svetlobi ali prehranskemu vnosu prekurzorjev kalcitriola. Čeprav je znano, da polimorfizmi v *VDR* genu lahko vplivajo na tvorbo proteinov, ki vežejo kalcij, in na gostoto kosti, zapletene interakcije med kalcitriolom, kalcijem in svincem—skupaj s polimorfno naravo gena *VDR*—prispevajo k dodatni kompleksnosti, kar pojasnjuje tudi nedosledne ugotovitve v literaturi.

Pri preučevanju genotipa *APOE* v populaciji nosečih žensk smo ugotovili, da so imele nosilke alela *APOE* ε2 višje vrednosti svinca in živega srebra v krvi, vendar le, če so bile noseče z deklico. Povišane vrednosti svinca so bile zaznane tudi v popkovni krvi. Nasprotno pa je bil *APOE* ε4 alel povezan z nižjimi vrednostmi svinca v krvi, ne glede na spol ploda. Te ugotovitve bi lahko podprle teorijo, da ima alel ε2 najmanj zaščitnega učinka pri ohranjanju kostne mase, kar bi lahko vplivalo na spremembe mineralne gostote kosti med nosečnostjo, medtem ko alel ε4 kaže antagonistični pleiotropizem, saj nudi zaščito v

mladosti ter reproduktivnem obdobju, vendar lahko povzroča negativne učinke v kasnejših letih življenja.

Pomembna ugotovitev dizertacije je tudi pomen upoštevanja spolnih razlik v prihodnjih študijah o toksikokinetiki svinca in živega srebra, ki vključujejo nosečnice in njihove novorojenčke. Poleg tega smo pokazali, da je smiselno v takšnih študijah upoštevati število predhodnih nosečnosti, saj so bili genetski vplivi izrazitejši pri ženskah, ki še niso rodile.

Ugotovljene povezave nakazujejo, da *ALAD* SNP-ji lahko vplivajo na toksikokinetiko svinca, *APOE* SNP-ji pa na toksikokinetiko svinca in živega srebra, tudi pri nizkih ravneh izpostavljenosti, medtem ko se zdi, da učinke SNP-jev gena *VDR* pri teh stopnjah izpostavljenosti zasenčijo drugi dejavniki.

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Abbreviations

ALAD	...	δ-aminolevulinic acid dehydrogenase
APOE	...	apoprotein E
ATSDR	...	Agency for Toxic Substances and Disease Registry
BMD	...	bone mineral density
DALYs	...	disability-adjusted life years
DBP/Gc	...	vitamin D-binding protein
DNA	...	deoxyribonucleic acid
EP	...	erythrocyte protoporphyrin
HBM	...	human biomonitoring
HEALS	...	Health and Environment-Wide Associations based on Large population Surveys
ICP-MS	...	inductively coupled plasma mass spectrometry
LD	...	linkage disequilibrium
Lrp2	...	low density lipoprotein receptor-related protein 2 / megalin
MAF	...	minor allele frequency
mRNA	...	messenger ribonucleic acid
PAHs	...	polycyclic aromatic hydrocarbons
PBSG	...	porphobilinogen synthase
PHIME	...	Public Health Impact of Long-term, Low-level Mixed Element Exposure in Susceptible Population Strata
POPs	...	persistent organic pollutants
SES	...	socioeconomic status
SG	...	specific gravity
SNP	...	single nucleotide polymorphism
SOP	...	standard operating procedure
TEs	...	trace elements
VDR	...	vitamin D receptor
WHO	...	World health organization

Chapter 1

Introduction

1.1 Human Biomonitoring Related to Metal and Metalloid Exposure

Human Biomonitoring (HBM) represents an efficient tool for evaluating human exposure to chemicals arising from the environment, occupation, and lifestyle, with the potential to induce adverse health effects. This scientific approach measures chemicals, their metabolites, or reaction products in human fluids and tissues, extending to the study of their effects and the consideration of individual susceptibility as a modulator of responses (CDC, 2005). Initially employed in occupational settings, HBM has evolved into a global tool for monitoring exposures of the general population to environmental pollutants, such as potentially toxic non-essential trace elements, bisphenols, polycyclic aromatic hydrocarbons (PAHs), perfluorinated alkyl substances, etc. with special consideration for vulnerable groups (WHO, 2015).

In Slovenia, first national HBM program started in 2007, including vulnerable populations; breast-feeding *primiparous* mothers and their partners in child-bearing age (18-49 years) and so far their exposure to metals, bisphenols, parabens, triclosan, phthalates, 1,2-Cyclohexane dicarboxylic acid diisononyl ester, PAHs, and persistent organic pollutants (POPs) (Joksić et al., 2022; Runkel et al., 2021, 2022; Snoj Tratnik et al., 2019). Similarly, in Italy, the initial national HBM program also focused on a vulnerable group, targeting adolescents aged 13 to 15 years. This program examined their exposure to metals and was initiated in 2008 (Pino et al., 2017). Whereas in Croatia, the first national HBM program started in 2019, including a non-occupationally exposed adult population aged 28-39 years, in which exposure to cadmium (Cd), PAHs and bisphenols was reported (Gilles et al., 2022). However, before this, Croatia had actively participated in international HBM projects including the Public Health Impact of Long-term, Low-level Mixed Element Exposure in Susceptible Population Strata (PHIME) project (2006-2011). This project included Italian, Croatian, Slovenian, and Greek pregnant women and their newborns (Miklavčič et al., 2013; Valent et al., 2013). The research presented in this thesis utilized samples from the PHIME project including only Italy, Croatia, and Slovenia participants as well as Slovenia's first HBM study including only male participants.

Exposure to environmental pollutants occurs through diverse routes, including inhalation, ingestion, and dermal absorption. The body burden of specific pollutants depends on factors such as pollutant concentration, physical and chemical properties, length and frequency of exposure, and individual factors influencing liberation, absorption, distribution, metabolism, and excretion rates of chemicals (WHO, 2015). Samples collected from various human tissues and fluids can serve as suitable materials for human biomonitoring, such as blood, urine, hair, breast milk, cord blood, placenta, nails, teeth,

adipose tissue, semen, etc. Nevertheless, the predominant choices for sampling typically include urine and blood (Santonen et al., 2015), with cord blood also being commonly included in prenatal exposure studies (Smolders et al., 2009).

Central to HBM is the utilization of biomarkers, acting as measurable indicators of exposure in biological systems. The World Health Organization (WHO) defined biomarkers in 1993 within the context of risk assessment, encompassing measurements reflecting interactions between biological systems and environmental agents—chemical, physical, or biological (WHO, 1993). Three classes of biomarkers were identified (WHO, 2015):

- Biomarkers of exposure measure chemical residues, metabolites, or the products of its interaction with target molecule (or cell) in human tissues or body fluids. Selection of appropriate analytes considers the kinetics of biomarkers, with different matrices reflecting exposure over varying time periods.

- Biomarkers of effect signify quantifiable changes in biochemical, physiological, behavioral or other parameters resulting from exposure. These biomarkers range from biomolecules in tissues or fluids to physiological measurements, aiding in the assessment of early reversible changes in organisms.

- Biomarkers of susceptibility reflect inherent or acquired characteristics making an organism more or less susceptible to a specific chemical exposure.

Biomarkers of exposure are the most widely accepted and used biomarkers in the context of human biomonitoring. Only a limited number of effect biomarkers have been validated and biomarkers of susceptibility have not been widely adopted for routine use in metal toxicology thus far (Santonen et al., 2015) even though a new concept named the "exposome" emerged already in 2005. The exposome is a concept that encompasses the totality of environmental exposures throughout an individual's life, including external factors such as pollutants, lifestyle, diet, socio-economic influences, and psycho-social factors. The exposome approach recognizes that genetic, environmental, and psycho-social factors are interconnected, and their interplay can influence health and disease outcomes. By considering these diverse factors, researchers aim to unravel the complex mechanisms underlying the development of diseases and identify potential areas for intervention and prevention. One of the studies in Europe that followed the "exposome" approach was the "Health and Environment-Wide Associations based on Large population Surveys" (HEALS) study (Dennis et al., 2017).

One of the objectives of HBM-based studies is identifying vulnerable subpopulations and ensure their inclusion in future HBM research. Vulnerable populations, such as children, pregnant women, the elderly, men in reproductive age, and individuals with pre-existing health conditions, have distinct physiological and metabolic characteristics that increase their susceptibility to toxicants (WHO, 2015). Pregnant women and their fetuses are particularly at risk, as toxic substances can cross the placental barrier, potentially affecting fetal development. Exposure to specific chemicals (lead, methylmercury, polychlorinated biphenyls, arsenic, etc.) during early fetal development can result in brain injury at much lower doses than those affecting adult brain function (Grandjean & Landrigan, 2006). Therefore, the focus of the presented studies is on pregnant women and their fetuses/newborns, as well as men in reproductive age, and their exposure to non-essential toxic trace elements, with a main emphasis on lead (Pb). However, focusing solely on vulnerable populations limits the generalizability of findings to broader groups, such as those with occupational exposures or lower susceptibility. Additionally, HBM often targets low-exposure populations and provides only a snapshot of exposure, which may not capture cumulative risks.

1.2 Trace Elements

Trace elements (TEs) are chemical elements present in minute quantities in biological systems but are essential for various physiological functions. The WHO defines TEs as "elements that are required in small amounts (less than 100 mg/day) and are essential for maintaining health" (WHO, 1996). However, this definition has evolved over time. In a 2022 article, Wolfgang Maret argues that defining trace elements solely based on daily intake is insufficient. He emphasizes the need to consider the broader context of bioelements, focusing on their unique properties and interactions within biological systems to fully understand their significance (Maret, 2022).

Trace elements are naturally and widely distributed in the environment, resulting in continuous human exposure. Human activities such as mining, industrial operations, and agriculture can significantly elevate their environmental concentrations locally or globally, leading to increased human exposure (Prashanth et al., 2015, Nordberg et. al., 2022a). Trace elements were studied and recognized as essential or non-essential, potentially toxic or non-toxic. General factors influencing essentiality and potential toxicity of metal(loid)s are: dose, exposure conditions, bioavailability and chemical species (Nordberg & Nordberg, 2016). According to the WHO (WHO, 1996), metals considered essential for human health include chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), molybdenum (Mo), selenium (Se), zinc (Zn), and the later addition of manganese (Mn) (Aggett et.al., 2015). Adequate intake of these essential trace elements through food is necessary to prevent deficiency states. However, excessive exposure may lead to toxic effects (Aggett et.al., 2015, Nordberg & Nordberg, 2016). Therefore, the dose-response relationship is U-shaped, with low and high doses having adverse health effects (Moffett et al., 2014).

For some assumed non-essential TEs their status is questionable as they might be essential for humans (e.g., Cd, nickel (Ni), aluminum (Al), chromium (Cr), while for others (arsenic (As), mercury (Hg), Pb) their function at very low levels is possibly still not known, but might change with the future studies (Prashanth et al., 2015). So far increased exposure to them has been associated with various adverse effects on human health, including an increased risk of diseases such as age-related neurological disorders, cardiovascular diseases, liver and kidney dysfunction, and cancer, usually at higher exposure rates (Nordberg et. al., 2022a). The US Agency for Toxic Substances and Disease Registry (ATSDR) ranks in its 2022 Priority List of Hazardous Substances those four as first, second, third and seventh, respectively (ATSDR, 2022). Notably, essential elements can, through various metabolic interactions, impact the kinetics of non-essential elements and vice-versa (Nordberg & Nordberg, 2016). Known for their protective properties, essential elements like Se, Zn and Fe can mitigate metal(loid)-induced toxicity, offering a means to reduce the risk to human health (Rahman et al., 2019). Therefore, when following exposure to potentially toxic metalloids by measuring their levels in human body, it is important to follow essential elements as well. At the same time, we should not overlook the so-called co-exposure to other potentially toxic non-essential trace elements, especially in the cases of long-term low-level exposure (Nordberg et al., 2014).

1.3 Lead

Lead is a naturally occurring, dense, soft and ductile metal and the history of Pb pollution is very long. For centuries it has been an economic commodity of major importance, with the first reports of its use dating all the way back to 3500 BC. The industrial and economic momentum which has cemented its role way into the future started during the Roman Empire, with the mining and smelting of Pb and subsequent usage in water distribution

systems. With the start of the industrial revolution there was a significant surge in Pb production and emission, which peaked around 1970-80 (Bergdahl & Skerfving, 2022).

To date, the main sources of Pb exposure have been lead-based paint in old homes, leaded gasoline (introduced in 1923), lead-containing pipes and solder frequently found within the plumbing of older buildings, lead-glazed food containers, certain cosmetics, cigarette smoke (including second and third hand exposure), Pb contaminated soil, water and air (street and attic dust) near the sites of historic or ongoing mining operations or smelters and near the roads with heavy traffic (Skerfving & Bergdahl, 2015). As knowledge of Pb toxicity continues to increase and improve, several efforts to reduce its presence within materials utilized in everyday life were made (Mani et al., 2019). An important step in reducing Pb exposure was a phase out of leaded gasoline, which started in the 1970's and is nearly complete worldwide. Leaded gasoline was banned in Slovenia in 2001 (OECD: Slovenia, 2012), in Italy in 2002 (OECD: Italy, 2002), and in Croatia in 2006 (Zorana et al., 2016). However, usage of Pb is still in place due to its practicality and versatility. Interestingly, Pb consumption reached 12 million tons in 2019, up from 6.5 million tons in 2001 (Bergdahl & Skerfving, 2022). Today, the primary use of Pb (80 %) is in batteries, particularly for vehicles, as well as for backup power systems and industrial batteries. Additionally, Pb is used in pigments (5 %) and ammunition (3 %). For general population, the main sources of Pb, especially in Europe, are drinking water and foods. In the diet, vegetables and cereals usually have the highest concentrations of Pb, which is mostly a result of deposition from air (Bergdahl & Skerfving, 2022).

Despite efforts to reduce exposure to Pb toxicity, it remains a persistent global health issue. According to recent data, Pb exposure continues to pose significant health risks worldwide, mostly in low- and middle-income countries. A recent review paper (Ericson et al., 2021), which analyzed data collected after 2005, the highest pooled mean blood Pb levels (B-Pb) were recorded in Pakistan's adult population (113.6 μg/L) and among children in Palestine (93.0 μg/L). However, data on B-Pb levels is unavailable for many countries (Ericson et al., 2021). In Italy, Croatia, and Slovenia mean Pb levels are substantially lower. In Italy, data from a study on adolescents revealed a B-Pb geometric mean of 9.60 μg/L (Pino et al., 2017). In Croatia, findings from postpartum, non-smoking women indicated a median B-Pb level of 7.0 μg/L (Grzunov Letinić et al., 2016). In Slovenia, data from an adult population including women and men, smokers and non-smokers showed a median B-Pb level of 15.1 μg/L (Štiglic et al., 2024).

1.3.1 Lead health effects at low-level of non-occupational exposure

For general non-occupationally exposure population, venous whole B-Pb concentrations of 100 μg/L have been established as the level of concern in the beginning of 2010's and were later revised to a reference value of 50 μg/L, as adverse health effects have been observed at B-Pb levels as low as 50 μg/L (ATSDR, 2020). For children and pregnant women, the threshold level of concern was further lowered to 35 μg/L, while it remains at 50 μg/L for non-pregnant adults (Ruckart et al., 2021; CDC, 2022).

When studying the toxic effects of Pb, most attention is given to the central nervous system. However, Pb toxicity also impacts the renal, cardiovascular, hematological, immunological, and reproductive systems (Mitra et al., 2017; ATSDR, 2020; Skerfving & Bergdahl, 2015). The Pb exposure levels associated with adverse health effects can vary depending on the specific health endpoint (summarized in Fig. 1.1). In the developed world, non-occupational exposure levels have significantly decreased in recent years, and they rarely reach the current reference values (Angrand et al., 2022), let alone the levels required to cause health effects (ATSDR, 2020). As aforementioned, for example in Slovenia, a study aiming to establish the population- and laboratory-specific reference intervals showed a

median B-Pb level of 15.1 µg/L in adult population. Clinical Pb poisoning is now primarily associated with occupational exposure, particularly among individuals working in lead mines, smelters, battery manufacturing, and the plastics industry, etc. (Skerfving & Bergdahl, 2015). Nevertheless, pregnant women and their fetuses, breastfeeding infants, and children remain at potential risk, as even low levels of Pb exposure could cause adverse health effects (ATSDR, 2020). This is why the recommended B-Pb threshold for pregnant women is lower than for non-pregnant adults (Ruckart et al., 2021). During pregnancy, Pb readily passes through the placenta, leading to prenatal exposure that can affect birth outcome and postnatal growth (ATSDR, 2020). Prenatal exposure is typically assessed by measuring Pb levels in cord blood (CB-Pb) (Bergdahl & Skerfving, 2022), and the reference value used for cord blood is generally the same as for maternal blood (CDC, 2022). Mothers can also pass Pb to their infants through breastfeeding and milk/maternal blood concentrations are usually <0.1 (ATSDR, 2020). Fetuses', infants' and children's developing organs are particularly sensitive to Pb, especially the central nervous system, as the blood-brain barrier is more permeable, increasing the risk of Pb entry (Sanders et al., 2009).

Special attention should be as well given to men of reproductive age, as studies suggested Pb negatively impacts male reproductive health. Pb exposure has been link to declines in various aspects of semen quality, including sperm count, motility, viability, structural integrity, along with increased morphological abnormalities and deoxyribonucleic acid (DNA) damage. The impact of Pb exposure becomes more pronounced as B-Pb levels increase. These effects become more pronounced as B-Pb levels rise, with harmful impacts observed even at levels of 100 µg/L or lower (ATSDR, 2020; Kumar, 2018).

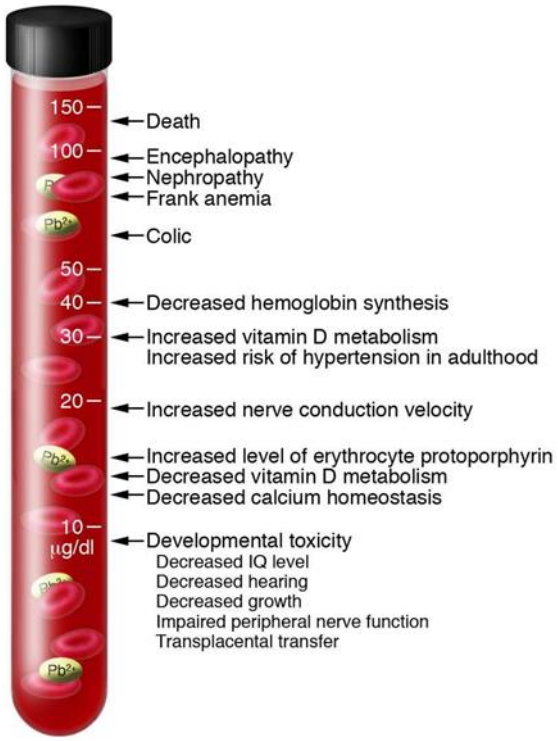

Figure 1.1: Pb exposure has been linked to specific physiological effects that impact major organ systems and their functions (Bellinger & Bellinger, 2006).

1.3.2 Lead toxicokinetics

The Pb toxicokinetic process includes liberation, absorption, distribution, accumulation/retention within one or more tissues and finally elimination of Pb from the body. Pb absorption differs between individuals based on age, health, nutritional status, route of exposure, particle size, etc. (Mushak, 2011). Absorption occurs mostly through the respiratory and gastrointestinal tract and only small portion through skin (Skerfving & Bergdahl, 2015).

Inhaled Pb deposition depends on the particle size, with particles of diameter >2.5 µm deposited in the upper part of respiratory tract and then swallowed, which adds additional burden load to the gastrointestinal absorption (Skerfving & Bergdahl, 2015). Inhaled particles less than 2.5 µm are deposited within the alveolar tract and can be absorbed after extracellular dissolution or ingestion by phagocytic cells (ASTDR, 2020). The extent and rate of gastrointestinal absorption of ingested Pb is affected by physiological factors (age, fasting state, nutritional levels of Ca and Fe, pregnancy, etc.), the physicochemical properties of the medium ingested (particle size, mineral composition, solubility) and ingested dose of Pb (ATSDR, 2020).

Absorbed Pb is first distributed to blood and then to various body compartment (ATSDR, 2020). Approximately 99 % of Pb within the blood is associated with erythrocytes. The reason lies in the high affinity of Pb for the ALAD protein, an enzyme found in all cells, especially erythrocytes. Only 1 % of blood Pb is linked with plasma (Bergdahl & Skerfving, 2022). However, at B-Pb levels exceeding 400 µg/L, the plasma fraction increases (Bergdahl et al., 1997). Pb turnover in plasma is rapid and Pb can then be distributed from plasma to soft and mineral tissue. More than 90 % of absorbed Pb is incorporated into the calcified tissues, bone accounts for 94 % of the total Pb body burden of adults and 73 % of the body burden in children (Skerfving & Bergdahl, 2015; ATSDR, 2020). Based on calcification rates the majority of Pb in children will accumulate in trabecular bone, while in adulthood accumulation will occur in both trabecular and cortical bone. Both types of bone contain inert and labile pools of Pb accumulation. While inert pools store Pb over longer periods, labile pools quickly exchange Pb with blood (Rabinowitz, 1991; Skerfving & Bergdahl, 2015). There is a constant turnover of the skeleton even in so-called human physiological base line. This causes Pb to be released from the skeleton and results in endogenous Pb exposure (Skerfving & Bergdahl, 2015). To illustrate the extent of such endogenous exposure, bone remodeling and modeling replace about 20-25 % of trabecular and 10 % of cortical bone annually (Kovacs, 2020a). In some circumstances such as pregnancy, lactation, menopause, kidney disease, etc. higher quantities of Pb can be triggered to be released from the bone, particularly in the case of malnutrition (Skerfving & Bergdahl, 2015). Pb levels in bones increase with age (ATSDR, 2020) and there are associations between bone Pb and B-Pb levels (Skerfving & Bergdahl, 2015).

The highest concentrations of Pb in soft tissues are found in the liver and kidney cortex. Relatively small volume of Pb is found in the brain in comparison to liver or kidney but it is of a high interest, particularly in developing children. Lead can pass the blood-brain barrier to some extent and becomes unevenly distributed within the brain with the highest concentrations in hippocampus, amygdala and choroids plexus (Skerfving & Bergdahl, 2015).

Lead is eliminated from the body mostly via urine and feces (ATSDR, 2020), to some extant is excreted in saliva and sweat and is present also in semen, the placenta, the fetus, milk, and hair (Skerfving & Bergdahl, 2015). Lead toxicokinetics in human body are summarized in Fig. 1.2.

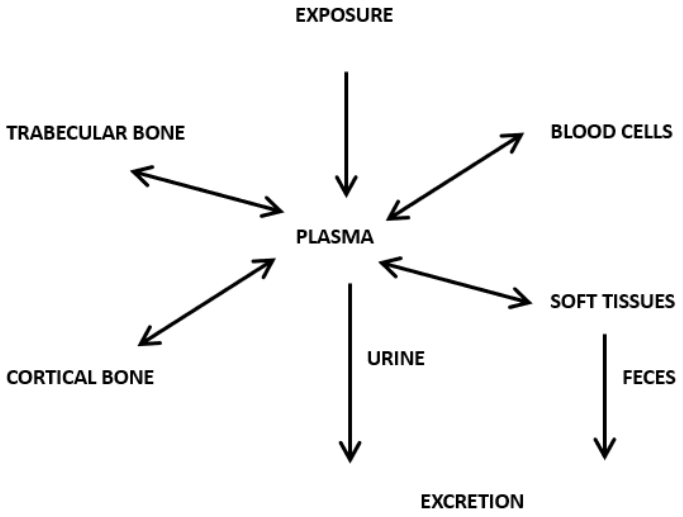

Figure 1.2: Lead toxicokinetics. Visual presentation of Pb distribution (modified from Bergdahl & Skerfving, 2020).

1.3.2.1 Lead toxicokinetics during pregnancy and lactation

During pregnancy, the fetus may be exposed to Pb released from the mother's bone stores or through increased maternal gastrointestinal Pb absorption due to pregnancy-related metabolic changes. This means that fetal Pb exposure can result not only from the mother's current exposure to external Pb during pregnancy but also from the mobilization of internal Pb that the mother accumulated before and during pregnancy (Bellinger, 2005).

Dietary Pb and previously stored maternal bone Pb can follow the same pathways as Ca in a competitive manner due to its affinity to similar ligands (Gulson et al., 2016; Téllez-Rojo et al., 2004). The fractional absorption of Ca in pregnant women begins to increase as early as the 12th week and continues until the end of pregnancy. This enhanced intestinal absorption is the primary maternal adaptation to meet the mineral needs of the growing fetus. By the middle of pregnancy, this increased absorption results in a positive Ca balance (Ryan & Kovacs, 2020; Kovacs, 2020b). Excess Ca is initially deposited in the maternal skeleton until fetal demand rises, leading to increased Ca release from maternal bones in the second part of pregnancy. Maternal bone resorption intensifies significantly, as approximately 80 % of that Ca is accreted during the third trimester (Kovacs, 2014). A study of 105 healthy women in Mexico City with averaged B-Pb levels of 70 μg/L observed that B-Pb levels followed a U-shaped pattern during pregnancy, with levels initially decreasing—likely due to factors such as hemodilution, organ growth, or increased glomerular filtration rate and Pb excretion (Bellinger, 2005)—and then increasing from 20 weeks of gestation onwards (Rothenburg et al., 1994). This increase continues postpartum (Kovacs, 2016). This pattern has been consistently observed in multiple studies (Bellinger, 2005), also in studies with comparable B-Pb levels (approximately 25 μg/L) (Gulson et al., 2003; Hertz-Picciotto et al., 2000) to our study populations from Italy, Croatia, and Slovenia.

Researchers are particularly interested in the potential mobilization of Pb from bone stores during the second part of pregnancy. Studies measuring stable Pb isotope ratios in pregnant women and cord blood have shown that up to 80 % of the Pb in fetal cord blood originates from maternal bone stores (ATSDR, 2020; Gulson et al., 2016). Gulson et al., 1997 also demonstrated that predominant mechanism for increase in B-Pb in last trimester

is not increased gastrointestinal absorption, but most likely resorption of the skeleton. The B-Pb levels were generally low, with a geometric mean of 30 µg/L. However, it is important to note the small sample size in this study (N=22). Furthermore, the increase of B-Pb during late pregnancy was more pronounced in older women, likely due to their longer history of Pb exposure and presumably higher levels of Pb stored in their bones (ATSDR, 2020). Women who experienced significant Pb exposure before pregnancy should be considered at higher risk (Bellinger, 2005).

1.3.3 Lead biomarkers at low level of exposure

1.3.3.1 Biomarkers of Pb exposure

1.3.3.1.1 Peripheral venous whole blood Pb levels

Lead measured in peripheral venous whole blood is the most commonly used marker of recent Pb exposure and Pb body burden. Additionally, long-term exposure can be determined using the mean of serial blood Pb levels. A significant portion of our understanding of human Pb exposure and its associated health effects is based on B-Pb measurements. Consequently, B-Pb results can be correlated with a vast array of toxicological data, giving B-Pb a major advantage over other Pb biomarkers by providing a more comprehensive and reliable source of information (Bergdahl & Skerfving, 2022).

The relationship between B-Pb levels and Pb exposure, intake, or uptake is not linear but curvilinear. At lower levels, B-Pb increases steadily with higher Pb uptake, but at elevated levels, the curve flattens, likely due to the saturation of Pb-binding sites in erythrocytes. For biological monitoring of Pb exposure, analyzing Pb in whole blood is particularly effective for detecting low and moderate levels (Bergdahl & Skerfving, 2008), which are typical in the subjects included in this thesis.

Pb in blood exists in at least two compartments: one with a half-life of about a month, reflecting recent exposure, and another related to bone and teeth stores, with a half-life spanning years to decades as lead is gradually released from bones. Thus, B-Pb concentration is an indicator of both recent exposure (over the last few months) and historical exposure (years prior) (Bergdahl & Skerfving, 2022). Evidence for the exchange of Pb between bone and soft tissue stores comes from analyzing stable Pb isotope signatures in bone and blood (ATSDR, 2020). A study comparing these isotope signatures found that bone Pb stores contributed to approximately 40-70 % of the Pb in blood. Notably, study included environmentally exposed population with low Pb exposure but did only include five patients. Their geometric mean of B-Pb levels was high (29 µg/L), which is important to acknowledge, as pharmacokinetics depend heavily on exposure levels (Smith et al., 1996). During pregnancy, aforementioned, the mobilization of bone Pb could increase even more (ATSDR, 2020). Among individuals, blood composition (e.g., plasma volume, cell amount) can vary slightly with age, sex, BMI, diet, and more significantly during specific physiological states, such as pregnancy—when plasma volume can increase by up to 50 %—or in cases of various diseases (e.g., hypertension, anemia) (Bergdahl & Skerfving, 2022; Kovacs, 2016). Since Pb is primarily bound to erythrocytes, B-Pb levels should ideally be normalized by hemoglobin concentration or hematocrit value to account for interindividual variability in cell/plasma proportion. Unfortunately, this normalization is still sometimes overlooked in HBM studies (Santonen et al., 2015).

While the invasive nature of blood sampling, typically through venipuncture, can be seen as a drawback, it does offer the advantage of minimizing sample contamination since only the arm, needle, and tube need to be kept clean. Measuring Pb levels in whole blood

is highly accurate, thanks to advanced analytical tools that ensure precise measurements (Bergdahl & Skerfving, 2008). The most commonly used are anodic stripping voltammetry, atomic absorption spectrometry and inductively coupled plasma mass spectrometry (ICP-MS) (Bergdahl & Skerfving, 2021), with the latter being the method used for measuring Pb levels in peripheral venous whole blood in our presented work.

1.3.3.1.2 Mixed cord blood Pb levels

When studying a birth cohort, identified as a type of longitudinal survey that assesses perinatal exposure and follows up with the children to evaluate associated health effects later in life, a very useful biomarker in the first step is cord blood (WHO, 2015). This biomarker reflects both the exposure history of the mother and the early exposure of the newborn infant. Cord blood offers the additional advantage of allowing the application of several well-defined and documented standard operating procedures (SOPs) used for peripheral blood. However, the volume of cord blood samples is usually limited, and their collection often faces the significant challenge of not always being practically feasible as during delivery, the primary focus is on the well-being of the mother and child, making the collection impossible (Smolders et al., 2009). However, in many birth cohort studies, even when collection is possible, mixed cord blood is used instead of arterial or venous cord blood, as obtaining the latter is extremely challenging. We must be aware of the drawback that using mixed cord blood results in random ratios of arterial and venous blood in the samples collected from different newborns. For metals like Pb, which are primarily stored in erythrocytes, this variation introduces a problem, as it can alter the measured concentrations of erythrocyte-accumulating elements (Trdin et al. 2020, Masoumi et al., 2017). Nevertheless, CB is a good screening biomarker for prenatal Pb exposure which happens as Pb can readily pass placenta. Cord blood concentrations are also a good representation of mother's current and pass exposure (ATSDR, 2020).

Studies examining the ratio of maternal blood to cord blood Pb concentrations revealed a correlation. Estimates of the maternal-to-fetal blood Pb ratio, based on CB-Pb measurements at delivery, range from 0.7 to 1.0, with mean maternal B-Pb levels ranging from 10 to 90 µg/L (ATSDR, 2020). It is important to note, that concentrations should be normalized by blood hemoglobin or hematocrit to get more reliable comparison and relevant ratios. In the presented thesis ICP-MS was used for measurements of CB-Pb levels.

1.3.3.1.3 Urine Pb levels

In biomonitoring studies, urine is the most frequently used matrix to quantify environmental or occupational exposure to pollutants, especially for substances with short biological half-lives. Its collection and analysis are risk-free, and large volumes can be obtained per individual. Spot collection is commonly used in biomonitoring programs, particularly for surveys requiring large sample numbers (Smolders et al., 2009). Furthermore, samples can be directly collected by the donors, simplifying the fieldwork. However, we have to be aware that due to uncontrolled nature of this collection method, it can introduce potential uncertainties (WHO, 2015).

Urine has been used to some extant in biomonitoring for Pb exposure, as U-Pb is associated with B-Pb, but the variation is too large to predict B-Pb from U-Pb reliably (Bergdahl & Skerfving, 2022; Sakai, 2000).

Urinary Pb concentrations respond more rapidly to changes in exposure compared to those in blood. Therefore, according to WHO (2015), U-Pb mainly reflects recent Pb absorption but may not serve as a reliable biomarker for long-term exposure. Nevertheless, for U-Pb is suggested to mostly reflect the filterable fraction of Pb in plasma, and the effect of day-to-day variation in U-Pb can be reduced by pooling repeated samples (Bergdahl &

Skerfving, 2022). Furthermore, recent studies involving 60 healthy, non-smoking individuals reported that, in addition to B-Pb, U-Pb adjusted for creatinine or specific gravity (SG) appears to be a useful biomarker for exposure assessment in epidemiological studies, as indicated by the intra-class correlation coefficient (Sallsten et al., 2022).The difficulty in predicting B-Pb from U-Pb is partly due to challenges in adjusting the dilution of urine, which in the past was usually done by creatinine excretion. The later depends on meat intake and muscle mass, so makes comparisons between different gender and ages doubtful. Specific gravity is less affected by diet, body size, and muscle mass than creatinine adjustment, therefore, it appears to be more suitable alternative in context of environmental exposure (Hoet et al., 2016; Stajnko et al., 2017). In this thesis, SG adjustment was applied to the U-Pb concentrations measured using ICP-MS.

1.3.3.2 Biomarkers of effect at low level of exposure

A biomarker of effect is any biological change that can qualitatively or quantitatively predict health impairment or the potential for health impairment resulting from exposure to a particular substance (NRC, 1987). Understanding the relationship between the dose of a substance and the resulting biological effect, known as the dose-effect relationship, is crucial for monitoring biological effects (Moffett et al., 2022). In the case of Pb exposure, the first critical tissues or organs to exhibit effects include the bone marrow, central and peripheral nervous systems, kidneys, and liver. Significant effects may also appear later in the cardiovascular and reproductive systems (ATSDR 2020). The most researched effect of Pb exposure is its impact on bone marrow which can be assessed by measuring disruptions in heme metabolism, including reduced enzyme activity or altered concentrations of pathway intermediates (Bergdahl & Skerfving, 2022). An elevated erythrocyte protoporphyrin (EP) level is one of the earliest and most reliable indicators of impaired heme biosynthesis. However, EP levels do not increase until B-Pb levels exceed 250 µg/L. Similarly, the activity of δ-aminolevulinic acid dehydrogenase (ALAD) enzyme has been used to determine Pb exposure and absorption, but to serve as a sensitive biomarker, B-Pb concentrations must be higher than 210 µg/L (ATSDR, 2020). According to a recent study, the possible threshold of B-Pb for begging to affect ALAD activity was 50 µg/L (Huang et al., 2020). Pb dose levels that cause measurable health effects in the aforementioned critical tissues and organs are today rarely reached in non-occupational populations, especially in the developed world (Bergdahl & Skerfving, 2022). This observation also applies to the populations included in the present work.

Numerous studies showing decrements in neurological function in children have been published. Collectively, these studies support the concept that Pb affects cognitive function in children prenatally exposed to PbB ≤100 µg/L, with some studies providing evidence for effects at PbB ≤50 µg/L. These effects include reduced cognitive abilities (e.g., IQ, learning, memory), altered mood and behavior (e.g., attention deficits, hyperactivity, impulsivity), and neuromotor and sensory changes (e.g., visual-motor integration, dexterity, hearing). Some studies also suggest that early Pb exposure may contribute to lasting neurobehavioral and neuroanatomical changes into adulthood. However, several factors can introduce bias in estimating associations between B-Pb levels and neurobehavioral outcomes, potentially weakening or exaggerating observed relationships, especially when exposure levels are low. Neurological function is shaped by various influences that may also correlate with Pb exposure, particularly socioeconomic status (SES). High-quality studies typically control for confounders like maternal education and IQ, SES, and parental care (HOME score), while some also consider maternal substance abuse, maternal stress conditions (cortisol levels), prenatal alcohol exposure, birth weight, tobacco smoke, nutrition, and *ALAD* genotype (ATSDR, 2020). Besides, it is important to

note that blood lead levels do not directly correlate with Pb levels in soft tissues or bone, nor with the biologically active forms of Pb present in the body.

1.3.3.3 Biomarkers of susceptibility at low level of exposure

It is a well-established that interindividual diversity in sensitivity to potentially toxic TEs is significant, and genetic (inherent) differences can play an important role. Genetic variations can lead to different responses at the same level of exposure, affecting both toxicokinetics and toxicodynamics of these toxins. Consequently, such variations may affect the uptake, distribution, accumulation, retention and dose-related toxic effects of Pb (Skerfving & Bergdahl, 2015). Moreover, other factors, including acquired factors such as nutrition, medications and disease, as well as physiological changes like pregnancy, can also impact susceptibility (Sakai, 2000).

Genetic susceptibility to TEs is often studied through the lens of naturally occurring DNA sequence variations, which, when accumulated in populations, contribute to human variability. When a genetic variation occurs with a frequency of 1 % or more of a population, it is arbitrary termed a polymorphism; otherwise as mutation. Among various types of polymorphisms, such as copy number variations, duplications, and deletions, the most common is the single nucleotide polymorphism (SNP). SNPs represent a change in a single nucleotide that occurs approximately every 500 base pairs in the genome (Broberg & Pawlas, 2022). Therefore, genes can exist in different versions known as alleles. The allele that appears most frequently in the population is called the major or common allele, while its alternate form is termed the minor or variant allele. The frequency of these variant alleles, known as the minor allele frequency (MAF), can vary significantly among different populations (Reid-Lombardo & Petersen, 2010). Studying gene-environment interactions is challenging due to the difficulty in obtaining large enough sample sizes. Two key factors to consider are the prevalence of the SNPs in the population and the magnitude of effect modification. SNPs with high MAF are less likely to have a strong effect, but they offer more statistical power due to their higher frequency (Kelada et al., 2003).

SNPs can have diverse effects on gene function and phenotype, depending on their location within a gene. However, only a small portion of SNPs have significant effects on the phenotypes, while the majority have little to no impact. When located in coding regions, they may alter amino acid sequences, while those in non-coding regions can affect promoter activity (gene expression levels), alternative splicing, messenger ribonucleic acid (mRNA) conformation (stability) and translational activity (Robert & Pelletier, 2018; Kelada et al., 2003). Many genes contain multiple SNPs, which may be strongly correlated and inherited together in specific nucleotide sequences known as haplotypes, due to a phenomenon called linkage disequilibrium (LD). The latter is a non-random linkage of genetic variants that are inherited together in chunks (Reid-Lombardo & Petersen, 2010). Therefore, the association of a single SNP with a biochemical mechanism may not imply causation, as it could be linked to another variant within the same gene that is the actual causative factor (Broberg et al., 2015). Investigating haplotypes within candidate genes—genes chosen for study due to their known or suspected role in a particular biological pathway or disease—can often provide more insight than studying individual SNPs alone (Kelada et al., 2003). Besides haplotypes derived from analyzing LD, it may also be valuable to examine so-called ‘combinations’, which assess the potential combined effects of different SNPs regardless of their LD. However, particularly in non-disease state, this approach can sometimes identify connections by chance rather than true biological relevance. If these combinations are further validated as influential factors, rather than coincidental findings, they could serve as more reliable biomarkers of susceptibility to Pb exposure than single SNPs alone.

Numerous SNPs may alter susceptibility to Pb with *ALAD* and Vitamin-D receptor (*VDR*) SNPs being the most extensively studied. Less data is available for other SNPs, such as those in Apoprotein E (*APOE*), metallothionein genes, hemochromatosis gene, Dopamine receptor D4, Glutathione S-transferase mu 1, Peptide transporter 2, Cytochrome P45, etc. (ATSDR, 2020; Mani et al., 2019).

1.3.3.3.1 *ALAD*

ALAD was one of the first genes investigated for its role in susceptibility to Pb poisoning and remains the most studied in relation to Pb exposure. The initial discovery of variations in B-Pb levels related to the *ALAD* genotype was made nearly 35 years ago (Ziemsen et al., 1986). *ALAD* is a gene that codes the ALAD enzyme, which is also known as porphobilinogen synthase (PBGS), a multimer metalloenzyme that is involved in the hem biosynthetic pathway, with Zn-binding sites essential for its activity (Jaffe, 2020). When Pb binds to the enzyme it inhibits it by displacing Zn from its active site, as Pb has significantly higher affinity for this enzyme than Zn, making ALAD an important Pb-binding enzyme and primary binding ligand in erythrocytes (ATSDR, 2020).

As SNPs of the *ALAD* gene can influence the binding affinity of Pb towards ALAD or alter its expression, changes in the amount of B-Pb concentrations and bioavailable Pb within the body, may result. he most studied variant is the Lys59Asn substitution (rs18000435), with the two alleles referred to as *ALAD1* (the Lys variant) and *ALAD2* (the Asn variant), resulting in the *ALAD1-1*, *ALAD1-2*, and *ALAD2-2* genotypes. Although thus substitution does not occur anywhere near the Zn-binding site, where Pb also presumably binds, it effects enzyme affinity and stability for Pb, likely due through structural changes. Since Asn is a neutral amino acid and Lys is positively charged, this substitution results in a more electronegative enzyme. This raises the hypothesis that *ALAD2* may bind the positively charged Pb more tightly than *ALAD1*, potentially immobilizing it in the intravascular space, limiting its bioavailability for other tissues (Broberg & Pawlas, 2022).

Findings on B-Pb levels between *ALAD1* and *ALAD2* carriers are somewhat inconsistent, depending on the exposure levels. At higher exposure levels results are more coherent with variant allele *ALAD2* linked to higher B-Pb levels, possibly indicating a protective role by limiting toxic effects in other tissues (Bergdahl & Skerfving, 2022; Broberg & Pawlas, 2022; Kelada, et al., 2001; ATSDR, 2020). However, at lower exposure levels, results have been fairly inconsistent, with some studies reporting no effect or even lower B-Pb levels for *ALAD2* carriers (Stajnko et al., 2020; Tasmin et al., 2015; Skerfving & Bergdahl, 2015).

The vast majority of studies that have looked into Pb exposure and susceptibility biomarkers have researched *ALAD* SNPs, however most of them focused solely on rs1800435. Potential effects of other *ALAD* polymorphisms should also be considered (Broberg et al., 2015; Broberg & Pawlas, 2022). Some studies have tested and highlighted the possible independent impact of several other *ALAD* SNPs, particularly rs1805313, rs1139488, rs2228083, rs818708, and rs8177800, further emphasizing *ALAD* 's influence on Pb concentrations (Broberg & Pawlas, 2022; Mani et al., 2019; Mitra et al., 2017). Some of them were done on populations with occupational exposure (Shaik, 2018; Szymańska-Chabowska et al., 2015; Li, et al., 2017) and some on non-occupationally exposed (Pawlas et al., 2012; Stajnko et al., 2020; Warrington et al., 2015). However, only one study has examined *ALAD* haplotypes and their combined effects on B-Pb concentrations in 129 women with B-Pb median level of 48 µg/L (Rabstein et al., 2008), and another attempted to associate *ALAD* haplotypes with Pb exposure based on questionnaire data rather than

direct measurement of Pb in biological samples (Bemmel et al., 2011). None have explored the potential combined effects of different SNPs, regardless of their LD, that are independently associated with variations in Pb concentrations. Both aspects were thoroughly investigated in the present work. Additionally, unlike the populations studied in this thesis, the majority of previous research focused on occupational or Pb-contaminated environments, with only two studies including pregnant women (Akyuzlu et al., 2014; Yun et al., 2015).

1.3.3.3.2 *VDR*

Vitamin D is a conditionally essential vitamin that represents a group of (seco)steroid compounds. The two most important forms for humans are D2 (ergocalciferol) and D3 (cholecalciferol), both of which can be obtained from dietary sources—D2 primarily from plant-based items and D3 from animal-derived products—or as supplements. Additionally, D3 can be synthesized in the skin from 7-dehydrocholesterol upon exposure to ultraviolet (UV) light from the sun. Both forms are hydroxylated in the liver and subsequently in the kidney to produce ercalcitriol and calcitriol, the active metabolites (Fig. 1.3). Calcitriol, also known as 1,25-dihydroxyvitamin D3 (or 1,25-cholecalciferol), is more potent than ercalcitriol. It plays a vital role in regulating Ca and phosphate metabolism by enhancing their intestinal absorption, promoting renal reabsorption, and providing the necessary minerals for new bone formation. The circulating vitamin D hormone binds to vitamin D receptors in intestinal, kidney, and bone cells, activating genes that encode Ca-binding proteins to facilitate Ca transport. In conditions of Ca deficiency, vitamin D production increases, enhancing Ca-binding protein synthesis, which may also bind Pb due to its similar properties as a divalent cation, potentially altering Pb toxicokinetics. Thus, Ca deficiency can lead to increased intestinal Pb absorption and retention (Bergdahl & Skerfving, 2022; Lips, 2006; Onalaja & Claudio, 2000).

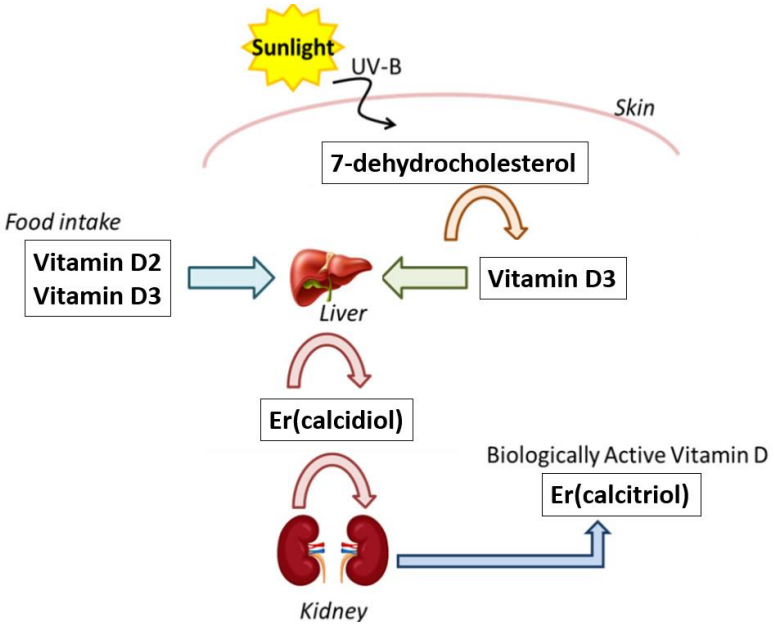

Figure 1.3: The vitamin D production pathway (modified from Aiello et al., 2024).

Interactions between calcitriol, Ca, and Pb are complex, and the *VDR* gene adds further complexity due to its polymorphic nature in humans. Multiple SNPs, including *FokI* (rs2228570), *BsmI* (rs1544410), *ApaI* (rs7975232), *TaqI* (rs731236) and BglI (rs739837),

have been identified. Some studies suggest that the *FokI* and *BsmI* variant alleles are associated with lower *VDR* expression, leading to diminished B-Pb levels (ATDRS, 2020). However, research on the effects of *VDR* SNPs on Pb toxicokinetics has yielded inconsistent results, particularly at lower exposure level. These discrepancies may be due to the highly polymorphic nature of the *VDR* gene and the presence of population-specific haplotypes, which can obscure associations when single polymorphisms are analyzed in relation to Pb biomarkers (Broberg et al., 2015). Additionally, associations studies at low exposure levels are influenced by various physiological pathways which could not be controlled.

1.3.3.3.3 *APOE*

Apolipoprotein E is a multifunctional glycoprotein synthesized by various tissues, including the liver, brain, bone, adipose tissue and other tissues. It plays a key role in lipid transport and metabolism, facilitating the distribution and uptake of cholesterol and other lipids essential for cell membrane maintenance, hormone production, and other cellular functions. The majority of APOE is found in the plasma and is primarily derived from the liver (Getz & Reardon, 2009; Giau et al., 2015).

APOE exists in three isoforms—APOE2, APOE3, and APOE4—encoded by alleles ε2, ε3, and ε4, respectively. These isoforms differ by single amino acid substitutions at residues 112 and 158: APOE2 has cysteine at both positions, APOE3 has cysteine at 112 and arginine at 158, while APOE4 has arginine at both sites. These structural variations influence lipid-binding, receptor-binding, antioxidative, and metal-binding properties differently (Berntsson et al., 2022; Egert et al., 2012; Jofre-Monseny et al., 2008; Kara et al., 2017; Mahley, 1988; Miyata & Smith, 1996; Tudorache et al., 2017).

APOE SNPs have diverse effects on human health across various stages of life and conditions. For instance, APOE4 demonstrates context-dependent effects on immune function. García et al. (2021) observed that in high-pathogen environments, APOE4 carriers exhibited lower baseline inflammation, potentially reducing the energetic burden of immune activation during infections. On the other hand, Gale et al. (2014) and Dose et al. (2018) noted that APOE4 can heighten pro-inflammatory responses under certain conditions, which may provide short-term benefits for pathogen defense but carry risks of chronic inflammation over time. Beyond its role in immunity, APOE4 has been linked to higher levels of vitamin D (Huebbe et al., 2011; Soares et al., 2021), and Ca (Huebbe et al., 2011), as well as increased progesterone levels and/or fertility in women of childbearing age (Jasienka et al., 2015; Trumble et al., 2023) and in experimental transgenic mice (Fagla et al., 2024). Conversely, in older adults, the ε4 allele is associated with a higher risk of 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). These contrasting effects make APOE4 a clear example of antagonistic pleiotropy, particularly in populations with Westernized lifestyles.

Recent studies highlight APOE important role in bone biology, suggesting it helps maintain bone density by promoting bone formation and reducing excessive bone resorption. The ε2 allele appear to have the least protective effect on preserving bone mass (Dieckmann et al., 2014), as well as having negative impact on the fracture risk (Zhang et al., 2014). The underlying mechanisms of different APOE isoforms on bone is still not completely clear with few possible explanations including–impaired lipoprotein-associated vitamin K delivery in APOE2 knock in mice to osteoblast (Dieckmann et al., 2014) because of reduced binding affinity of APOE2 to cell surface lipoprotein receptors (Niemeier et al., 2005, 2008) or the higher affinity of APOE4 to lipoprotein receptors (Niemeier et al., 2012) and higher serum vitamin D and Ca levels related to APOE4 (Huebbe et al., 2011). APOE appears to influence bone metabolism and may also affect B-Pb concentrations particularly

during pregnancy, when the Ca cycle intensifies, potentially increasing the uptake and release of Pb together with Ca.

In general, research on *APOE* SNPs often focuses only on the ε4 allele due to its association with adverse health outcomes in old age. However, the ε2 allele, probably less frequently studied due to its lower prevalence (about 0.08 in Caucasians compared to 0.77 for ε3 and 0.15 for ε4), also plays a distinct role. Therefore, it is important to compare not only carriers with non-carriers but also those with the common ε3/ε3 genotype against ε2 and ε4 carriers to fully understand the effects (Niemeier et al., 2012).

1.3.3.3.4 Venous blood used for DNA isolations

Blood is the preferred matrix for determining SNPs. Compared to other matrices like saliva or urine, blood provides a higher quantity and quality of isolated DNA, resulting in improved performance of molecular techniques (Philibert et al., 2008). In some circumstances decision to isolate DNA comes some years later than the blood was collected, same was the case for us. For Italian PHIME participants, DNA was isolated in 2018, for Croatian participants in 2015, and for the Slovenian HBM population in 2019. For Slovenian PHIME participants, venous blood was collected during the CROME-LIFE + project (Cross-Mediterranean Environment and Health Network) project between 2013 and 2017, and DNA was isolated in 2018. However, studies have shown that long-term storage of whole blood (up to 19 years) does not affect the integrity of the extracted nucleic acids. No correlation was found between the duration of storage and the total yield or quality of the DNA extracted (Chen et al., 2018).

1.3.4 Interactions in lead exposure

When researching Pb exposure, it is important to monitor other potentially toxic and essential TEs due to their complex interactions. Metals such as Cd, Hg, and As, alongside essential elements like Fe, Ca, Se, and Zn can influence Pb absorption, distribution and retention (Nordberg et al., 2022b). Several nutritional and physiological factors are important to follow as well. Age, gender, smoking, alcohol and seafood consumption, parity in the case of pregnant women, etc. (Nordberg et. al., 2022b; Bocca et al., 2020; Lewin et al., 2017) can affect the risk for toxic effect of Pb.

Adequate nutritional levels of Zn, Fe, Se, and Ca seem to reduce Pb absorption and toxicity. Adequate Zn levels can counteract Pb binding to the ALAD enzyme, while Fe deficiency has been shown to increase Pb absorption, with children experiencing 4-5 times greater Pb poisoning in the absence of sufficient Fe (Nordberg et al., 2022b). In a study of occupationally exposed population, it was shown that a group with lower blood Se had significantly higher B-Pb levels than a group of high blood Se (Pawlas et al., 2016). Moreover, a study conducted on children with low Pb exposure also demonstrated that B-Pb concentrations have a negative relationship with Se levels. This indicated that even at low B-Pb levels, Se status is important to incorporate into the studies (Nordberg et al., 2022b). The influence of Ca statues on B-Pb levels has also been widely researched, particularly in pregnant and lactating women, showing that Ca supplementation may reduce maternal B-Pb levels as well as Pb levels in breast milk (Bergdahl & Skerfving, 2022).

Co-exposure to mixtures of metals such as Pb, Hg, As, Cd often results in more severe effects, both at high and low dose levels (Wang & Fowler, 2008). Interactions between Pb, Cd, and As have been shown to alter the tissue deposition of these elements in animal studies (Nordberg et al., 2022b). Given the PHIME study's focus on Hg levels in

Mediterranean populations with seafood consumption (Valent et al., 2013), it is important to track Hg exposure as well.

In this research, Pb susceptibility biomarkers are main focus, including *ALAD* and *APOE* SNPs, which code for ALAD protein and APOE protein isoforms that have been shown to interact with other TE as well. In the case of ALAD, in addition to Pb also Cd, Hg and As can bind to the enzyme (Rocha et al., 2012). APOE isoforms may influence not only Pb, but also Hg, Se, and Ca levels (Huebbe et al., 2011; Snoj Tratnik et al., 2017; Trdin et al., 2020; Wright et al., 2003; ATSDR, 2020).

Chapter 2

Aims and Hypotheses

2.1 Specific Aims

The aim of the present dissertation was to assess the influence of *ALAD*, *VDR*, and *APOE* SNPs on Pb levels in blood, cord blood, and urine in susceptible general populations, including pregnant women, newborns, and men of reproductive age, who have been exposed to low to moderate levels of Pb, Hg, Cd, and As. The populations, characterized by good nutritional status (adequate levels of Zn and Se), were recruited from Italy, Croatia, and Slovenia as part of the PHIME project (pregnant women with newborns) and Slovenian HBM project (men of reproductive age). Firstly, we investigated the associations between the four most researched *ALAD* and *VDR* SNPs (individual SNPs, their haplotypes, and combinations) as well as less studied *APOE* genotype and Pb levels in (cord)blood of pregnant women and newborns. Possible associations with other potentially toxic and essential TEs were also examined. Secondly, we assessed the influence of APOE2, APOE3, and APOE4 protein isoforms on (cord)blood Pb levels with an emphasis on bone metabolism, incorporating the importance of fetal/newborn sex and parity. Thirdly, we analyzed the influence of ten different *ALAD* SNPs and five different *VDR* SNPs on blood and urine Pb levels in men of reproductive age.

2.2 Hypothesis

1. In populations with low to moderate Pb exposure, we predict that the *ALAD2* allele will be associated with lower Pb concentrations in blood than the *ALAD1* allele.
2. Additional *ALAD* SNPs as well as *VDR* SNPs, their haplotypes, and combinations, may also affect (cord)blood and urine Pb levels.
3. The *APOE* ε4 allele, which is thought to provide a protective effect in younger populations, is predicted to be associated with lower (cord)blood Pb levels.

Chapter 3

Publications

This dissertation comprises three published Articles: *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, Maternal *APOE* $\varepsilon 2$ as a possible risk factor for elevated prenatal Pb levels, Genetic susceptibility to low-level lead exposure in men: Insights from *ALAD* polymorphisms. These articles are presented in alignment with the Specific Aims and address the questions posed in the Hypotheses (Chapter 2).

The first hypothesis, proposing that the *ALAD2* allele is associated with lower B-Pb levels than the *ALAD1* allele, was tested in Articles 1 and 3. These Articles analyze the effect of the most researched *ALAD* SNP (rs1800435) (*ALAD1/2*) on (cord)blood Pb levels in a population of pregnant women and their newborns from costal region of Italy (PHIME study, Article 1), and on blood and urine in a population of men of reproductive age from different regions of Slovenia (HBM 1 study, Article 3).

The second hypothesis, examining the associations of additional *ALAD* SNPs and selected *VDR* SNPs with Pb levels, was tested in the same two Articles (Article 1 and Article 3) The influence of additional *ALAD* SNPs on (cord)blood and urine Pb levels was analyzed in both Articles, focusing on three SNPs (rs1805313, rs1139488, and rs818708) in Article 1, and nine SNPs (rs1805313, rs1139488, rs818708, rs2761016, rs8177812, rs2228083, rs1805312, rs8177796, and rs818684) in Article 3. Four *VDR* SNPs (FokI, BsmI, ApaI, and TaqI) were examined in both articles, with additional SNP, BglI, included in Article 3.

The third hypothesis, concerning the influence of *APOE* genotype on cord(blood) Pb levels, is addressed in Article 1 and explored more comprehensively in Article 2. Article 2 expands the sample size by including pregnant women and their newborns from Croatia and Slovenia, in addition to the Italian cohort, all from the PHIME study. This Article additionally emphasis the importance of fetal/newborn sex (already observed in Article 1) by stratifying participants accordingly.

3.1 Article 1: *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

The paper is authored by Neža Palir, Anja Stajnko, Janja Snoj Tratnik, Darja Mazej, Alenka Sešek Briški, Alenka France-Štiglic, Valentina Rosolen, Marika Mariuz, Elisa Giordani, Fabio Barbone, Milena Horvat, Ingrid Falnoga (2023). It has been published in Environmental Research.

Research on *ALAD* and *VDR* SNPs is extensive, and some SNPs, such as *ALAD* rs1800435 (*ALAD1/2*) and *VDR FokI* and *BsmI*, linked to influences on Pb concentrations, though mostly at high exposure levels. At lower exposure levels, findings have been inconclusive or, for some SNPs, nonexistent. Studies on pregnant women are especially rare. APOE isoforms have been suggested to influence TEs kinetics through various mechanisms, but research on its relationship to Pb toxicokinetics remains limited.

In this study, we examined the associations between specific SNPs in the *ALAD*, *VDR*, and *APOE* genes with biomarkers of exposure to Pb and Hg, including some other TEs. Monitoring other potentially toxic and essential TEs is beneficial when researching Pb exposure due to their complex interactions. Studied population included Italian pregnant women and their newborns, who were part of the PHIME project. The cohort consisted of 873 pregnant women aged 18-44 years and 619 newborns, with low-level mixed-element exposure (B-Pb GM: 11.0 ng/g, range: 3.09-60.5 ng/g; B-Hg GM: 2.16 ng/g, range: 0.05-39.6 ng/g; CB-Pb GM: 10.4 ng/g, range: 2.63-47.0 ng/g; CB-Hg: 3.88 ng/g, range: 0.12-54.8 ng/g) and adequate nutritional status of Zn and Se. We analyzed DNA from maternal blood for four *ALAD* SNPs (rs1800435, rs1805313, rs1139488, rs818708), four *VDR* SNPs (rs2228570, rs1544410, rs7975232, rs731236), and two *APOE* SNPs (rs429358, rs7412) SNPs using TaqMan SNP assays. Available trace elements levels for maternal blood, plasma, hair, and cord blood were evaluated. Multiple linear regression models were used to explore the relationships between gene SNPs and selected TEs concentrations while controlling for appropriate confounding variables.

Our study confirmed three *ALAD* SNPs (rs1800435 variant allele aka *ALAD2*, rs1805313 variant allele and rs1139488 common allele) and their combination were negatively associated with B-Pb levels. Their combination showed the most significant influence. No significant associations were found with other TEs in the blood or cord blood, including CB-Pb levels. Additionally, no association were identified between *VDR* SNPs and Pb levels. However, we confirmed the negative association of *APOE* ε4 allele on maternal and cord blood Hg levels compared to non-carriers, and the positive association of the maternal ε2 allele with CB-Pb levels in newborn girls compared to non-carriers. All models which included blood TEs were re-run for pregnant women sampled in the second trimester (78 % of population). By excluding the influence of advancing pregnancy on TE levels, we found that the strength of associations increased.

The results indicate potential modification effects of *ALAD* and *APOE* SNPs on Pb and Hg toxicokinetics, highlighting the importance of genetic factors in assessing susceptibility to metal toxicity, particularly in vulnerable populations such as pregnant women and their developing fetuses.

I contributed to the Article by genotyping the included SNPs, statistical analyses, preparation of tables, and writing of the Article.

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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 *ALAD* 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 $\epsilon 2$, $\epsilon 3$ and $\epsilon 4$, 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 $\epsilon 4$ 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 SpekıtAA-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 *ALAD* rs1800435 polymorphism, in which only allele categories were applied because of an insufficient number of participants with the *ALAD2/2* 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 $\pm$ 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.

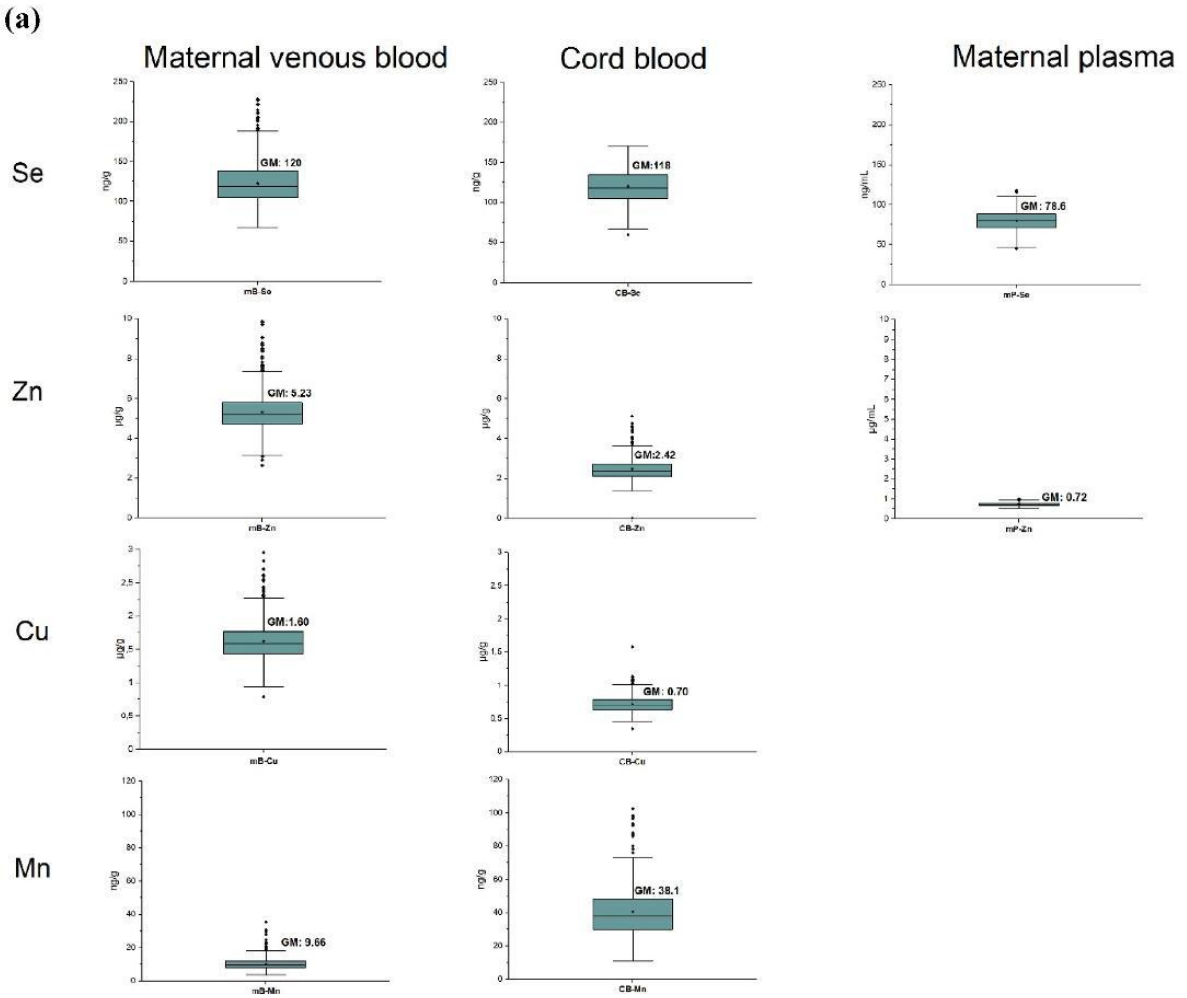
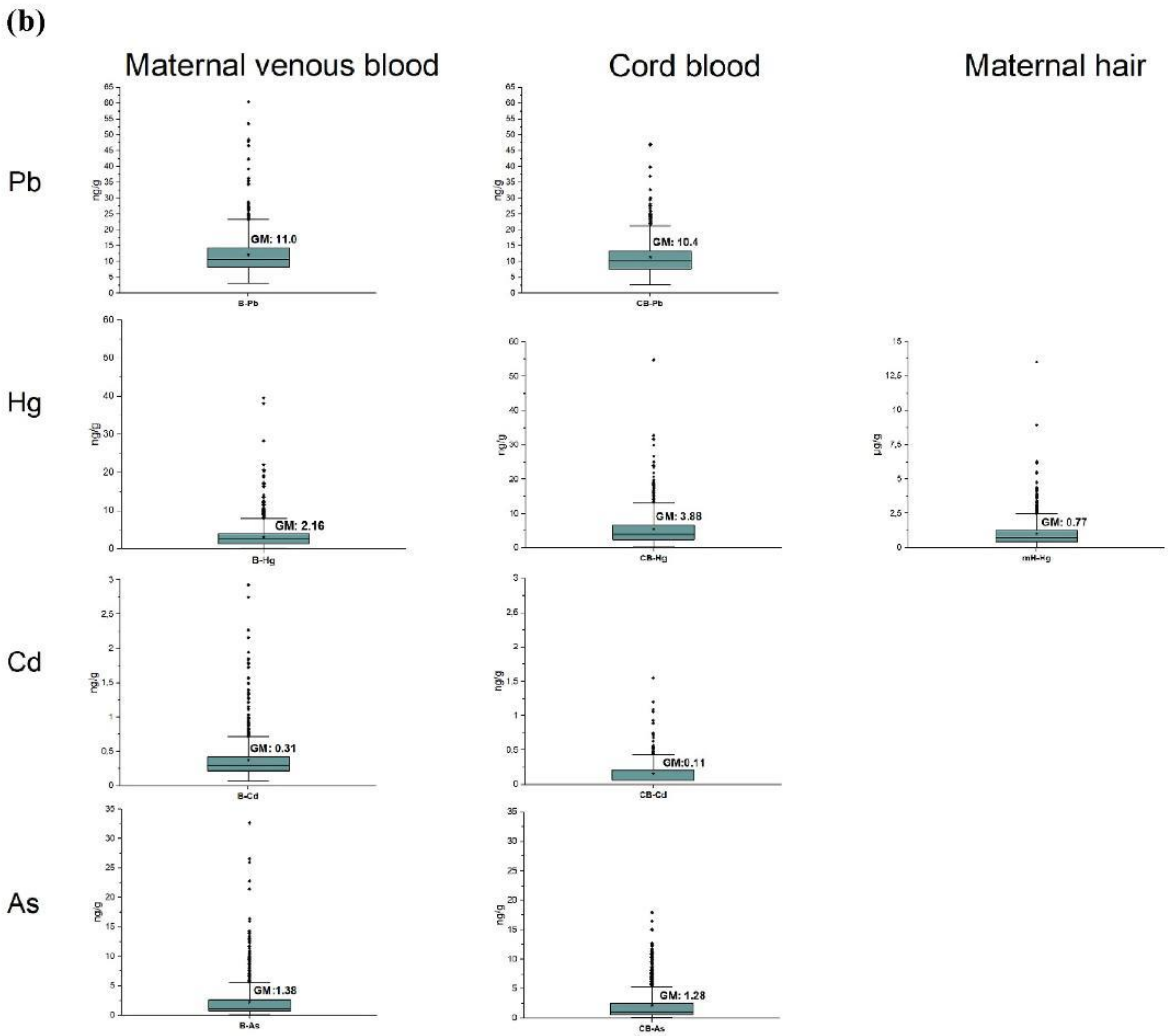


Fig. 1. Distribution of essential (a) and non-essential (b) TEs in maternal blood, plasma, hair and mixed umbilical CB presenting the median, AMs, 25th and 75th percentiles, outliers and GMs.



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 *ε4* 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 *ALAD2* allele, and a similar finding was reported by Yun et al. (2015), in which pregnant women carrying at least one *ALAD2* 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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3.2 Article 2: Maternal *APOE* ε2 as a Possible Risk Factor for Elevated Prenatal Pb Levels

The paper is authored by Neža Palir, Anja Stajnko, Darja Mazej, Alenka France Štiglic, Valentina Rosolen, Marika Mariuz, Luca Ronfani, Janja Snoj Tratnik, Agneta Annika Runkel, Veronika Tursunova, Janja Marc, Igor Prpić, Zdravko Špirić, Fabio Barbone, Milena Horvat, Ingrid Falnoga (2024). It has been published in Environmental Research.

Based on our findings regarding the sex-related influence of *APOE* ε2 allele on CB-Pb levels in Article 1, we further investigated the impact of APOE isoform (APOE2, APOE3, APOE4) on B-Pb and CB-Pb levels, together with B-Hg and CB-Hg levels. We extended our study population to include Croatian and Slovenian participants, in addition to Italian, all from the PHIME project. This allowed us to exclude smokers and stratify the population by fetal/newborn sex. The study was conducted on a cohort of 817 pregnant women aged 18-44 years and 772 newborns, all of whom had adequate levels of Zn and Se and low Pb and Hg exposure (B-Pb GM: 11.1 ng/g, range: 3.58–87.6 ng/g; B-Hg GM: 2.17 ng/g, range: 0.11-39.6 ng/g; CB-Pb GM: 9.31 ng/g, range: 1.82–47.0 ng/g; CB-Hg GM: 3.05 ng/g, range: 0.12-32.8 ng/g). We analyzed DNA from maternal blood for *APOE* (rs429358, rs7412) SNPs using TaqMan SNP assays, and multiple linear regression models were applied. Given the PHIME study cohort's expected seafood consumption and our previous findings of associations between Hg and *APOE*, we also conducted association analysis on Hg.

We confirmed sex differences and APOE isoform influences on (cord)blood Pb levels. The maternal ε2 allele was positively associated with B-Pb and CB-Pb levels when the fetal/newborn sex was female. The maternal ε4 allele showed a negative link with B-Pb levels regardless of the fetal sex and, with CB-Pb levels specifically in female newborns. These genetic influences were more pronounced in *nulliparous* women, where we also observed a negative association between the maternal ε4 allele and B-Hg levels when the fetal sex was female.

These findings highlight the importance of considering maternal genetics, fetal/newborn sex, and parity in understanding prenatal Pb exposure, and suggest that the maternal ε2 allele may increase the risk of Pb exposure during pregnancy.

I contributed to the Article by genotyping the *APOE* SNPs, statistical analyses, preparation of tables and figures, and writing of the Article.

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Maternal *APOE* ε2 as a possible risk factor for elevated prenatal Pb levels☆

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(cord)blood
Maternal *APOE* ε2 allele
Bone turnover
Fetal sex
Parity

ABSTRACT

Lead (Pb) is a global contaminant associated with multiple adverse health effects. Humans are especially vulnerable during critical developmental stages. During pregnancy, exposure to Pb can occur through diet and release from maternal bones. Apolipoprotein E gene (*APOE*) variants (ε2, ε3, ε4 alleles) may influence sex steroid hormones, bone metabolism, and Pb kinetics.

We examined the interplay among maternal *APOE* (m*APOE*) genotypes, fetal sex, parity, and Pb in maternal and cord blood (mB-Pb, CB-Pb) using linear regression models. Our study involved 817 pregnant women and 772 newborns with measured adequate levels of zinc and selenium. We compared carriers of the ε2 and ε4 alleles to those with the ε3/ε3 genotype.

The geometric means (range) of mB-Pb and CB-Pb were 11.1 (3.58–87.6) and 9.31 (1.82–47.0) ng/g, respectively. In cases with female fetuses, the maternal m*APOE* ε2 allele was associated with higher, while the m*APOE* ε4 allele was associated with lower mB-Pb and CB-Pb levels. *Nulliparity* increased the strength of the observed associations. These findings highlight the significance of m*APOE* genetics, fetal sex, and parity in prenatal Pb kinetics. Notably, the maternal ε2 allele may increase the risk of Pb exposure.

Research dealt with human subject; therefore, this paragraph is included in the article

In Italy, the protocol was approved by the Ethics Committees of the University of Udine and the Institute for Maternal and Child Health, IRCCS Burlo Garofolo in Trieste. The Ethics Committee of the University Hospital Centre Rijeka approved the protocol for the Croatian participants; in Slovenia, the Ethics Committee of the University Medical Centre of Ljubljana, and in Greece, the Ethics Committee of the Institute of Child Health of Athens. Research was conducted in accordance with the Declaration of Helsinki, and all participants signed an informed consent form.

1. Introduction

In recent decades, the general population has been exposed to relatively low levels of lead (Pb), primarily through diet and internally from bones, which have accumulated Pb from lifelong past exposure. In

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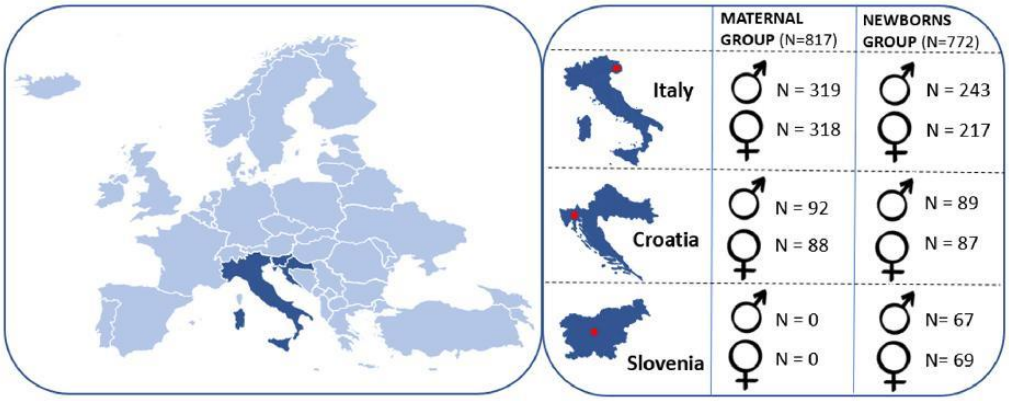

Fig. 1. Study area and study design: Participants stratified by country of residence and fetal/newborn sex.

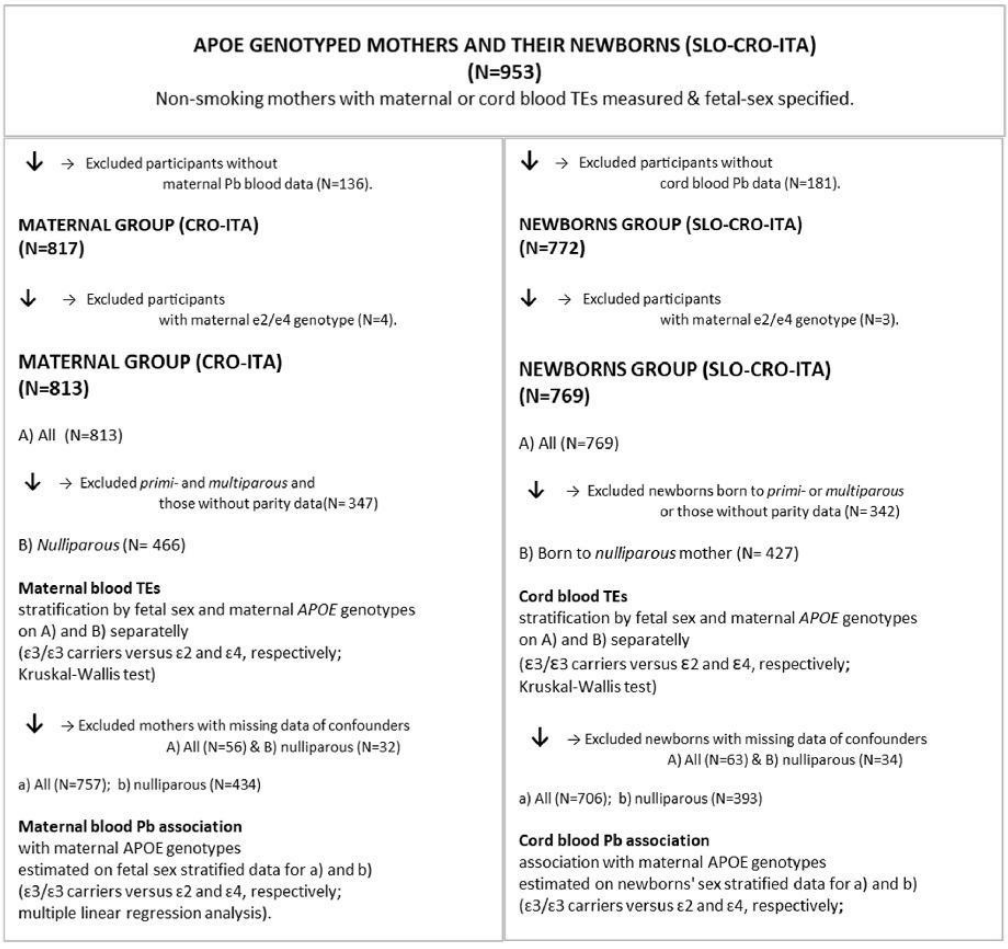

Fig. 2. Flowchart depicting the selection of participants for statistical analyses.

adults, the majority (94%) of absorbed Pb is distributed to the bones, where it can be retained for decades, with slow desorption representing internal Pb exposure (ATSDR, 2020; Bergdahl and Skerfving, 2022). To illustrate the extent of endogenous exposure, bone remodeling and modeling replace about 20–25% of trabecular and 10% of cortical bone annually (Kovacs, 2020). The degree of Pb intake through the

Table 1
m*APOE* genotype and allele frequencies in **Maternal group** and **Newborns group,** stratified by fetal/newborn sex.

Maternal group	Frequency N (%)	Frequency N (%) ♂	Frequency N (%) ♀	p
Genotype	817 (100)	411 (100)	406 (100)	0.138
ε2/ε2	5 (0.61)	4 (0.97)	1 (0.25)	
ε2/ε3	77 (9.42)	41 (9.98)	36 (8.87)	
ε2/ε4*	4 (0.49)	3 (0.73)	1 (0.25)	
ε3/ε3	607 (74.3)	306 (74.5)	301 (74.1)	
ε3/ε4	119 (14.6)	57 (13.9)	62 (15.3)	
ε4/ε4	5 (0.61)	0 (0)	5 (1.23)	
Allele	1634 (100)	822 (100)	812 (100)	0.208
ε2	91 (5.57)	52 (6.33)	39 (4.80)	
ε3	1410 (86.3)	710 (86.4)	700 (86.2)	
ε4	133 (8.14)	60 (7.30)	73 (8.99)	
Newborns group				
Genotype	772 (100)	399 (100)	373 (100)	0.112
ε2/ε2	5 (0.65)	4 (1.00)	1 (0.27)	
ε2/ε3	68 (8.81)	36 (9.02)	32 (8.58)	
ε2/ε4*	3 (0.39)	3 (0.75)	0 (0)	
ε3/ε3	583 (75.5)	302 (75.7)	281 (75.3)	
ε3/ε4	109 (14.1)	54 (13.5)	55 (14.8)	
ε4/ε4	4 (0.52)	0 (0)	4 (1.07)	
Allele	1544 (100)	798 (100)	746 (100)	0.342
ε2	81 (5.25)	47 (5.89)	34 (4.56)	
ε3	1343 (87.0)	694 (87.0)	649 (87.0)	
ε4	120 (7.77)	57 (7.14)	63 (8.45)	

N – number of observations; ♂ – fetal sex is male; ♀ – fetal sex is female; * – participants with ε2/ε4 were not included in statistical analysis as its function can resemble that of ε3/ε3.

gastrointestinal tract is affected by many factors (Mushak, 1991), including age, pregnancy, and nutrient-Pb interactions, such as competition with calcium (Ca) and iron (Fe) for absorption. During pregnancy, the proportion of Ca absorbed from the intestines into the bloodstream doubles by the 12th week of gestation, and this heightened absorption rate is maintained until term. The positive Ca balance is initially deposited in the maternal skeleton until fetal demand rises, leading to increased Ca release from maternal bones in the second part of pregnancy. This release peaks during the third trimester when 80% of fetal skeleton's mineral content is accreted (Ryan and Kovacs, 2021; Arnold et al., 2021). Dietary Pb and previously stored maternal bone Pb can follow the same pathways as Ca in a competitive manner due to its affinity to similar ligands, potentially contributing to elevated Pb levels in maternal blood (mB-Pb) during pregnancy (Gulson et al., 2016; Téllez-Rojo et al., 2004). mB-Pb can then readily pass through the placenta, resulting in prenatal Pb exposure (Skerfving and Bergdhal, 2015; ATSDR, 2020; Bergdahl and Skerfving, 2022). As Pb from bone can add to the mB-Pb burden, it is vital to understand and study gene polymorphisms that could also influence Pb mobilization from the bone. One such gene is the apolipoprotein E gene (*APOE*), which has been studied in relation to bone metabolism (bone turnover/remodeling) including bone mineral density (BMD) and bone fractures (Niemeier et al., 2012; Dieckmann et al., 2014; Zhang et al., 2014; Noguchi et al., 2018), as well as its effect on bone in combination with bone-lipids (Wang et al., 2023) and hormones like estrogen (Qi et al., 2023).

Apolipoprotein E (APOE) is a multifunctional lipid-binding glycoprotein expressed in various tissues and cells, including osteoblasts. It plays an important role in general and neuronal lipid metabolism (Tudorache et al., 2017). Moreover, emerging evidence suggests its role in preserving bone mass, presumably by delivering lipids and vitamin K to osteoblasts (Dieckmann et al., 2014; Niemeier et al., 2012) and influencing cholesterol, Ca, and vitamin D levels (Huebbe et al., 2011). The *APOE* gene is polymorphic, with two single nucleotide polymorphisms (SNPs; rs429358 and rs7421) forming three distinct protein isoforms: APOE2, APOE3, and APOE4, encoded by the ε2, ε3, and ε4 alleles, respectively. These isoforms have different binding affinities for lipids, receptors, oxidants, and some metals, leading to different functional properties and disease risks (Kara et al., 2017; Tudorache et al., 2017; Lumsden et al., 2020). The isoallelic ε2 genotype potentially has the lowest impact on maintaining bone mass (Dieckmann et al., 2014) and protection against bone fracture (Zhang et al., 2014). Accordingly, the ε2 allele has been identified as a potential genetic risk factor for skeletal disorders. However, most research on *APOE* SNPs focusses on ε4 allele, often overlooking the effect of ε2 (Niemeier et al., 2012).

Another frequently overlooked aspect in *APOE* studies is the impact of sex, despite the well-documented sex-related effects of *APOE* and its interaction with sex hormones (Belloy et al., 2019; Gamache et al., 2020). Equally important is the influence of fetal sex on fetal and maternal physiology, the functioning of the placenta, and the maternal-placental-fetal response to environmental toxicants (Al-qaraghouli et al., 2017; Enninga et al., 2015; O'Tierney-Ginn, 2020; Clifton, 2010). The influence of fetal sex on associations between maternal *APOE* genotypes and Pb levels in cord blood (CB–Pb) was also evident in our recent research on pregnant Italian women (2nd to 3rd trimester) and their newborns participating in the PHIME (Public Health Impact of Long-Term, Low-Level Mixed Element Exposure in Susceptibility Population Strata) study (Palir et al., 2023). We noted that girls born to ε2 allele carriers had higher CB-Pb levels than girls born to mothers without this allele.

In the present study, we extended our previous work by including participants from three countries (i.e., Slovenia, Italy, and Croatia) which were all part of the PHIME project and followed the same study protocol. This substantially increased the sample size, allowing us to exclude smoking participants and stratify both mothers and newborns by fetal/newborn sex. We aim to estimate whether the maternal ε2 allele, presumably associated with higher bone remodeling (Dieckmann et al., 2014), is linked to elevated Pb levels in blood during the second half of pregnancy when the fetal Ca accretion is elevated, although increased skeletal resorption of minerals predominates during lactation period (Ryan and Kovacs, 2021). The associations of maternal *APOE* (m*APOE*) genotypes with mB-Pb and CB-Pb were tested separately according to fetal/newborn sex. To eliminate potential confounding effects of parity on placental detoxification functioning (Prior et al., 2014), we also conducted separate analyses on *nulliparous* women and their newborns. Zinc (Zn) and selenium (Se) levels were as well followed to assess the nutritional status of pregnant women, as Zn and Se deficiency can impact gastrointestinal absorption of Pb (Ahamed and Siddiqui, 2007) and the integrity of the skeleton (Zofkova et al., 2017). Additionally, given that the PHIME study was designed to assess mercury (Hg) levels in this Mediterranean cohort with expected seafood consumption, and considering our prior findings of associations between Hg and *APOE* (Snoj Tratnik et al., 2017; Trdin et al., 2020; Palir et al., 2023), we conducted stratified association analysis on Hg as well. This can serve as an important indicator, considering Hg does not accumulate in the bone.

2. Material and methods

2.1. Study population

During the years 2006–2009, pregnant women from four Mediterranean countries - Italy, Croatia, Slovenia, and Greece - were recruited as participants in the PHIME study (Miklavčič et al., 2013; Valent et al., 2013). In Italy, the protocol was approved by the Ethics Committees of the University of Udine and the Institute for Maternal and Child Health, IRCCS Burlo Garofolo in Trieste. The Ethics Committee of the University Hospital Centre Rijeka approved the protocol for the Croatian participants; in Slovenia, the Ethics Committee of the University Medical Centre of Ljubljana, and in Greece, the Ethics Committee of the Institute of Child Health of Athens. The research was conducted in accordance with the Declaration of Helsinki, and all participants signed an informed consent form. The recruitment process, detailed study protocol, and the

Table 2a
Maternal group general characteristics and trace elements stratified by fetal sex.

Maternal group participants	All		♂		♀		p
	AM ± SD (min-max)	**N (%)**	**AM ± SD (min-max)**	**N (%)**	**AM ± SD (min-max)**	**N (%)**	
MOTHERS (m)		817		411		406	
mAge (years)	32.2 ± 4.57 (18–44)	813	32.1 ± 4.88 (18–44)	410	32.2 ± 4.25 (20–44)	403	0.979
mPre-pregnancy BMI (kg/m^2)	22.7 ± 3.86 (15.6–46.7)	815 (100)	22.7 ± 3.95 (15.6–42.4)	409 (100)	22.7 ± 3.76 (16.9–46.7)	406 (100)	0.873
Underweight (<18.5)		58 (7.12)		35 (8.56)		23 (5.66)	0.539
Normal (18.5 – < 25)		596 (73.1)		289 (70.7)		307 (75.6)	
Overweight (25 – < 30)		113 (13.9)		59 (14.4)		54 (13.3)	
Obesity (30 – < 40)		44 (5.40)		24 (5.87)		20 (4.93)	
Severe obesity (≥40)		4 (0.49)		2 (0.49)		2 (0.49)	
mParity	0.52 ± 0.68 (0–4)	815 (100)	0.51 ± 0.68 (0–4)	411 (100)	0.52 ± 0.68 (0–4)	404 (100)	0.621
nulliparous		467 (57.3)		239 (58.2)		228 (56.4)	0.336
primiparous-1		287 (35.2)		137 (33.3)		150 (37.1)	
multiparous-2		52 (6.38)		32 (7.79)		20 (4.95)	
multiparous-3		6 (0.74)		2 (0.49)		4 (0.99)	
multiparous-4		3 (0.37)		1 (0.24)		2 (0.50)	
mEducation		810 (100)		410 (100)		400 (100)	0.530
Elem. or high school		523 (64.6)		269 (65.6)		254 (63.5)	
University or higher		287 (35.4)		141 (34.4)		146 (36.5)	
mSeafood intake frequency (150 g portion/day)	0.36 ± 0.26 (0–2.21)	809	0.36 ± 0.27 (0–2.21)	407	0.37 ± 0.26 (0–1.56)	402	0.651
mEGW (weeks)		770		384		386	
2nd trimester (14–26)	20.5 ± 0.58 (19–24)	509 (66.1)	20.5 ± 0.58 (19–23)	262 (68.2)	20.6 ± 0.58 (19–24)	247 (64.0)	0.816
3rd trimester (27–40)	34.2 ± 3.43 (28–41)	261 (33.9)	34.2 ± 3.47 (28–41)	122 (31.8)	34.2 ± 3.42 (28–41)	139 (36.0)	0.991
mTrace elements	**AM ± SD (min-max) GM (95%CI)**	**N (%)**	**AM ± SD (min-max) GM (95% CI) ♂**	**N (%) ♂**	**AM ± SD (min-max) GM (95%CI) ♀**	**N (%) ♀**	
mB-Pb (ng/g)	12.3 ± 6.99 (3.58–87.6) 11.1 (10.7–11.4)	817	12.2 ± 6.13 (3.86–55.5) 11.1 (10.6, 11.5)	411	12.4 ± 7.77 (3.58–87.6) 11.0 (10.6, 11.5)	406	0.558
mB-Hg (ng/g)	3.13 ± 3.34 (0.11–39.6) 2.17 (2.04, 2.30)	815	2.98 ± 3.00 (0.11, 22.0) 2.06 (1.89, 2.25)	411	3.28 ± 3.65 (0.12, 39.6) 2.28 (2.10, 2.48)	404	0.228
mB-Zn (μg/g)	5.56 ± 1.14 (2.92–11.0) 5.45 (5.38, 5.52)	817	5.54 ± 1.08 (2.92–10.8) 5.44 (5.34, 5.54)	411	5.57 ± 1.19 (3.08–11.0) 5.46 (5.36, 5.57)	406	0.893
mP-Se (ng/mL)	73.9 ± 15.3 (33–118) 72.2 (71.1, 73.4)	786	73.5 ± 15.3 (35–115) 71.8 (70.2, 73.4)	397	74.4 ± 15.3 (33–118) 72.7 (71.1, 72.6)	389	0.427
mP-Zn (μg/mL)	0.73 ± 0.09 (0.46–1.08) 0.73 (0.72, 0.73)	784	0.74 ± 0.10 (0.46, 1.08) 0.73 (0.72, 0.74)	396	0.73 ± 0.09 (0.49, 1.05) 0.73 (0.72, 0.74	388	0.653

AM – arithmetic mean; SD – standard deviation; min – minimum; max – maximum; m – maternal; B – blood; P – plasma; GM – geometric mean; CI – confidence interval; ♂ – male fetal sex; ♀ – female fetal sex; p – values indicate statistically significant difference between the fetal sexes; EGW – estimated gestation week of pregnancy at maternal blood sampling.

number of participants from each country have been described elsewhere (Miklavčič et al., 2013; Valent et al., 2013).

The main inclusion criteria for mothers were a singleton, low-risk pregnancy, aged 18 years or older, and residency in the same region for at least 2 years. Recruitment and sampling took place at local maternity hospitals. Maternal fasting peripheral venous blood samples (whole blood, plasma, and serum) were collected during the prenatal period in Italy and Croatia only. Of those, 65% were taken during the second trimester (19–24 weeks), with 99% of those collected in the second half of the second trimester (20–24 weeks), and the remaining 35% during the third trimester of pregnancy (28–41 weeks). At delivery, mixed cord blood (whole blood, plasma, and serum) and cord tissue were collected in all countries.

In the present study, we introduced additional exclusion criteria and included only non-smoking participants with data on the m*APOE* genotype, fetal sex, and Pb concentrations in either maternal or cord blood. Accordingly, our study population comprised 953 mothers and 772 newborns from three countries: Italy, Croatia, and Slovenia. As previously mentioned, blood Pb levels were not available for Slovenian mothers. Therefore, the maternal group included 817 blood samples from mothers in Italy and Croatia, while the newborns group consisted of 772 cord blood samples from Italy, Croatia, and Slovenia. Detailed information on the number of participants with measured Pb levels in blood or cord blood, stratified by country of residence and fetal/newborn sex, is shown in Fig. 1. Participants' demographic, lifestyle, and personal information were obtained through interviews with the mothers conducted during the study.

2.2. Determination of trace elements

Measurements of Pb, Hg, and Zn in maternal blood and cord blood were performed at the Jožef Stefan Institute, Ljubljana, Slovenia, and Zn and Se in plasma at the University Medical Centre Ljubljana, Institute of Clinical Chemistry and Biochemistry, Ljubljana, Slovenia.

Concentrations of Pb and Zn in blood were determined via inductively coupled plasma mass spectrometry (ICP-MS), following the method described by Jagodic et al. (2017). The LOD for Pb was 1.30 ng/g, and for Zn 20.0 ng/g.

Blood Hg concentrations were determined via atomic absorption spectrometry using a direct mercury analyzer (Milestone, USA) (Miklavčič et al., 2013) and the LOD was 0.02 ng/g.

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

For all measurements, strict quality control procedures were followed, and blank samples, control samples, and reference materials were measured together with the samples on a daily basis, as described in the above-listed references.

2.3. DNA isolation and genotyping

DNA for *APOE* genotyping was isolated from maternal peripheral venous blood in the Croatian (Trdin et al., 2020) and Italian (Palir et al.,

Table 2b
Newborns group general characteristics and trace elements of match-paired mothers and newborns stratified by newborn sex.

Newborns group participants	All		♂		♀		
	AM ± SD (min-max)	**N (%)**	**AM ± SD (min-max)**	**N (%)**	**AM ± SD (min-max)**	**N (%)**	**p**
MOTHERS (m)		772		399		373	
mAge (years)	31.8 ± 4.49 (18–44)	767	31.8 ± 4.80 (18–44)	398	31.9 ± 4.13 (20–44)	369	0.709
mPre-pregnancy BMI (kg/m^2)	22.3 ± 4.09 (13.4–46.7)	770 (100)	22.3 ± 4.12 (15.6–42.4)	397 (100)	22.3 ± 4.07 (13.4–46.7)	373 (100)	0.947
Underweight (<18.5)		106 (13.8)		60 (15.1)		46 (12.3)	0.725
Normal (18.5 – < 25)		518 (67.3)		261 (65.7)		257 (68.9)	
Overweight (25 – < 30)		101 (13.1)		52 (13.1)		49 (13.1)	
Obesity (30 – < 40)		42 (5.45)		22 (5.54)		20 (5.36)	
Severe obesity (≥40)		3 (0.39)		2 (0.50)		1 (0.27)	
mEducation		766 (100)		398 (100)		368 (100)	0.332
Elem. or high school		455 (59.4)		243 (61.2)		212 (57.6)	
University or higher		311 (40.6)		155 (38.9)		156 (42.4)	
mParity	0.55 ± 0.70 (0–4)	770 (100)	0.54 ± 0.69 (0–3)	399 (100)	0.56 ± 0.72 (0–4)	371 (100)	0.640
nulliparous		428 (55.6)		225 (56.4)		203 (54.7)	0.363
primiparous-1		276 (35.8)		139 (34.6)		137 (37.0)	
multiparous-2		56 (7.28)		32 (8.02)		24 (6.49)	
multiparous-3		7 (0.91)		4 (1.00)		3 (0.81)	
multiparous-4		3 (0.39)		0 (0)		3 (0.81)	
mSeafood intake frequency (150 g portion/day)	0.35 ± 0.26 (0–2.21)	764	0.35 ± 0.27 (0–2.21)	396	0.35 ± 0.25 (0–1.56)	368	0.846
NEWBORNS (c)		772		399		373	
cLength (cm)	50.7 ± 2.26 (42–58)	760	51.1 ± 2.26 (43–58)	393	50.2 ± 2.17 (42–55)	367	**0.000**
cWeight (g)	3452 ± 482 (1450–5140)	763	3519 ± 472 (1790–4930)	396	3380 ± 483 (1450–5140)	367	**0.001**
cEGA (weeks)	39.5 ± 1.43 (28–42)	727 (100)	39.5 ± 1.37 (35–42)	375 (100)	39.4 ± 1.50 (28–42)	352 (100)	0.351
Pre-term (<37)		16 (2.20)		5 (1.33)		11 (3.13)	0.110
Full term (37–42)		711 (97.8)		370 (98.7)		341 (96.9)	
Post-term (> 42)		–		–		–	
cTrace elements	**AM ± SD (min-max) GM (95%CI)**	**N (%)**	**AM ± SD (min-max) GM (95% CI)** ♂	**N (%)** ♂	**AM ± SD (min-max) GM (95%CI)** ♀	**N (%)** ♀	
CB-Pb (ng/g)	10.4 ± 5.28 (1.82–47.0) 9.31 (9.01, 9.63)	772	10.3 ± 4.88 (1.82–34.1) 9.33 (8.92, 9.75)	399	10.5 ± 5.67 (2.63–47.0) 9.30 (8.85, 9.77)	373	0.785
CB-Hg (ng/g)	4.56 ± 4.40 (0.12, 32.8) 3.05 (2.85, 3.26)	766	4.70 ± 4.66 (0.12, 32.8) 3.10 (2.81, 3.41)	395	4.42 ± 4.10 (0.14, 26.3) 3.00 (2.73, 3.30)	371	0.727
CB-Zn (µg/g)	2.36 ± 0.60 (1.16–7.24) 2.29 (2.26, 2.33)	771	2.33 ± 0.56 (1.18–4.76) 2.23 (2.22, 2.32)	399	2.39 ± 0.65 (1.16–7.24) 2.32 (2.26, 2.37)	372	0.403

AM – arithmetic mean; SD – standard deviation; min – minimum; max – maximum; m – maternal; c – child; CB –cord blood; GM – geometric mean; CI – confidence interval; ♂ – male newborn sex; ♀ – female newborn sex; p – values indicate statistically significant difference between the fetal sexes; Length – newborn length at birth; Weight – newborn weight at birth; EGA – estimated gestational age at delivery.

2023) cohorts of the PHIME study, whereas for the Slovenian cohort, blood for DNA isolation was obtained during their recruitment in the PHIME follow-up study within the CROME-LIFE + project (Cross-Mediterranean Environment and Health Network) (2013–2017) (Stajnko et al., 2019).DNA extraction was performed using the FlexiGene® DNA kit (Qiagen, Hilden, Germany), following the manufacturer's protocols. The quantity and quality of the isolated DNA were determined using an ultraviolet–visible (UV-VIS) spectrophotometer NanoDrop 2000c (Thermo Fisher Scientific, USA).

Single nucleotide polymorphism (SNP) genotyping for *APOE* (rs7412 and rs429358) was performed using predesigned TaqMan SNP Genotyping Assays (Applied Biosystems, USA), as described in previous studies (Snoj Tratnik et al., 2017; Trdin et al., 2020; Palir et al., 2023). SNP frequencies were tested using Pearson's chi-squared test for deviation from the Hardy–Weinberg equilibrium (HWE) ($p > 0.05$). The basic SNP characteristics are given in the appendix (Table A1).

2.4. Statistics

Descriptive statistics were employed to evaluate participant characteristics derived from questionnaires (maternal age, pre-pregnancy body mass index [BMI], parity, education, daily seafood consumption, estimated gestation week [EGW] at venous blood sampling, newborn estimated gestation age [EGA] at delivery, newborn sex, length, and weight). Continuous variables were expressed as arithmetic means ± standard deviation (AM ± SD) with minimum and maximum values, while continuous variables were presented as frequency and percentage distributions. Additionally, concentrations of trace elements (TEs) were presented as AM ± SD and as geometric means with a 95% confidence interval (GM, 95% CI).

General characteristics were stratified by fetal/newborn sex, and differences between sexes were assessed using Chi-squared test for categorical variables and the Mann-Whitney *U* test for continuous variables. Parity was defined as the number of pregnancies reaching a gestation period of 20 weeks or more, with *nulliparity* indicating the first pregnancy surpassing 20 weeks and *multiparity* indicating 1 to 4 previous pregnancies extending beyond 20 weeks. Seafood intake frequency was determined based on participant responses regarding the consumption of 150 g of various seafood types at different frequencies (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., 2013). EGW at blood sampling and EGA at delivery were calculated based on menstrual history.

Concentrations of Pb were further expressed as GM with 95% CI, stratified by fetal/newborn sex, with the statistical comparison between m*APOE* genotypes conducted using the Kruskal-Wallis test.

The associations between m*APOE* genotype and mB-Pb and CB-Pb as well as mB-Hg and CB-Hg were estimated using fetal/newborn-sex-stratified linear regression models with an adjustment for different confounding variables introduced in our previous work (Palir et al., 2023). Maternal blood zinc (mB-Zn) and cord blood zinc (CB–Zn) values were utilized as approximate substitutes for unmeasured hemoglobin and iron values, according to Gibson et al. (2008) and Houghton et al. (2016). Carriers of the ε2 (genotype: ε2/ε2 and ε2/ε3) and ε4 (genotype:

Table 3
mB-Pb and CB-Pb levels (GM, 95% CI) stratified by fetal sex and m*APOE* genotype.

	GM (95% CI) N			p
	ε3/ε3	ε2/ε2, ε2/ε3	ε3/ε4, ε4/ε4	
Maternal group				
♂				
All, N=408				
mB-Pb ng/g	11.2 (10.6, 11.8) 306	11.4 (10.0, 13.1) 45	10.2 (9.38, 11.1) 57	0.404
Nulliparous, N=238				
mB-Pb ng/g	11.2 (10.5, 12.0) 178	11.2 (9.56, 13.2) 26	9.82 (8.77, 11.0) 34	0.311
♀				
All, N=405				
mB-Pb ng/g	*10.9 (10.4, 11.4) 301*	*12.2 (10.6, 14.0) 37*	*10.9 (9.66, 12.3) 67*	*0.102*
Nulliparous, N=228				
mB-Pb ng/g	***10.8 (10.1, 11.5) 178***	***13.2 (11.1, 15.8) 20***	***10.6 (8.61, 12.9) 30***	***0.062***
Newborns group				
♂				
All, N=396				
CB-Pb ng/g	9.28 (8.80, 9.79) 302	9.23 (8.05, 10.6) 40	9.75 (8.83, 10.8) 54	0.659
Nulliparous, N=224				
CB-Pb ng/g	9.35 (8.71, 10.0) 172	9.37 (8.02, 11.0) 20	10.1 (9.01, 11.4) 32	0.366
♀				
All, N=373				
CB-Pb ng/g	9.35 (8.82, 9.91) 281	10.1 (8.46, 12.0) 33	8.65 (7.69, 9.73) 59	0.349
Nulliparous, N=203				
CB-Pb ng/g	**9.49 (8.75, 10.3) 156**	**12.2 (10.3, 14.5) 18**	**8.81 (7.37, 10.5) 29**	**0.046**

♂ – fetal/newborn sex is male; ♀ – fetal/newborn sex is female; GM – geometric mean; CI – confidence interval; m – maternal; B – blood; CB – cord blood; N – number of observations; p – statistically significant difference between any of the three groups; significant results ($p \leq 0.05$) are in bold, and marginally significant results ($p > 0.5$ and < 0.10) are in bold italics.

ε3/ε4 and ε4/ε4) allele were compared to the common m*APOE* genotype (ε3/ε3), and additional comparisons were made between ε4 carriers and ε2 carriers. Participants with ε2/ε4 were not included in statistical analysis as its function can resemble that of ε3/ε3.

The effect of m*APOE* genotypes on mB-Pb and mB-Hg concentrations was studied while controlling for mother's age, pre-pregnancy BMI, parity (*nulliparous/multiparous*), education (high school or lower/university or higher), EGW, seafood intake frequency, country of residence, and mB-Zn levels **(Model 1)**. To eliminate the influence of parity, which has been reported to affect blood Pb levels (Lewin et al., 2017; Bocca et al., 2020), the same model was run including only *nulliparous* women **(Model 2)**. The effect of m*APOE* on CB-Pb and CB-Hg was tested in a model adjusted for mother's age, pre-pregnancy BMI, parity (*nulliparous/multiparous*), education (high school or lower/university or higher), seafood frequency intake, country of residence, CB-Zn levels, newborn length and weight, and EGA **(Model 3)**. Again, to exclude the influence of parity, the model including only the newborns born to *nulliparous* mothers was run **(Model 4)**. All models were run separately based on fetal/newborn sex (♂ – male, ♀ – female). A flowchart depicting the selection of participants for statistical analyses is shown in Fig. 2 and statistical models' formulas are provided in the appendix (Equations A1 - A4).

Statistical analyses were conducted using **STATA12/SE** and R version 3.6.0 with **RStudio version February 1, 1335**. Natural log-transformation was applied to dependent variables (mB-Pb, CB-Pb, mB-Hg, and CB-Hg) to approximate a normal distribution. The estimation coefficients (b) of the independent variables were exponentiated (exp(b)) for easier interpretation. If the independent variables were log-transformed, their effects were presented with estimation coefficients (b), as in the case of mB-Zn and CB-Zn. Statistical significance (p-value) was set at a p-value of ≤0.05, with marginal significance defined as p-values between > 0.05 and ≤ 0.1.

3. Results and discussion

3.1. mAPOE genotype and allele frequencies

The frequencies of m*APOE* genotypes and alleles for the Maternal and Newborns groups are listed in Table 1. We did not observe significant differences in allele frequencies between the groups or between the fetal sexes. The frequencies were geographically consistent with those reported in the literature (Giau et al., 2015). Although the frequencies of *APOE* genotypes can vary even between neighboring countries, our study locations were geographically close and showed similar percentages of each genotype (data not shown).

The presence of the isoallelic genotypes ε2/ε2 and ε4/ε4 was exceptionally rare, accounting for less than or equal to 1% across all groups examined. Notably, the ε2/ε2 genotype was observed in only one mother with a female offspring and in four mothers with male offspring, while no instances of the ε4/ε4 genotype were detected among mothers with male offspring and only five instances among those with female offspring. Overall, there were no statistically significant differences in m*APOE* genotypes based on fetal/newborn sex (Table 1).

3.2. General characteristics and trace elements levels

Tables 2a and 2b presents the general characteristics and TEs concentrations of the Maternal and Newborns groups, respectively. These tables include data for all participants within the groups and are further stratified by fetal/newborn sex. Notably, no significant differences in general characteristics between the groups based on fetal/newborn sex were observed, with the expected exception of newborns' weight and length ($p < 0.001$, Table 2b).

As previously reported (Barbone et al., 2019; Miklavčič et al., 2013; Palir et al., 2023; Snoj Tratnik et al., 2017; Trdin et al., 2020), the participants exhibited very low to moderate levels of potentially toxic TEs, including Pb and Hg, in their maternal blood and cord blood samples. In this study, participants consistently showed low levels of Pb, with GM (95%CI) in maternal blood being 11.1 (10.7, 11.4) ng/g and in cord blood 9.31 (9.01, 9.63) ng/g. Only four women exceeded the recommended blood Pb threshold for pregnant women, which is set at 50 ng/mL (CDC, 2010; Taylor et al., 2014), and 13 women exceeded the recently updated CDC threshold of 35 ng/mL (Ruckart et al., 2021). None of the newborns' CB-Pb levels exceeded these thresholds, which is particularly noteworthy given the higher volume of erythrocytes in cord blood compared to venous blood. The observed Pb levels are

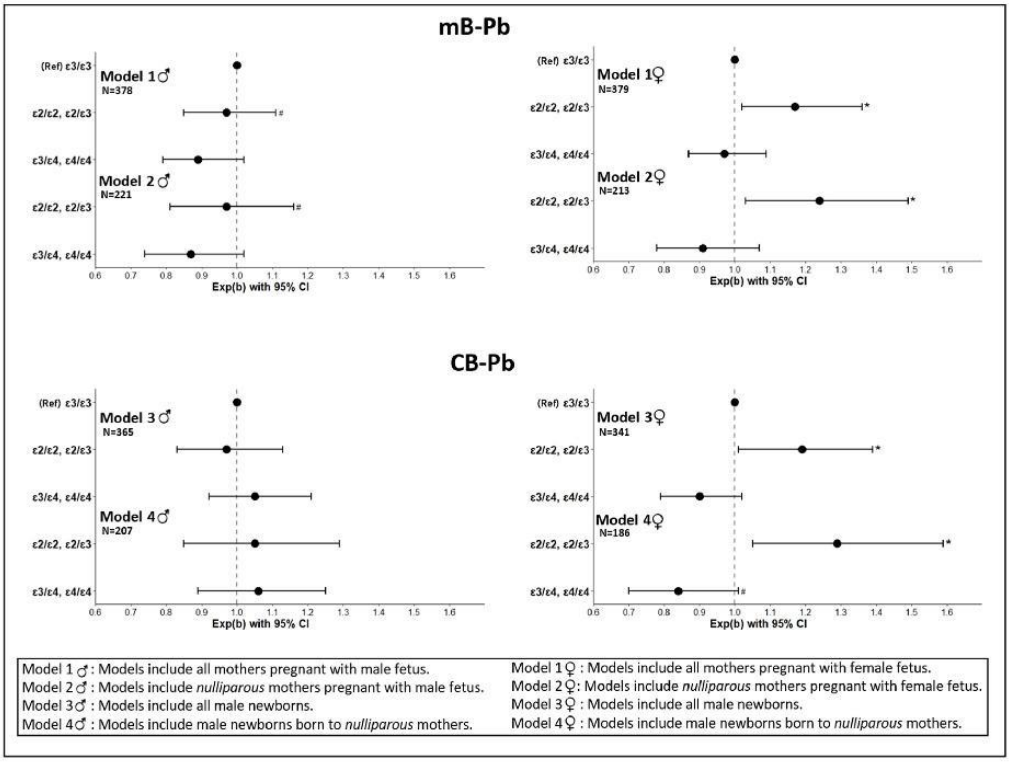

Fig. 3. The influence of *APOE* genotypes on mB-Pb and CB-Pb levels based on linear regression models, comparing the **ε2/ε2, ε2/ε3 and ε3/ε4, ε4/ε4 genotypes to the ε3/ε3 (reference genotype).**

♂ – fetal/newborn sex is male; ♀ – fetal/newborn sex is female; m – maternal; B – blood; CB – cord blood; exp(b) – exponentiation of the B coefficient; #p ≤ 0.1, *≤0.05. Models were adjusted for.

Model 1: mAge, mBMI, mParity (nulliparity/multiparity), mEducation (high school or lower/university or higher), mSeafood consumption, mEGW, mCountry (ITA/CRO), mB_Zn_log; m – maternal; c – child.

Model 2: as for Model 1 but without mParity.

Model 3: mAge, mBMI, mParity (nulliparity/multiparity); mEducation (high school or lower/university or higher), mSeafood consumption, mCountry (ITA/CRO/SLO), CB_Zn_log, cEGA, cWeight, cLength; m – maternal; c – child.

Model 4: as for Model 4 but without mParity.

Number of observations (N), statistical significance (p), the percentage of variability of Pb level explained by the model (R^2) and estimates of above listed variables for each model are given in tables A1 and A2 (Appendix).

representative of the post-industrial countries with an early ban of leaded gasoline (Poropat et al., 2018). In recent decades, environmental exposure to Pb has been gradually decreasing, largely due to the elimination of leaded gasoline and lead pipes used in plumbing (Bergdahl and Skerfving, 2022). Leaded gasoline was banned in Italy in 2002 (OECD, 2003), in Croatia in 2006 (Zorana et al., 2016), and in Slovenia in 2001 (OECD, 2012). Consequently, all participants were born significantly before the phase-out of leaded gasoline and could have potentially been exposed to it for 11–41 years until their inclusion in the PHIME project (2006–2009). Therefore, mB-Pb and CB-Pb in our study reflect both current external exposure and past internally accumulated exposure.

Consistent with prior reports on Italian and Croatian participants (Palir et al., 2023; Trdin et al., 2020), adequate levels of mP-Se and mP-Zn were also observed among the participants, with levels of 72.2 (71.1, 73.4) ng/mL and 0.73 (0.72, 0.73) μg/mL, respectively (Table 2a). These finding indicate good micronutrient status (Abbassi-Ghanavati et al., 2009; Thomson, 2004; Varsi et al., 2017), with no significant differences among groups stratified by fetal sex (Table 2a).

3.3. Lead concentrations in maternal blood and newborns' cord blood stratified by mAPOE genotype and fetal sex (Bivariate analysis)

We conducted a simple comparison of mB-Pb and CB-Pb among m*APOE* genotypes, stratified by fetal/newborn sex, including all mothers or only *nulliparous*, and all newborns or only newborns born to *nulliparous* mothers (Table 3). Statistically significant or marginally significant differences in mB-Pb and CB-Pb concentrations between m*APOE* genotypes were observed only for *nulliparous* women with female offspring and in female newborns. In both cases, ε2 genotypes were associated with higher Pb levels. A similar trend was noted for mB-Pb in mothers carrying female fetuses, regardless of parity status (referred to as All). However, since blood Pb levels can be influenced by various factors (age, education, seafood intake, country of residence, EGW, etc.), particularly when concentrations are low, we accounted for these potential confounding variables using multiple linear regression models.

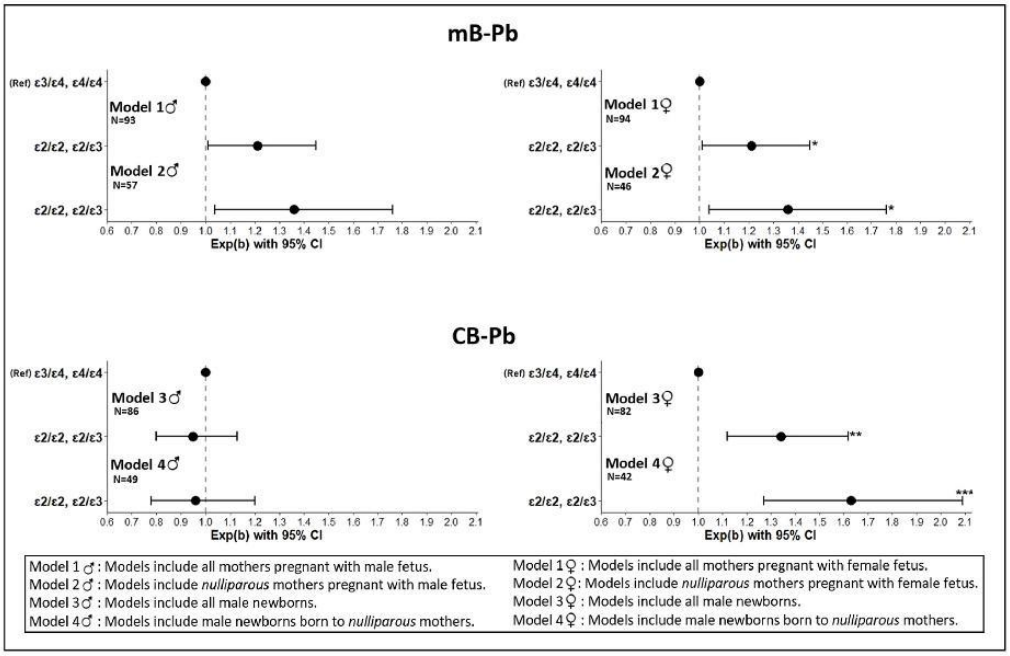

Fig. 4. The influence of *APOE* genotypes on mB-Pb and CB-Pb levels based on linear regression models, comparing the ε2/ε2, ε2/ε3 genotype to the ε3/ε4, ε4/ε4 (reference genotype).
♂ – fetal/newborn sex is male; ♀ – fetal/newborn sex is female; m – maternal; B – blood; CB – cord blood; exp(b) – exponentiation of the B coefficient; *p ≤ 0.05, **p ≤ 0.01, **p ≤ 0.001. Models were adjusted for.
Model 1: mAge, mBMI, mParity (nulliparity/multiparity), mEducation (high school or lower/university or higher), mSeafood consumption, mEGW, mCountry (ITA/CRO), mB_Zn_log; m – maternal; c – child.
Model 2: as for Model 1 but without mParity.
Model 3: mAge, mBMI, mParity (nulliparity/multiparity); mEducation (high school or lower/university or higher), mSeafood consumption, mCountry (ITA/CRO/SLO), CB_Zn_log, cEGA, cWeight, cLength; m – maternal; c – child.
Model 4: as for Model 4 but without mParity.
Number of observations (N), statistical significance (p), the percentage of variability of Pb level explained by the model (R^2) and estimates of above listed variables for each model are given in tables A3 and A4 (Appendix).

3.4. Associations between mAPOE genotypes and Pb (Hg) levels in maternal (cord)blood stratified by fetal/newborn sex and parity (multiple linear regression models)

We employed fetal/newborn-sex-stratified multiple linear regression models to estimate the associations between m*APOE* genotypes and mB-Pb and CB-Pb (Fig. 3 – 4, Table A2-A5). Firstly, we ran the models with all participants (Model 1, Model 3) and then focused on *nulliparous* women and their newborns (Model 2, Model 4). We tested the impact of the ε2 or ε4 genotypes versus the "neutral" ε3 homozygotes, as well as ε4 compared to ε2. Fig. 3 – 4 present the estimates for Pb, while the estimates for additional explanatory variables are presented in the Appendix (Tables A2 – A5).

The results confirmed the association between the presence of the ε2 allele and higher concentrations of mB-Pb and CB-Pb, but only when the fetal/newborn sex was female. Mothers carrying the ε2 allele had 17% (95%CI 2%, 36%) higher mean mB-Pb levels than mothers with the ε3/ε3 genotype (Fig. 3, Model 1♀, Table A2). In cord blood, we observed a slightly higher increase of 19% (95CI 1%, 39%) in mean Pb levels if m*APOE* genotype was ε2/ε2 or ε2/ε3 in comparison to ε3/ε3 (Fig. 3, Model 3♀, Table A3). As aforementioned in the introduction, several studies suggest that *APOE* is involved in bone homeostasis, including maintaining bone mass by regulating bone metabolism, including turnover (Dieckmann et al., 2014; Noguchi et al., 2018; Wang et al., 2023). Some studies highlight the isoallelic ε2 genotype as having the weakest effect on maintaining bone mass (Dieckmann et al., 2014; Niemeier et al., 2012; **S. Q.** Zhang et al., 2014). This aligns with our findings; if bone turnover is indeed higher among ε2 carriers (Dieckmann et al., 2014), it could influence blood Pb concentrations. Notably, the majority of accumulated Pb, both historically and during the first half of pregnancy, is stored in bones (ATSDR, 2020) and can be released along with Ca during the second half of pregnancy when sampling was performed. Furthermore, our models reveal modest yet statistically significant positive associations between age and mB-Pb, as well as CB-Pb (which were consistently slightly higher when the fetal/newborn sex was female) (Table A3 - A4). This suggests that part of the Pb measured in maternal and cord blood originates from historically accumulated bone-Pb, as demonstrated by isotopic measurements in previous studies (Gulson et al., 1997; Kovacs, 2016).

In our research, when fetal/newborn sex was male, no association was found between mB-Pb and CB-Pb levels when comparing carriers of the ε2 allele to ε3/ε3 (Fig. 3, Model 1♂, Model 3♂, Table A2 – A3). We believe these sex-based differences can be at least partially attributed to sex hormones. Jasienska et al. (2015) reported interactions between sex hormones and *APOE* genotypes in fertile females, and Glynn et al. (2016) observed higher levels of serum sex hormones in mothers carrying female fetuses. Moreover, in an ongoing study involving mothers in the third trimester and their newborns from Kyrgyzstan (N = 91), we observed significantly higher levels of testosterone, progesterone, estradiol, and cholesterol in mothers pregnant with female fetus

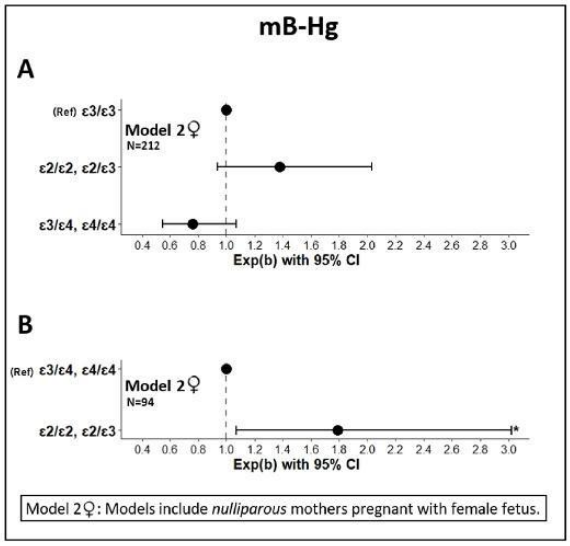

Fig. 5. The influence of *APOE* genotypes on mB-Hg levels based on linear regression models, comparing (A) the ε2/ε2, ε2/ε3 and ε3/ε4, ε4/ε4 genotypes to the ε3/ε3 (reference genotype) and (B) comparing the ε2/ε2, ε2/ε3 genotype to the ε3/ε4, ε4/ε4 (reference genotype). ♂ – fetal/newborn sex is male; ♀ – fetal/newborn sex is female; m – maternal; B – blood; CB – cord blood; exp(b) – exponentiation of the B coefficient; *p ≤ 0.05. Models were adjusted for.
Model 2: mAge, mBMI, mEducation (high school or lower/university or higher), mSeafood consumption, mEGW, mCountry (ITA/CRO), mB_Zn_log; m – maternal; c – child.Number of observations (N), statistical significance (p), the percentage of variability of Hg level explained by the model (R^2) and estimates of above listed variables for each model are given in tables A6 and A7 (Appendix).

compared to those pregnant with a male; the difference was even higher if the mothers carried the ε4 allele (Tursunova et al., 2023; unpublished data). In the elderly population, increasing evidence suggests the involvement of sex hormones (particularly estradiol but also progesterone) in APOE effects related to AD, menopause-associated bone loss, dyslipidemia, and the risk of cardiovascular disease (Belloy et al., 2019; Gamache et al., 2020, Valencia-Olvera et al., 2022). It is evident that various sex-based hormonal-APOE interactions occur in (patho)physiological, metabolic processes during early life and later, with different effects at different life stages and health conditions (Valencia-Olvera et al., 2022; Jasienska et al., 2015). The absence of associations between mB-Pb and CB-Pb levels with *APOE* genotypes could also be attributed to the larger size, higher bone mineral density, and therefore presumed increased mineral flux into the skeletal system of male babies. This enhanced mineral flux might obscure any association between the low concentrations of Pb observed in this study and *APOE* genotype.

The influence of the ε2 allele on mB-Pb and CB-Pb became even more pronounced when only *nulliparous* mothers and their newborns were included. The difference in Pb concentrations between ε3/ε3 and ε2/ε2, ε2/ε3 increased to 24% (95%CI 3%, 49%) in blood (Fig. 3, Model 2♀, Table A2) and to 29% (95%CI 5%, 59%) in cord blood (Fig. 3, Model 4♀, Table A3) when nulliparity was considered as an inclusion factor. It is important to note, that in the Maternal group, the average age was 32.2 years for all participants **(Model 1)** and 31.2 years for *nulliparous* women **(Model 2)** and in the Newborns group, the corresponding values were 31.8 **(Model 3)** and 30.9 years **(Model 4)**. As the age distribution of mothers was similar between the 'All' and '*Nulliparous*' groups, our findings may indicate the modulating effects of the ε2 allele and *nulliparity* on Pb kinetics, leading to an increased transfer of Pb from maternal blood to cord blood. The protective effect of parity, or parity as a negative predictor for Pb, Cd and/or Hg exposure in maternal and cord blood, has already been reported (Lewin et al., 2017; Bocca et al., 2020; Trdin et al., 2020). Lewin et al. (2017) measured several toxicants and found that chemical concentrations are generally higher in *nulliparous* women than in *uniparous* or *multiparous* women, likely due to the enhanced placenta's ability to detoxify toxic elements. Parity can affect metal kinetics through various processes (Gundacker and Hengstschläger, 2012), and *nulliparous* women are generally more vulnerable due to less effective placental detoxification functioning (Prior et al., 2014), whereas *multiparity* (however below five or more pregnancies), signifies a protective metabolic adaptation of the placenta. A recent study by Punshon et al. (2019) reported that "parity was positively associated with placental weight, efficiency increased for every previous pregnancy and placental disc symmetry was greater among women with higher number of previous pregnancies". It should also be noted that in our study, only 1% of the participants had three or four previous pregnancies, and none had five or more (Tables 2a and **2b**), which could signify a higher-risk pregnancy (Bai et al., 2002).

When conducting the statistical analysis, we also identified an association between the m*APOE* ε4 allele and mB-Pb concentrations. The m*APOE* ε4 allele showed a protective effect against Pb exposure, although the associations did not reach statistical significance in all groups (Fig. 3, Table A2 – A3). Among mothers pregnant with a male fetus, ε4 carriers had mean mB-Pb levels that were 11% (95%CI -21%, +2%) or 13% (95%CI -26%, +2%) lower than those in the ε3/ε3 group, depending on whether all participants were included or only *nulliparous* women (Fig. 3, Model 1♂, Model 2♂, Table A2). Furthermore, in female newborns, a trend towards lower CB-Pb in the ε4 allele group compared to the ε3/ε3 group was found (p = 0.104) (Fig. 3, Model 3♀, Table A3). This trend became marginally significant (p = 0.063) when only newborns born to *nulliparous* women were included (−16%, 95%CI -30%, +1%) (Fig. 3, Model 4♀, Table A3). These observations could indicate the protective function of maternal ε4 for pregnant women and their newborns. Despite the ε4 allele being considered a risk factor for age-related diseases like late-onset AD, it has been proposed to have beneficial effects in early life and on fertility (Tudorache et al., 2017; Jasienska et al., 2015; Oria et al., 2020; Trdin et al., 2020). Huebbe et al. (2011) demonstrated, in targeted replacement mice and humans, that the *APOE* ε4 allele is associated with higher levels of cholesterol and vitamin D, and more efficient intestinal Ca absorption, leading to higher Ca levels.

To further examine the impact of the ε2 allele, we conducted an additional step by directly comparing the effects of the ε2 and ε4 alleles, which in previous steps displayed opposite effects on mB-Pb and CB-Pb. As expected, significant differences in Pb levels were only observed when the fetal/newborn sex was female. Carriers of the ε2 allele demonstrated 21% (95%CI 1%, 45%) higher mean mB-Pb levels than ε4 when considering all participants regardless of parity (Fig. 4, Model 1♀, Table A4). When focusing specifically on *nulliparous* women, this difference increased to 36% (95%CI 4%, 76%) (Fig. 4, Model 2♀, Table A4). In cord blood, if m*APOE* genotype included ε2, mean Pb levels were 34% (95%CI 12%, 62%) higher compared to ε4 genotypes when analyzing all newborns (Fig. 4, Model 3♀, Table A5) and 63% (95%CI 27%, 109%) when analyzing only those born to *nulliparous* women (Fig. 4, Model 4♀, Table A5). These results support the possible impact of the ε2 allele on increased (cord)blood Pb levels, which could be driven by various multifactorial mechanisms, including increased bone remodeling.

In our study, we also tested associations between m*APOE* genotype and Hg (cord)blood levels using the same models. Similar to the findings for mB-Pb, we observed a 38% (95%CI -6%, 103%) higher mean mB-Hg in ε2 carriers in comparison to ε3/ε3 genotype when fetal sex was female and mothers were *nulliparous*; at the same time, ε4 carriers were associated with lower mB-Hg levels (Fig. 5A, Table A6, Model 2♀). Hence, significant associations emerged when comparing ε2 allele carriers to ε4 carriers, demonstrating that ε2 carriers exhibited 79% (95%CI 7%, 202%) higher mB-Hg levels (Fig. 5B–Table A7, Model 2♀). However, in

other models analyzing m*APOE* genotypes and mB-Hg or CB-Hg, no associations were found (data not shown). Knowing that Hg does not readily store in the bones, this finding suggests that ε2 may be acting through various pathways. Experimental data on different interactions such as APOE/selenoprotein P (Jin et al., 2020), selenoprotein P/Pb (Bi et al., 2019), APOE/metallothioneins (Augsten et al., 2011; Graeser et al., 2012), and metallothioneins/Pb (He et al., 2014) suggest that APOE has a complex impact on Pb on various levels. Thus, our results point to the influence of the m*APOE* ε2 allele on increased mB-Pb and CB-Pb levels; however, determining the primary mechanisms behind this influence remains a challenge, particularly at low exposure levels where accurately estimating external dietary or environmental Pb exposure and distinguishing it from internal bone Pb exposure can be difficult. Future mechanistic studies are necessary to ascertain the significance of the *APOE* ε2 allele.

3.5. Study strengths and limitations

Our main strength is the relatively large sample size, which enabled us to compare ε2 allele carriers with ε4 allele carriers and those with the 3/3 genotype. Many studies involving *APOE* often diminish the significance of the ε2 allele by combining it with ε3/ε3 or excluding ε2 carriers altogether. Another notable strength is our stratification of all statistical analyses based on fetal/newborn sex, whereas in many studies, sex is merely added as a confounder. Furthermore, our study's robustness extends to the inclusion of additional trace elements such as Zn and Se, offering insights into nutritional status and their potential impact on gestational Pb absorption and bone integrity. Additionally, we investigated the influence of m*APOE* on Hg, recognizing that Hg in contrast with Pb, is not stored in the bone.

However. there are some limitations in the study that increase uncertainty in data and limit the refinement of statistical associations. The first is a common issue in epidemiological and human biomonitoring studies: reliance on self-reported data on smoking status, dietary habits, etc. Another limitation is the lack of data on hemoglobin/hematocrit levels, which we partly compensated for by including mB-Zn and CB-Zn concentrations in the statistical models. Additionally, another challenge of the study is the sampling of mixed CB instead of arterial or venous CB, resulting in random ratios of arterial and venous CB in samples. Hemoglobin values vary between arterial and venous CB (Masoumi et al., 2017), which affects the concentration of erythrocytes-accumulating elements like Pb. It is also known that fetal demand for TEs and other nutrients varies greatly depending on the week of gestation; therefore, it would be extremely beneficial to sample women for prenatal blood analysis in the same week of pregnancy. This could eliminate another bias due to changing lipid status during pregnancy (Zheng et al., 2017). Having data on hormonal and lipid status would also improve the observed associations.

4. Conclusions

We discovered fetal/newborn-sex-based associations between m*APOE* genotypes and blood Pb levels in pregnant women and newborns, who had low blood Pb levels and adequate plasma Zn and Se levels. The presence of the maternal ε2 allele was significantly associated with higher mean mB-Pb and CB-Pb levels when the fetal/newborn sex was female. This may be linked to heightened bone turnover among ε2 carriers, a factor potentially amplified by the unique calcium dynamics of pregnancy and modifying effects between sex hormones and *APOE* genotypes. Conversely, the ε4 allele was linked with lower mB-Pb regardless of fetus sex and lower CB-Pb in female newborns, suggesting a protective effect of ε4 against Pb exposure and proposing beneficial effects of *APOE* ε4 early in life. Notably, genotype-based observations were contingent on fetus/newborn sex and were more pronounced in the case of *nulliparity*. These findings highlight the importance of considering sex, genetic polymorphisms, and parity as influencing factors in future studies.

CRediT authorship contribution statement

Neža Palir: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Anja Stajnko:** Writing – review & editing, Visualization, Investigation, Formal analysis, Data curation. **Darja Mazej:** Writing – review & editing, Formal analysis, Data curation. **Alenka France Štiglic:** Data curation. **Valentina Rosolen:** Writing – review & editing, Resources, Data curation. **Marika Mariuz:** Resources. **Luca Ronfani:** Resources. **Janja Snoj Tratnik:** Writing – review & editing, Methodology. **Agneta Annika Runkel:** Writing – review & editing, Methodology. **Veronika Tursunova:** Resources. **Janja Marc:** Resources, Methodology. **Igor Prpić:** Writing – review & editing, Resources, Methodology. **Zdravko Špirić:** Writing – review & editing, Project administration, Methodology. **Fabio Barbone:** Writing – review & editing, Resources, Project administration, Methodology. **Milena Horvat:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Formal analysis. **Ingrid Falnoga:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgments

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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.2024.119583.

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3.3 Article 3: Genetic Susceptibility to Low-Level Lead Exposure in Men: Insights from *ALAD* Polymorphisms

The paper is authored by Anja Stajnko, Neža Palir, Janja Snoj Tratnik, Darja Mazej, Alenka Sešek Briški, Agneta Annika Runkel, Milena Horvat, Ingrid Falnoga (2024). It has been published in International Journal of Hygiene and Environmental Health.

Extensive research has explored *ALAD* SNPs as biomarkers of Pb susceptibility, with the most studies focusing on rs1800435. However, other *ALAD* SNPs, particularly at low environmental exposures, may also impact Pb toxicokinetics. Few studies have explored these additional *ALAD* SNPs, however incorporating LD analysis and analyzing *ALAD* haplotypes in relation to Pb concentrations is especially rare.

This study investigates genetic susceptibility to low to moderate level Pb exposure in men of reproductive age, focusing on the role of SNPs in the *ALAD* and *VDR* gene. Conducted on 281 men from Slovenia aged 18-49 years, including 20 individuals from a Pb-contaminated area, the research examines how the associations between ten selected *ALAD* SNPs (rs1800435, rs1139488, rs1805313, rs818708, rs2761016, rs8177812, rs2228083, rs1805312, rs8177796, and rs818684) influence B-Pb and U-Pb levels.

Using data from the Slovenian HBM program, this research assessed the impact of individual SNPs, SNP combinations, and haplotypes on Pb levels. While no significant associations were found for haplotypes, certain SNPs showed notable effects. The variant alleles of SNPs rs1800435 and rs1805312 were negatively associated with B-Pb levels, while rs1139488 was positively associated. Additionally, the variant allele of rs1800435 was also negatively associated with U-Pb levels. Combination analyses revealed that specific SNP pairs explained significantly more variability in B-Pb concentrations than individual SNPs.

Five *VDR* SNPs (*FokI*, *BsmI*, *ApaI*, *TaqI*, and *BglI*) were as well tested on their associations with B-Pb and U-Pb levels; however, no significant influence on Pb concentration were discovered.

These findings underscore the importance of *ALAD* SNPs in determining individual susceptibility to Pb exposure. Furthermore, the study suggests that specific combinations of *ALAD* SNPs could serve as effective biomarkers for assessing susceptibility to Pb exposure, pending further validation in other populations and mechanistic studies.

I contributed to the Article by genotyping all of the included SNPs and was partly included in the conceptualization and the writing of the Article.

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Genetic susceptibility to low-level lead exposure in men: Insights from ALAD polymorphisms

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ABSTRACT

The genetic susceptibility to low-level lead (Pb) exposure in general populations has been poorly investigated and is limited to the single nucleotide polymorphism (SNP) rs1800435 in the delta-aminolevulinic acid dehydratase gene (ALAD). This study explored associations between ten selected ALAD SNPs with Pb concentrations in blood (BPb) and urine (UPb) among 281 men aged 18–49 years from Slovenia, including 20 individuals residing in a Pb-contaminated area. The geometric mean (range) of BPb and UPb were 19.6 (3.86–84.7) μg/L and 0.69 (0.09–3.82) μg/L SG, respectively. The possible genetic influence was assessed by examining SNP haplotypes, individual SNPs, and the combination of two SNPs using multiple linear regression analyses. While no significant associations were found for haplotypes, the presence of variant alleles of rs1800435 and rs1805312 resulted in an 11% and 13% decrease in BPb, respectively, while the presence of variant allele of rs1139488 (homozygous only) exhibited significant 20% increase in BPb, respectively. Additionally, variant allele of rs1800435 resulted in lower UPb. Individual SNPs in the model explained only around 1 additional percentage point of BPb variability. In contrast, combination analyses identified six combinations of two SNPs, which significantly explained 3–22 additional percentage points of BPb variability, with the highest explanatory power observed for the rs1800435-rs1139488 and rs1139488-rs1805313 combinations. Moreover, excluding participants from the Pb-contaminated area indicated that exposure level influenced SNPs-Pb associations.

Our results confirm the importance of the *ALAD* gene in Pb kinetics even at low exposure levels. Additionally, we demonstrated that identifying individuals with specific combinations of *ALAD* SNPs explained a larger part of Pb variability, suggesting that these combinations, pending confirmation in other populations and further evaluation through mechanistic studies, may serve as superior susceptibility biomarker in Pb exposure compared to individual SNPs.

1. Introduction

Environmental exposure to lead (Pb), which is present in the environment mainly due to current and historical (legacy site) emissions, has been substantially reduced due to the phase-out of leaded gasoline and lead-based plumbing (Bergdahl and Skerfving, 2022). Nevertheless, Pb continues to be of great concern for public health due to its environmental persistence, transportability, and cumulative/retentive properties in humans (particularly in bones). Today, exposure in the general population mainly occurs in urban and industrial areas through air dust inhalation, ingestion via diet (food and drinking water), and smoking. Exposure to both high and relatively low levels of Pb has been linked to detrimental effects on various systems in the body, including the neurological, renal, cardiovascular, haematological, skeletal, and endocrine systems. Importantly, it is crucial to note that no safe level of Pb exposure has been established to date (ATSDR, 2020; Canada, 2021; Mitra et al., 2017; Bergdahl and Skerfving, 2022). A weak association with cognitive effects in children was reported at concentrations as low as <10 μg/L; however, it is important to keep in mind the methodological uncertainties at low levels (Bergdahl and Skerfving, 2022). Children and pregnant women are considered the most susceptible groups due to the high gastrointestinal absorption of Pb (ATSDR, 2020).

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However, Pb's effects on reproduction might also be an important issue for men and women in their childbearing age (Kumar, 2018). Recently, Balachandar et al. (2020) pointed out the susceptibility of adult men to Pb-induced endocrine disruption in occupational exposure, highlighting that results may be cautiously extended to environmental exposure.

Through various epidemiological and experimental studies, it has become clear, that toxicokinetics and toxicodynamics of Pb, and consequently one's susceptibility towards health effects, are influenced by various factors, such as age, nutritional status (levels of iron, calcium, zinc, and vitamin D), (patho)physiological state (pregnancy, lactation, fasting, chronic diseases etc.), lifestyle, and with increasing literature evidence also by genetic predisposition (Bergdahl and Skerfving 2022; ATSDR, 2020; Broberg and Pawlas, 2022). Concerning the latter, delta-aminolevulinic acid dehydratase (ALAD) or porphobilinogen synthase (PBGS) – a metalloenzyme crucial in the biosynthesis of porphyrins and haeme in various cells – represents the primary and major binding site for Pb in the erythrocytes (Bergdahl and Skerfving, 2022; ATSDR, 2020). The *ALAD* gene is the earliest and main target in investigations of gene-Pb interactions (Broberg et al., 2015). It is highly polymorphic, with dozens of identified single nucleotide polymorphisms (SNPs); however, most studies on Pb, have been focused on rs1800435 SNP. It is a C > G transversion (5–3′ strand) resulting in a Lys > Asn substitution, which is characterized traditionally by *ALAD1* and *ALAD2* alleles, respectively (Broberg et al., 2015; Mani et al., 2019). Based on comprehensive literature data, with the first study dating almost 35 years back, the findings on the different Pb levels between *ALAD1* and *ALAD2* allele carriers are inconclusive (Broberg et al., 2015; Scinicariello et al., 2007; Zhao et al., 2007). Similarly, the potential implications of rs1800435 for human health remain inconclusive. Some studies related to occupational exposure suggest a potential protective effect of ALAD2 (due to reduced bioavailability of Pb) against Pb neurotoxicity (e.g. motor dexterity function and cognitive defects) and in preserving sperm counts. In other studies, the presence of this allele has been associated to a higher risk of hypertension and detrimental effect on kidney function (summarized by Skerfving and Bergdahl, 2015).

One potential explanation for these contradicting findings may be attributed to the variation in Pb exposure levels. Meta-analyses have consistently demonstrated that higher Pb levels in ALAD2 carriers are typically observed in populations with high exposure, such as occupational settings or Pb-polluted regions (Zhao et al., 2007; Scinicariello et al., 2007). Conversely, studies examining non-occupational low environmental exposures have shown an opposite trend (Hu et al., 2001; Stajnko et al., 2020; Wu et al., 2003). Furthermore, population size, ethnicity, psycho-socio-economic disparities, and wider (epi)genetic background – including possible effects of other *ALAD* polymorphisms, polymorphisms in other genes, or their combinations – might also explain the observed heterogeneity in results (Scinicariello et al., 2007; Broberg et al., 2015; Broberg and Pawlas, 2022). Within the last decade, various studies have tested and highlighted the possible independent impact of several other *ALAD* SNPs – especially rs1805313, rs1139488, rs2228083, rs818708, and rs8177800 – further demonstrating that *ALAD* is a relevant gene influencing Pb concentrations (Mitra et al., 2017; Mani et al., 2019; Broberg and Pawlas, 2022). However, the possible linkage disequilibrium (LD) of *ALAD* SNPs and the influence of haplotypes on Pb concentrations and/or related health effects, was to the best of our knowledge, studied only in three studies so far (Rabstein et al., 2008; Bemmel et al., 2011; Palir et al., 2023). Moreover, only one study has investigated the possible combined effect of different SNPs (irrespective of their LD) that were each significantly associated with Pb concentrations (Palir et al., 2023).

Most of the aforementioned studies primarily concentrate on populations exposed to high levels of Pb, such as occupational or Pb-contaminated environments. However, there is a notable scarcity of research conducted on individuals experiencing long-term low-level environmental exposures, which is a prevalent scenario for a significant part of the global population today. Moreover, among those studies, adult men are underrepresented.

Accordingly, the present study aimed to test the possible influence of 10 selected SNPs in the *ALAD* gene on the distribution of Pb in blood and urine within the non-occupationally exposed population of adult men (18–49 years old) from Slovenia. The influence of SNPs was tested on the level of estimated haplotypes, individual SNPs, and combinations of significantly associated SNPs.

2. Materials and methods

2.1. Study design and selection of participants

In this study, we included a subset of male participants who initially participated in the Slovenian Human Biomonitoring program. The original study aimed, to estimate trace element levels and persistent organic pollutants in a population of 548 men and 536 *primiparous lactating* women recruited between 2008 and 2014 from 12 rural, urban, and known or potentially contaminated areas. All participants were recruited at least 13 years after the phase out of leaded petrol in Slovenia in 1995, however possibly exposed to Pb-contaminated dust from petrol during their childhood. The recruitment protocol, sampling procedures, geographical areas, and exposure assessments were previously described (Snoj Tratnik et al., 2019). Briefly, all participants provided a random spot urine sample and a non-fasting sample of venous blood on the same day, and they completed questionnaires covering their general characteristics, socio-economic status, lifestyle, and dietary habits. All participants signed an informed consent form, and the Republic of Slovenia National Medical Ethics Committee approved the study protocol (numbers of accordance 42/12/07, 53/07/09, and 70/02/11).

To reuse biobanked samples for genetic analyses, we obtained additional ethical approval (number of accordance 0120–431/2018/4). Approximately 50% of the participants provided their signed informed consent, while 510 participants dropped out of the study (no reason stated or due to the changed place of residency). Owing to the possible enhanced release of bone-stored Pb within the state of lactation (ATSDR, 2020; Health Canada, 2021) and the possibility of "masked" or changed gene-environment associations due to the dominant influence of changing physiology during lactation (Gardner et al., 2012; Stajnko et al., 2019), only male participants were selected for the assessment of SNPs-Pb associations in the present study (Fig. 1A). As such, available archived blood samples of 281 men were used to isolate genomic DNA.

When comparing the distribution of demographic variables (e.g., age, BMI, smoking, residency location) in the selected population compared to those reported in the study by Snoj Tratnik et al. (2019), no selection bias was observed. The geographical distribution of sampling areas for selected male participants is presented in Fig. 1B. The study also included 20 participants from the Pb-contaminated Upper Mezica Valley (UMV, characterized by past Pb/Zn mining activity and still ongoing recycling of Pb-containing batteries; Miler and Gosar, 2012; Žibret et al., 2018).

2.2. Determination of Pb and Zn

Elements were determined in 0.3 mL of whole blood (Pb and Zn) and 1 mL of spot urine (Pb) with an Octopole Reaction System (ORS) Inductively Coupled Plasma Mass Spectrometry (ICP-MS; 7500ce, Agilent Technologies) equipped with an ASX-510 autosampler (Cetac) at the Department of Environmental Sciences, Jožef Stefan Institute Ljubljana, Slovenia. The details on both analytical and quality control procedures were described previously by Miklavčič et al. (2013) and Snoj Tratnik et al. (2019). The limit of detection (LOD) for Pb was 0.4 μg/L in blood and 0.3 μg/L in urine and for Zn in blood was 30 μg/L.

2.3. Determination of haemoglobin and specific gravity

Haemoglobin (Hb) in blood samples was obtained as a part of

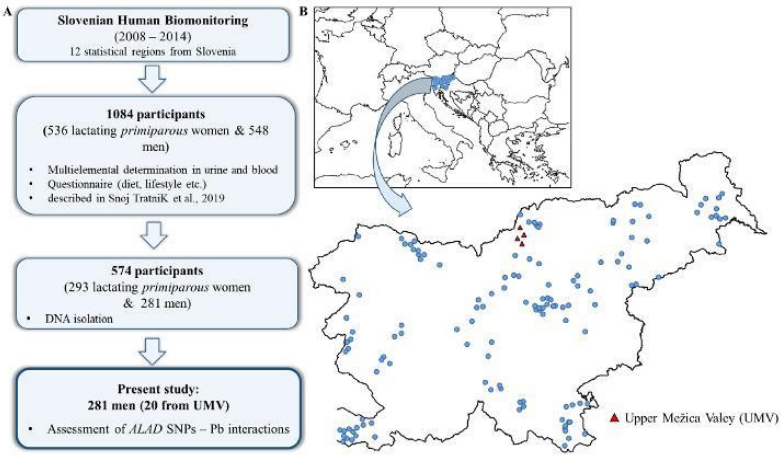


Fig. 1. Study population selection (A) and sampling areas (settlements of study participants' residencies) including Pb polluted areas (marked with a red triangle) (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

haemogram analyses using the standard routine method by Beckman-Coulter GEN-S haematology analyzer at the University Medical Centre Ljubljana, Slovenia.

Specific gravity (SG), which was used to adjust urine Pb concentrations for inter-individual difference in diuresis, was determined by a PAL-10S refractometer (Atago®, Japan; measurement range of 1.000–1.060) at the Department of Environmental Sciences, Jožef Stefan Institute Ljubljana, Slovenia.

2.4. DNA isolation and genotyping

An aliquot of 0.5 mL archived whole blood was used for genomic DNA isolation by the FlexiGene® DNA kit (Qiagen, Germany) following the manufacturer's instructions. The quantity and quality of isolated DNA were evaluated by UV-VIS spectrophotometer NanoDrop 2000c (ThermoFisher Scientific, USA). DNA isolates were stored at −80 °C before genotyping.

The criteria for the SNP selection were: i) evidence of a significant association between the SNP and Pb from existing literature, ii) a minor allele frequency (MAF) above 5%, and iii) the availability of pre-designed TaqMan genotyping assays. Accordingly, isolated DNA was genotyped for 10 SNPs in the *ALAD* gene: rs1805313, rs818708, rs1800435, rs8177812, rs8177796, rs2228083, rs1139488, rs1805312, rs818684, and rs2761016. Basic information on selected SNPs is shown in Table 1.

For the determination of genotypes, pre-designed TaqMan SNP Genotyping Assays (Applied Biosystems, USA; Table 1) were used. The 5 μL reaction consisted of 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. For amplification and fluorescence detection LightCycler® 480 Instrument II and LightCycler480® Software version 1.5.1 (Roche, Germany) was used. PCR cycling included the following steps: pre-PCR step (1 cycle: 50 °C for 2 min), activation step (1 cycle: 95 °C for 10 min), annealing and amplification step (50 cycles: 95 °C for 15 s and 61 °C for 1 min), and post-PCR step (1 cycle: 40 °C for 30 s). For each SNP, a subset of randomly selected samples was repeated as a control (~30%).

All the analyses were conducted at the Department of Environmental Sciences, Jožef Stefan Institute Ljubljana, Slovenia.

2.5. Linkage disequilibrium and haplotype analyses

SNPs within the *ALAD* gene were tested for possible linkage disequilibrium (LD; which measures non-random co-occurrence of SNPs' alleles with each other) and for haplotype blocks (regions in which SNPs are in LD) using the Haploview software (version 4.2, Day Lab at the Broad Institute Cambridge, USA). The LD or the "strength of the SNPs co-occurrence" is presented in greyscale (darker colour indicates stronger co-occurrence) and correspondingly by its pairwise r^2 value (100 = maximum disequilibrium, and 0 = no disequilibrium). SNPs are shown in 5′–3′ order based on their position in the gene.

Table 1
Basic information on studied SNPs.

dbSNP ID	Alternative Name	Chr/location	Nucleotide change[a]	Amino acid Change	TaqMan assay ID
rs1800435	ALAD1/2	9/exon	C (ALAD1) > G (ALAD2)	Lys > Asn	C_11495146_10
rs1139488		9/exon	A > G	Tyr > Ter	C__3045785_10
rs1805313		9/intron	A > G		C_11495186_1_
rs818708		9/3′UTR	G > A		C__1632155_20
rs2761016		9/intron	C > T		C_15929257_20
rs8177812		9/intron	G > A		C_25767045_10
rs2228083		9/exon	G > A	Asn > Asn	C_16170526_10
rs1805312		9/intron	C > G		C_11495150_30
rs8177796		9/intron	G > A		C__1632161_20
rs818684		9/intron	C > T		C__1632165_10

[a] The nucleotide changes are reported based on the forward or 5′-3′ strand.

2.6. Statistical analyses

Central tendency parameters were calculated for the dataset, including the arithmetic mean (±SD), minimum and maximum values (min and max), geometric mean (GM), median (P50), and the 25th, 75th, and 95th percentiles (P25, P75, and P95). Concentrations of Pb in urine are presented as un-adjusted (μg/L) and as SG adjusted (μg/L SG) by applying the previously described calculation (Suwazono et al., 2005) and using 1.019 (population's mean) as a standard SG.

Differences in the distribution of BPb and UPb concentration between SNPs groups were tested using the one-way ANOVA test with the post hoc Tukey HSD test (for differences between three groups). The SNP-Pb associations were further tested by multiple linear regression analyses with BPb or UPb as the dependent variable and SNPs as the independent variable. Each SNP-Pb association was tested individually in a separate model with the adjustment for possible confounding by age (years), height (cm), current smoking (yes vs. no), alcohol consumption (≥1 per week vs. <1 per week; considering 1 glass off wine (125 ml), liquor (30 ml) or beer), source of drinking water (private vs. public or bottled; regardless the geographical region), living in the Pb-contaminated UMV area (yes vs. no), blood-Zn (μg/L), Hb (g/L; for BPb only), and SG (UPb only). The confounders were chosen according to our previous study by Snoj Tratnik et al. (2019), which identified the determinants of Pb exposure for the whole Slovenian HBM population, and according to other literature data (De Silva, 1984; Bergdahl et al., 1997; Skerfving and Bergdahl, 2015). Zn concentrations in blood were used as a proxy of nutritional status, which is known to influence the gastrointestinal absorption of Pb (Ahamed and Siddiqui, 2007; Rahman et al., 2019; Skerfving and Bergdahl, 2015). We also tested education, weight, BMI, dietary habits (consumption of game, nuts, poultry, and seafood), passive smoking, distance to the main road, intake of supplements or medicine, the season and the year of sampling, and presence of illness or chronic disease but excluded them from the final model, due to insignificant influence on Pb concentrations.

All SNP-Pb associations were tested as follows.

i) SNP haplotypes identified within *ALAD* (0 copies vs. at least one copy or two copies),
ii) individual SNPs (based on genotype and/or allele stratification),
iii) combinations of two SNP genotypes and/or alleles were formed based on the observed trend of Pb concentrations associated with each individual SNP. Moreover, the change in R^2 (ΔR^2) was calculated between models that included the SNPs combination as a covariate and models that excluded it. This allowed for a rough estimation of the "strength" of these combinations's influence on Pb concentrations in blood and urine. In other words, we tested how various SNP combinations affected the percentage of variability in the Pb level explained by the models.

Moreover, we conducted a sensitivity analysis to evaluate the potential influence of UMV participants on the SNP-Pb associations by excluding those participants from the models (presented in supplementary material).

The performance of the models was assessed by diagnostic analyses (testing for linearity, normality, homoscedasticity, and multicollinearity). The estimation coefficients are presented as exponentiated b coefficients (Exp(b)). The level of statistical significance (p-value) was set to ≤ 0.05. Values below the LOD were replaced with a value of LOD/2, and when appropriate, non-normally distributed data were log-transformed to approximate normal distribution. Statistical analyses and visualisations of the results were carried out in the statistical software R version 3.6.0 with RStudio version February 1, 1335, and the QGIS program version 3.16.15.

3. Results

3.1. Basic characteristics and exposure biomarkers

The descriptive statistics of the participants' basic characteristics and Pb exposure biomarkers are presented in Table 2 and Table 3, respectively. The participants were, on average, 31 years old, and 38% held at least a university degree. Current smoking was reported by 6% of participants while the intake of alcohol "one drink per week or more" was reported in 56% and "one drink per day" in only 1% of participants. Twenty participants (7%) lived in the Pb-contaminated area, and 6% reported a private drinking water supply.

The geometric mean (GM) of Pb in the study population was 19.6 μg/L (range 3.86–84.7 μg/L) in blood and 0.69 μg/L SG (range 0.15–4.40 μg/L SG) in urine. GM of Zn and Hb in blood were 6612 μg/L (range 4515–10300 μg/L) and 153 g/L (range 127–179 g/L), respectively. Pb in blood and urine were statistically significantly correlated ($r_s = 0.528$ with $p < 0.001$ data not shown). Participants from the Pb-contaminated area ($n = 20$) had, on average, two times higher Pb concentrations in blood and urine (GM: 41.1 μg/L and 1.35 μg/L SG, respectively) than the rest of the population (18.5 μg/L and 0.66 μg/L SG, respectively) (Figure SP1).

3.2. Pb exposure, sociodemographic variables, and Zn levels

Multiple linear regression models explained 32–34% and 43–44% of the variability (R^2) in BPb and UPb, respectively. Residing in the Pb-polluted area of UMV was a crucial factor affecting Pb concentrations, particularly BPb. Additional statistically significant determinants of Pb were age, height, current smoking, alcohol intake, type of water supply, and BZn concentration (data not presented). On average one unit change in age (year) resulted in an approximately 1% increase in BPb and UPb, whereas a one unit change in height (cm) resulted in a 1% decrease in BPb (no influence on UPb). Current smokers, on average, had 25% higher BPb than non-smokers, with no effect on UPb. The consumption of alcohol at least once per week resulted in approximately 20% and 16% higher BPb and UPb, respectively. Furthermore, the usage of a private water supply for drinking was, on average, associated with approximately 40% higher BPb and UPb (marginally significant) than public supply or bottled water usage. BZn was significantly positively associated with BPb and negatively with UPb; a 1% increase in BZn resulted in around a 0.6% increase in BPb and a 0.9% decrease in UPb.

Table 2
Basic characteristics of the studied population.

Basic characteristics	N	$\bar{x} \pm$ SD	min - max
Age (years)	281	31 ± 5	18–49
Height (cm)	279	180 ± 6	160–200
Weight (Kg)	278	84 ± 13	50–140
BMI (Kg/m^2)	278	26 ± 3	18–37
	N	**%**	
Education			
< university	170	62	
≥ university	106	38	
Living in upper Mežica (UMV)			
Yes	20	7	
No	261	93	
Current smoking			
Yes	16	6	
No	263	94	
Water supply			
Private	17	6	
Public or bottled	263	94	
Alcohol intake			
< 1 per week	124	44	
≥ 1 per week	157	56	

N- number of participants.

Table 3
Descriptive statistics of measured parameters in blood and/or urine of the study population.

	N	GM	Median	min	P25	P75	P95	max
BPb (µg/L)	281	19.6	19.0	3.86	14.1	26.8	46.8	84.7
UPb (µg/L)	226	0.64	0.68	0.15	0.40	1.13	2.31	4.40
UPb (µg/L SG)	224	0.69	0.72	0.09	0.48	1.09	1.93	3.82
BZn (µg/L)	280	6612	6633	4515	6142	7201	8087	10300
Hb (g/L)	265	153	154	127	148	159	159	179

BPb-blood Pb, BZn – blood Zn; UPb – urine Pb; N- number of participants, GM – geometric mean.

Nevertheless, additional interaction models (including BZn-SNP interaction as a covariate) showed that Zn levels did not modify the investigated SNP-Pb associations (data not presented).

3.3. SNPs frequency distributions, linkage disequilibrium, and haplotypes

The genotype and allele frequency distribution of analysed *ALAD* SNPs within the study population are summarized in Table 4. All SNPs followed the Hardy-Weinberg equilibrium ($p > 0.05$) and were successfully genotyped in 89%–100% of cases. The range of minor allele frequencies (MAFs) in the Slovenian male population was 8–48% for ten *ALAD* SNPs.

In the case of six of them (rs8177812, rs2228083, rs1805312, rs1800435, rs8177796, and rs818684), only ten or fewer individuals were homozygous for the variant allele. For whom further statistics were performed based on the allele stratification only (presence vs absence of variant allele (e.g. G+ vs G-); + for heterozygotes & variant homozygotes and – for common homozygotes). For other SNPs, the allele and genotype stratification were used.

The results of LD analyses and haplotypes are presented in Fig. 2. A weak co-occurrence was observed between three SNPS - rs1805313 (A > G), rs8177812 (G > A), and rs2228083 (G > A) SNPs (pairwise r2 = 18–51) - resulting in the identification of one haplotype block with four haplotypes with frequencies between 7 and 60% occurring in our population (Fig. 2).

Frequencies of SNPs and haplotypes did not differ after the exclusion of UMV individuals (data not presented).

3.4. Haplotypes and Pb concentrations

No significant differences in BPb and UPb concentrations were observed between carriers and non-carriers for each identified *ALAD* haplotype based on group comparisons or after the adjustment for confounding variables (supplementary material, Table SP1). Furthermore, no significant difference in Pb concentrations was observed among different haplotypes (data not presented). Consequently, the possible influences of SNPs on Pb concentrations were further investigated at the level of individual SNPs.

3.5. Individual SNPs and Pb concentrations

Possible SNPs-Pb relations were tested by the stratification of BPb and UPb concentrations among genotype and/or allele groups (supplementary material Table SP2 for BPb and SP3 for UPb; summarized in Fig. 3), and by multiple linear regression analyses with the adjustment for possible confounding variables (Supplementary material Table SP4 for BPb and SP5 for UPb; summarized in Fig. 4).

Among the SNPs, significant or marginally significant SNP-Pb associations within participants were observed only for three *ALAD* SNPs namely rs1800435 (C > G or ALAD1>ALAD2), rs1805312 (C > G), and rs1139488 (A > G) (Figs. 3 and 4). The presence of variant alleles (G+) of rs1800435 and rs1805312 resulted in lower BPb concentrations compared to its absence (G-), as can be seen from the group comparison (GM: 17.4 vs. 20.1 µg/L and $p = 0.072$, for rs1800435; GM: 17.6 vs 20.2 µg/L and $p = 0.050$ for rs1805312; Fig. 3) and from the multiple linear regression analyses which showed 11% ($p = 0.100$) and 13% ($p = 0.050$) lower BPb in variant carriers for the respective SNP (Fig. 4).

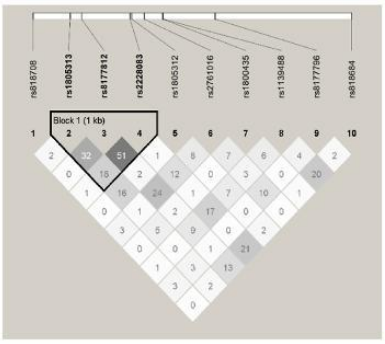
In contrast, variant homozygotes (GG) of rs1139488 exhibited significantly higher BPb concentrations compared to heterozygotes (GC) or common homozygous (AA). This was evident from the group comparisons (GM: 24.1, 18.7, and 19.0 µg/L, respectively; $p = 0.009$; Fig. 3) and after the adjustment for possible confounding, which showed 20% higher BPb in GG carriers (Fig. 4). Notably, these differences were observed exclusively at the genotype level, as allele stratification did not yield significant differences in BPb levels.

For UPb, an association was observed only for rs1800435, with the variant allele carriers (G or ALAD2) having lower concentrations than non-carriers (GM: 0.59 vs. 0.71 µg/L SG, respectively; $p = 0.100$). The association for rs1800435 was marginally significant based in the whole population. However, after excluding the 20 UMV participants, the association with BPb and UPb became stronger and statistically significant (Table SP3-5). Additionally, excluding those participants also resulted in the significant influence of rs818708 on BPb, with the variant allele showing 13% higher levels (Table SP4).

Table 4
SNPs genotypes and alleles distribution in the study population.

SNP	Common homozygous	Heterozygous	Variant homozygous	MAF[b]	MAF EU[a]	HWE	genotyped individuals
	N (%)	N (%)	N (%)	%	%	p-value	%
rs1800435 (C > G)[c]	230 (82)	47 (17)	2 (1)	9	8	0.811	99.3
rs1139488 (A > G)	100 (36)	135 (48)	46 (16)	40	38	0.969	100
rs1805313 (A > G)	94 (33)	148 (53)	39 (14)	40	31	0.110	100
rs818708 (G > A)	70 (25)	151 (54)	60 (22)	48	45	0.482	100
rs2761016 (C > T)	92 (33)	135 (48)	54 (19)	43	47	0.722	100
rs8177812 (G > A)	190 (68)	79 (28)	10 (4)	18	12	0.617	99.3
rs2228083 (G > A)	220 (79)	54 (20)	3 (1)	11	8	0.877	98.6
rs1805312 (C > G)	227 (81)	50 (18)	2 (1)	10	6	0.674	99.3
rs8177796 (G > A)	241 (86)	36(13)	3 (1)	8	9	0.219	99.6
rs818684 (C > T)	173 (62)	97 (35)	9 (3)	21	17	0.297	99.3

MAF – minor allele frequency.
HWE – Hardy-Weinberg equilibrium.
[a] European population (n = 1006) from 1000 Genomes project (NCBI, 2022).
[b] The frequencies did not differ significantly after the exclusion of UMV participants a also known as ALAD1>ALAD2.
[c] ALAD1>ALAD2



ALAD Haplotype

rs1805313 (A>G)	rs8177812 (G >A)	rs2228083 (G>A)	Frequency
A	G	G	60%
G	G	G	22%
G	A	A	10%
G	A	G	7%

Fig. 2. Linkage disequilibrium plot (LD, left) and frequencies of identified haplotype patterns (Block 1, right) for investigated SNPs within the whole study population. The greyscale colour indicates the strength of the correlation (the darker the stronger) and, correspondingly, its pairwise r^2 value (100 = the strongest or maximum disequilibrium and 0 = no disequilibrium). SNPs are shown in 5′–3′ order based on their position in the gene. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

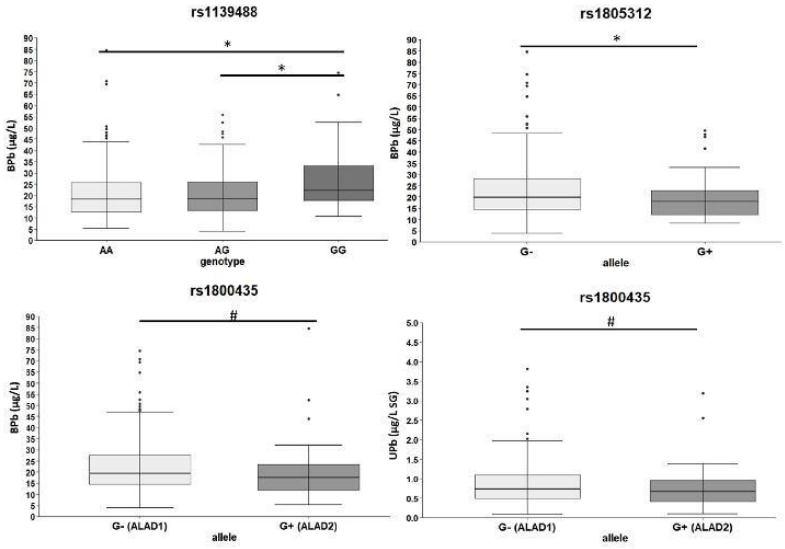


Fig. 3. Distribution of BPb (µg/L) based on the rs1139488 (A > G) genotypes, and presence or absence of variant alleles of rs1800435 (C > G, ALAD1>ALAD2), rs1805312 (C > G)and of UPb (µg/L SG) based on the presence or absence of variant alleles of rs1800435 (C > G, ALAD1>ALAD2) (*p < 0.05 and #p < 0.1).

3.6. SNP combinations and Pb concentrations

A significant association with BPb was observed for six SNPs combinations and one of them also with UPb, as summarized in Table 5.

As expected, based on the assessment of individual SNPs, the significant combinations were those between rs1139488, rs1800435 (C > G; ALAD1>ALAD2), rs1805312 (C > G), and rs818708 (G > A) SNPs, and additionally with rs1805313 (A > G) SNP (Table 5). A statistically significant decrease in BPb was observed for the carriers of the following six two-allele-combinations: G(ALAD2)-A for rs1800435-rs1139488, G (ALAD2)-G for rs1800435-rs818708, G(ALAD2)-G for rs1800435-rs1805313, A-G for rs1139488-rs1805312, A-G for rs1139488-rs1805313 and G-G for rs818708-rs1805312 – when compared to non-carriers; (decrease for: 33-20%, $p < 0.05$; Table 5). Those results were confirmed in sensitivity analyses exluding UMV participants, with slightly stronger influences (Table SP6).

Moreover, the inclusion of those combinations (based on the allele and/or genotype combination) into the models explained, on average, an additional 9% (range 3–22 %) points (i.e. ΔR^2) in the variability of BPb concentrations. The highest ΔR^2 was observed for the rs1139488-rs1805313 combination (AA-GG vs GG-AA; $\Delta R^2 = 20$) and for the rs1800435-rs1139488 combination (G+(ALAD2)-AA vs CC(ALAD1/1)-GG; $\Delta R^2 = 19\%$ points; Table 5).

For UPb, a statistical significance was observed only for the rs1800435-rs1805313 combination with G-G carriers, resulting in 18% lower UPb compared to non-carriers (Table 5).

4. Discussion

The investigation of SNP-Pb associations in general populations with low non-occupational exposures has been limited, particularly when considering the testing of SNPs on the basis of haplotypes, as well as their individual and combined effects. In this context, the present study addressed these gaps and contributed new insights to the field.

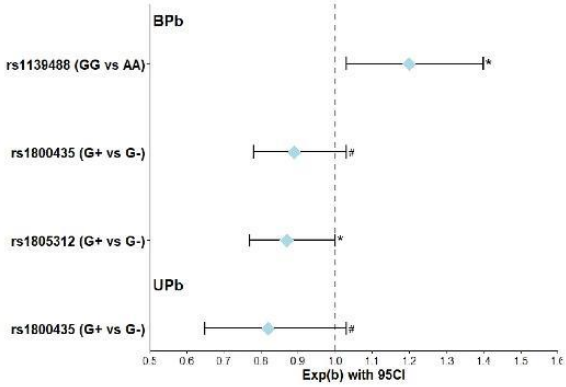


Fig. 4. Summarized influences of significant SNPs on BPb (μg/L) and UPb (μg/L) based on the linear regression models. Presented are estimation coefficients (Exp(b)) based on genotype stratification (variant homozygotes vs. common homozygotes; for rs1139488) or allele stratification (presence of variant allele (+i.e. heterozygotes + variant homozygotes) vs. its absence (-, i.e. common homozygotes), for other SNPs); #$p < 0.1$, *$p < 0.05$; **$p < 0.01$). Models were adjusted for age, height, current smoking, alcohol consumption, source of drinking water, living in Pb-contaminated UMV area, BZn, Hb (for BPb only), and SG (for UPb only).

4.1. Exposure

In the present study, we assessed Pb concentrations in Slovenian men from uncontaminated regions, revealing generally low environmental exposure to Pb (GM, BPb 19.6 μg/L). However, the levels for individuals residing in the Pb-contaminated area were higher (GM, BPb 41.1 μg/L). The elevated concentrations among UMV participants can be attributed to both higher external factors, such as elevated Pb levels in the living environment, including house dust, soil, and locally produced food (NIJZ, 2016), as well as internal factors, such as the continuous and gradual release of historically accumulated Pb from bone (ATSDR, 2020). Five participants (all from UMV) exceeded the reference value of 64 μg/L in the blood set for adult Spanish men (n = 962; recruited in 2009–2010) (Cañas et al., 2014). Although there are currently no known safe or recommended BPb levels, the concentration below 50 μg/L was set as a reference value for adults (ATSDR, 2020). The same value was recently set as a threshold for Pb inhibition of the ALAD catalytic activity (Huang et al., 2020). In the present study, only nine participants (from those seven from UMV) exceeded this value. The concentrations observed for most of participants are generally believed not to present an elevated health risk; however, the possibility for some health effects in men from the UMV area could not be excluded.

4.2. ALAD SNPs – Pb associations

In the present study, a weak co-occurrence was observed only for rs1805313, rs8177812, and rs2228083 SNPs, which resulted in four haplotypes with frequencies >1% within the Slovenian population of men (Fig. 2). Similar observations were reported in previous studies (Neslund-Dudas et al., 2014; Palir et al., 2023; Rabstein et al., 2008). In the present study, none of the haplotypes significantly influenced Pb concentrations, as indicated in Table SP1. This finding aligns with a study conducted on Romanian women residing in a Pb-polluted region affected by historical mining activities (aged 20–65 years; BPb P50: 48

Table 5
ALAD SNPs combinations with significant influence on BPb (μg/L) and UPb (μg/L) based on the multiple linear regression analyses.

Genotype or allele combination of two SNPs		N	GM	P50	Exp(B) 95CI, (N) R^2	R^{2a}	ΔR^2
		BPb (μg/L)			Dependant variable: BPb (μg/L)		
rs1800435 (C > G)	rs1139488 (A > G)						
CC (ALAD1)	GG	46	24.1	22.2	1.00		
G+ (ALAD2)	AA	31	**17.7****	17.9	**0.74 (0.61–0.89)***** (70) 0.52	*0.33*	0.19
G+(ALAD2)	A+	49	**17.4*****	17.5	**0.77 (065–0.91)***** (86) 0.49	0.33	0.16
rs1800435 (C > G)	rs818708 (G > A)						
CC (ALAD1)	AA	51	19.8	19.0	1.00		
G+ (ALAD2)	GG	16	**15.3****	14.8	**0.67 (0.51–0.87)***** (60) 0.48	*0.38*	0.10
G+ (ALAD2)	G+	41	17.3	15.7	**0.77 (0.63–0.93)***** (79) 0.47	*0.42*	0.05
rs1800435 (C > G)	rs1805313 (A > G)						
CC (ALAD1)	AA	65	21.8	21.3	1.00		
G+ (ALAD2)	G+	20	18.5	18.2	**0.76 (0.60–0.95)**** (80) 0.40	*0.35*	0.05
rs1139488 (A > G)	rs1805312 (C > G)						
GG	CC	46	24.1	22.2	1.00		
AA	G+	35	**18.7****	18.1	**0.79 (0.66–0.97)****(75) 0.38	*0.33*	0.05
A+	G+	52	**17.6****	17.9	**0.77 (0.64–0.91)***(92) 0.41	*0.34*	0.07
rs1139488 (A > G)	rs1805313 (A > G)						
GG	AA	19	24.8	23.1	1.00		
AA	GG	16	**18.3****	17.3	**0.80 (0.71–0.91)***** (33) 0.64	*0.44*	0.20
A+	G+	160	**18.7****	18.1	**0.75 (0.61–0.93)***** (166) 0.33	0.30	0.03
rs818708 (G > A)	rs1805312 (C > G)						
AA	CC	47	19.6	19.0	1.00		
GG	G+	14	17.0	17.0	**0.73 (0.54–0.97)**** (55) 0.33	*0.26*	0.07
G+	G+	39	16.7*	17.5	**0.75 (0.61–0.91)***** (78) 0.37	*0.29*	0.08
		UPb (μg/L SG)	**Dependant variable: UPb (μg/L)**				
rs1800435 (C > G)	rs1805313 (A > G)						
CC (ALAD1)	AA	51	0.80	0.85	1.00		
G+ (ALAD2)	G+	16	0.69	0.67	**0.72 (0.53–1.00)**** (65) 0.62	*0.59*	0.03

Statistically significantly different from reference group: ***< 0.01, **$p < 0.05$; *$p < 0.1$; Underlined are variant alleles; When less than 10 participants per group, the comparison, and multiple regression analyses were not tested.
Adjustment for: age, height, current smoking, alcohol consumption, type of water supply, Hb, BZn, living in Upper Mežica Valley, and SG (for urine only).
[a] with the exclusion of SNP combination as covariate, ΔR^2– the difference in R^2 between models with or without SNP combination; N – number of participants.

µg/L) (Rabstein et al., 2008). However, a case-control study investigating the interactions between 19 *ALAD* SNPs, BPb exposure, and the risk of renal cancer reported a significant association of haplotype (G-C-T-G-G for rs818687-rs2792818-rs8177796-rs8177800-rs2761016, respectively) with an increased risk for cancer. Unfortunately, due to the low statistical power, the authors could not evaluate the possible role of haplotype-Pb interaction (Bemmel et al., 2011). The lack of strong co-occurrences between the *ALAD* SNPs, especially those with previously observed significant influences on Pb concentrations (Broberg et al., 2015; Broberg and Pawlas, 2022), could explain the dominating investigation of individual SNPs over haplotypes in the literature.

The most widely studied SNP is rs1800435 (C > G, ALAD1>ALAD2). A previous hypothesis on the dose-dependent influence of *ALAD2* on BPb (high exposure: Pb in *ALAD2>ALAD1;* low exposure: Pb in *ALAD2<ALAD1)* (Zhao et al., 2007; Scinicariello et al., 2007), was supported by the results of our study. As such, at relatively low Pb exposure *ALAD2* carriers had lower not only BPb but also UPb concentrations compared to *ALAD1* carriers, although the difference was only marginally significant. Lower Pb levels in both blood and urine point to an internal ALAD2-related Pb sequestration different from blood erythrocytes. As expected, after the exclusion of UMV participants, with the highest Pb concentrations, the differences were more pronounced and statistically significant. Firstly, ALAD is beside erythrocytes, also highly expressed within cells of the liver, endocrine tissues, kidney, proximal digestive tract, and gastrointestinal tract (GI) (https://www.proteinatlas.org). Its involvement in the production of heme is vital for all human organs, as it is an essential component of several iron-containing proteins (haemoproteins), including haemoglobin and various cytochromes. Accordingly, a part of ingested Pb is most probably being sequestered by ALAD in the cells of other tissues before entering the central bloodstream. Secondly, the Lys > Asn amino acid substitution of rs1800435 leads to a more electronegative charge of the ALAD2 isoform and consequently in its increased binding capacity and accumulation of Pb compared to ALAD1 (Wetmur et al., 1991). Taken together, at low exposure levels, the presence of ALAD2 may contribute to an increased accumulation of Pb in GI cells and hepatocytes leading to lower BPb levels compared to ALAD1. However, at higher exposure levels, the impact of these primary tissues might be less pronounced, and larger amounts of Pb enter the bloodstream, where once again it is more likely to accumulate in the presence of ALAD2 compared to ALAD1.

Nevertheless, it is important to keep in mind the relevance of several other independent *ALAD* SNPs that can influence the kinetics/dynamics of Pb (Broberg et al., 2015) - especially at low environmental exposures. For *ALAD*, besides rs1800435, the second two most studied SNPs are rs1139488 (A > G) and rs1805313 (A > G). In the present study, a significant association with Pb was observed for SNP rs1139488 (A > G) – closely located to rs1800435 – which indicated higher BPb for homozygous carriers of the variant allele. This observation has been consistently, but not always with statistical significance, observed by several other studies conducted in occupational or Pb-contaminated settings (Chia et al., 2005; Rabstein et al., 2008; Shaik et al., 2018; Szymańska-Chabowska et al., 2015) and recently also at low environmental exposure of pregnant women (Palir et al., 2023). In the case of rs1805313 (A > G), the genome-wide analyses on populations with non-occupational Pb exposure conducted by Warrington et al. (2015) identified this intronic SNP as the most significantly associated with BPb concentrations; the variant allele determined lower BPb, which was further confirmed in our previous studies in low-level exposed populations (Stajnko et al., 2019; Palir et al., 2023). In the present study, we observed the same trend, however, it was without statistical significance (Table SP2 and SP5). At the same time, studies conducted on populations with higher exposures (occupational or Pb-contaminated settings) reported either no influence of SNP on Pb (Rabstein et al., 2008; Neslund-Dudas et al., 2014; Callahan et al., 2019) or contrary, higher BPb among variant allele carriers (Szymańska-Chabowska et al., 2015). Experimental studies showed that rs1805313 might affect *ALAD* expression in non-transformed blood cells; however, whether this is the mechanism behind the SNP-BPb association is unclear (Warrington et al., 2015).

For other *ALAD* SNPs included in the present study (Table 1), only a few studies exist in the literature, with some reporting a significant influence of rs818708, rs2228083, rs2761016, and rs818684 on BPb concentrations (Li et al., 2017; Szymańska-Chabowska et al., 2015; Neslund-Dudas et al., 2014; Bemmel et al., 2011). We did not observe a significant influence of those SNPs in the present study. However, in a sensitivity analysis excluding UMV participants, a SNP rs818708 (G > A) showed significantly higher BPb in carriers of the variant allele. A study by Li et al. (2017) indicated that rs818708 might influence ALAD expression through modifying effects in epigenetic regulation (i.e., disturbance in the binding of some miRNAs to ALAD). Additionally, we observed significantly lower BPb for carriers of the rs1805312 (C > G) variant allele. This SNP was previously investigated only in one study on Romanian women from a Pb-contaminated area and showed no significant influence on Pb (Rabstein et al., 2008).

As previously highlighted, only a small proportion of the Pb variation in blood can be explained by individual SNPs (Broberg et al., 2015), particularly at non-occupational exposure. This was observed also in the present study, where the presence of either above mentioned SNP in the model explained at most an additional 1% point in Pb variability (data not shown). Accordingly, we further tested whether combinations of two ALAD SNPs might explain more variability in Pb. Indeed, six combinations between 5 above-discussed SNPs (rs1800435, rs1139488, rs1805312, rs818708, and rs1805313) showed a significant influence on BPb and one on UPb and, on average, explained an additional 9% (range 3–22%) (ΔR^2) in the variability of BPb and/or UPb. The highest explanation of the variability in BPb was observed for the combinations of rs1139488-rs1805313 and rs1139488-rs1800435 ($\Delta R^2 = 22$ and 19%, respectively). In the case of the latter, a higher effect of the combination compared to single SNPs ($R^2 = 24\%$ and 12%, respectively) on BPb was also observed in a study on Italian pregnant women with low Pb exposure (n = 873, GM BPb, 11 µg/L) (Palir et al., 2023). Such results indicate that susceptibility to Pb exposure might be better estimated through selected combinations of two or more *ALAD* SNPs if a biological mechanistic background exists. Therefore, additional research is necessary to investigate the molecular mechanism of *ALAD* SNPs, including potential changes in the gene expression and activity of the ALAD enzyme.

Genetic polymorphisms in various other genes, such as vitamin D receptor (*VDR*), homeostatic iron regulator (*HFE*), transferrin (*TF*), etc., were previously associated with Pb kinetics and toxicity, although mostly at high environmental or occupational exposures (Broberg and Pawlas, 2022; Mani et al., 2017). Accordingly, in the context of the present study selected polymorphisms in *VDR* (rs739837, rs731236, rs7975232, rs1544410, and rs2228570) and HFE (rs12346, rs1799945) were additionally tested but did not significantly influence Pb concentrations (unpublished data).

Additionally, although BPb and UPb were in the present study significantly correlated, SNPs influences were mostly observed for BPb. Blood Pb is the most commonly used biomarker of Pb exposure in environmental and occupational settings, and it reflects both body burden and recent exposure. Contrary, UPb is less widely employed and is suggested to mostly reflect the filterable fraction of Pb in plasma. In addition to the known impact of diuresis, Pb concentrations in urine respond more rapidly to changes in exposure compared to those in blood. (Bergdahl and Skerfving, 2022; Sallsten et al., 2022). The correlation would be better estimated by comparing Pb concentrations in fasting morning blood plasma samples with those in fasting morning urine. However, it is interesting that the simultaneous SNP influence on blood and urine Pb was visible only for the most characterised and studied SNP (rs1800435).

4.3. Study limitations

The most important limitation of the study is the small sample size, which hinders the investigation of SNPs with lower frequencies and leads to a limited number of individuals with variant alleles, thereby reducing the statistical power of the analysis. Additionally, another limitation is the lack of parameters, such as Fe, Ca, Se, and vitamin D concentrations, which are known to interfere with Pb kinetics in the human body. Furthermore, uncertainties arise from self-reported data, potential missed/hidden diet Pb sources, and undefined internal exposure resulting from the possible slow release of Pb accumulated in the bone.

5. Conclusions

In the present study, we investigated the effects of individual SNPs, their combinations, and haplotypes in the *ALAD* gene on Pb levels in blood and urine in non-occupationally exposed Slovenian men aged 20–40 years. The assessment of the influence of *ALAD* haplotypes on Pb concentrations in participants showed no significant associations. However, at such low Pb exposure, the individual investigation of *ALAD* SNPs showed significantly lower BPb and/or UPb concentrations in variant allele carriers of rs1800435 (C > G or ALAD1>ALAD2) and rs1805312 (C > G), and higher BPb in variant allele carriers of rs1139488 (A > G). As previously suggested, our findings support the notion that ALAD-Pb associations, particularly in the case of rs1800435, may be influenced by the exposure dose. Nevertheless, it is important to note that the individual contribution of each SNP to the variability of Pb concentrations was relatively modest. Interestingly, our study revealed the identification of individuals with specific combinations of *ALAD* SNPs, which explained a larger part of BPb variability. If these combinations are further validated as influential factors, rather than coincidental findings, they could serve as more reliable susceptibility biomarkers for Pb exposure yhan single SNPs. Future mechanistic studies are warranted to confirm their significance.

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CRediT authorship contribution statement

Anja Stajnko: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Neža Palir:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Janja Snoj Tratnik:** Writing – review & editing. **Darja Mazej:** Writing – review & editing. **Alenka Sešek Briški:** Resources. **Agneta Annika Runkel:** Writing – review & editing, Data curation. **Milena Horvat:** Writing – review & editing, Project administration, Funding acquisition. **Ingrid Falnoga:** Writing – review & editing, Supervision, Investigation, Conceptualization.

Declaration of competing interest

The authors report no conflict of interest.

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Appendix A. Supplementary data

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Chapter 4

Discussion and Summary

The three Articles (3.1-3.3) collectively provide insight into the complex interplay between genetic factors, environmental exposure, and physiological processes in determining Pb toxicokinetics. By focusing on the influence of SNPs in key genes (*ALAD*, *VDR*, and *APOE*), this work enhances our understanding of how genetic variability affects Pb kinetics in vulnerable populations such as Italian, Croatian, and Slovenian pregnant women and their developing fetuses (PHIME project, 2006-2011), as well as Slovenian men in reproductive age (1st Slovenian HBM, 2008-2014). All groups were exposed to low to moderate Pb levels in the recent post-leaded gasoline environment. The official end of leaded gasoline use occurred in 2002 in Italy, 2006 in Croatia, and 2001 in Slovenia. The main findings are summarized below, organized according to the specified hypothesis.

4.1 *ALAD* rs1800435 (*ALAD1/2*)

The study found that among 873 Italian pregnant women with low Pb exposure (B-Pb GM: 11.0 ng/g; range: 3.09-60.5 ng/g) and adequate Zn and Se levels, those carrying the *ALAD2* allele had significantly lower maternal B-Pb levels in comparison to *ALAD1/1* carriers (Fig. 4.1). However, this variant allele was not significantly associated with CB-Pb levels in 619 of their newborns (Article 1).
Similarly, in a study conducted on 281 men of reproductive age with low to moderate Pb exposure (B-Pb GM: 19.6 µg/L; range: 3.86-84.7 µg/L) and adequate levels of Zn and Se (Snoj Tratnik et al., 2019), *ALAD2* allele carriers has as well lower B-Pb levels than those with *ALAD1/1* genotype (Fig. 4.2). Additionally, U-Pb levels followed the same trend, with *ALAD2* as well having lower U-Pb levels; however, the difference was only marginally significant (Fig. 4.2) (Article 3).

For the *ALAD2* allele is proposed to have a dose-dependent effect on Pb levels, where higher Pb exposure leads to increased B-Pb levels, and lower Pb exposure to decreased levels compared to the *ALAD1/1* genotype, a hypothesis supported by both our studies. ALAD is primarily found in the liver and red blood cells but it is also present in other tissues, such as the kidneys, and the gastrointestinal tract. Some Pb is likely sequestered by ALAD in the cells of other tissues before it enters the central bloodstream (Sulinskiene et al., 2015). The ALAD2 isoform has a more electronegative charge, which increases its binding affinity for Pb compared to ALAD1 (Broberg & Pawlas, 2022; Kelada et al., 2001). Consequently, at low exposure levels, ALAD2 may lead to greater Pb accumulation in gastrointestinal cells and hepatocytes, resulting in lower B-Pb levels compared to ALAD1. However, at higher exposure levels, the influence of these initial tissues may diminish, allowing more Pb to enter the bloodstream, where it is more likely to accumulate in the presence of ALAD2 than ALAD1.

The dose-dependent effect is further supported by findings in Article 3. When data from the "Upper Mezica Valley" region, which had the highest Pb concentrations, were excluded, more pronounced and statistically significant differences between *ALAD2* allele carriers and *ALAD1/1* carriers were observed. This emphasizes that Pb exposure levels are crucial when analyzing *ALAD* SNP rs1800435.

Additionally, *ALAD2* allele carriers were found to have lower urine Pb levels compare to those with *ALAD1/1* genotype. This finding could support the theory that the *ALAD2* isoform has a higher binding affinity for Pb, leading to greater accumulation in tissues and reduced Pb excretion in urine.

4.2 *ALAD* and *VDR* SNPs, Their Haplotypes, and Combinations

4.2.1 Ten *ALAD* SNPs

In the Italian population of pregnant women and newborns (PHIME) (Article 1), in addition to rs1800435, three other *ALAD* SNPs were tested: rs1805313, rs1139488, and rs818708. The variant allele of rs1805313 and the common allele of rs1139488 were negatively associated with maternal B-Pb levels, while rs818708 showed no statistically significant associations. Associations were stronger when including only women sampled in the second trimester (78 % of sampled population) (Fig. 4.1), minimizing the influence of changing TEs due to physiological changes from advancing pregnancy. Although no LD was found between analyzed *ALAD* SNPs, combining them base on their influence on B-Pb levels produced much higher R^2 values (24-31 %) in multiple linear regression models compared to models including only individual *ALAD* SNPs (12-14 %). The associations were not reflected in CB-Pb levels.

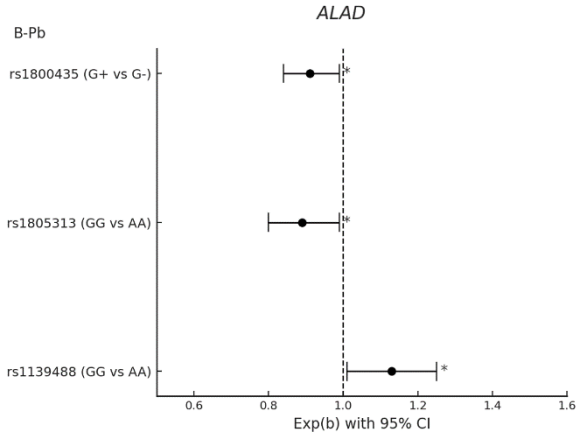


Figure 4.1: Summarized influences of significant SNPs on B-Pb based on the multiple linear regression models in the Italian population of pregnant women (PHIME). Presented are estimation coefficients (Exp(b)) based on genotype or allele stratification; # p <0.1, *p< 0.05; **p < 0.01; ***p < 0.001). Models were adjusted for: mAge, mBMI, mParity, mEducation, mSeafood consumption, mSmoking, mEGW at blood sampling, mB-Zn-log, cSex; included only women sampled in second trimester. B-blood, m-maternal, c-child; exp(b) – exponentiation of the B coefficient.

Given the possible interaction of ALAD with other trace elements (Rocha et al., 2012; Sulinskiene et al., 2015), multiple linear regression models were also tested to assess associations between Hg, Cd, Zn, Se, Cu, and Mn (all within reference levels) and *ALAD* SNPs. No statistically significant associations with (cord)blood levels were found. However, when blood Cd was included as a confounding variable in a model analyzing the associations between *ALAD* SNPs and B-Pb levels, the strength of the associations slightly increased. This may suggest that Cd exposure could (synergistically) modify the association between *ALAD* SNPs and B-Pb levels, possibly due to shared metabolic pathways.

In the Slovenian men population (HBM) (Article 3), 10 *ALAD* SNPs, besides rs1800435 also rs1139488, rs1805313, rs818708, rs2761016, rs8177812, rs2228083, rs1805312, rs8177796, and rs818684 were analyzed for their impact on B-Pb and U-Pb levels. The homozygous variant allele of rs1139488 significantly increased B-Pb concentrations, while the variant allele of rs1805312 was associated with a decrease in B-Pb levels (Fig. 4.2). Linkage disequilibrium analysis revealed only a weak co-occurrence among rs1805313, rs8177812, and rs2228083, forming four haplotypes, though none were associated with B-Pb or U-Pb levels.

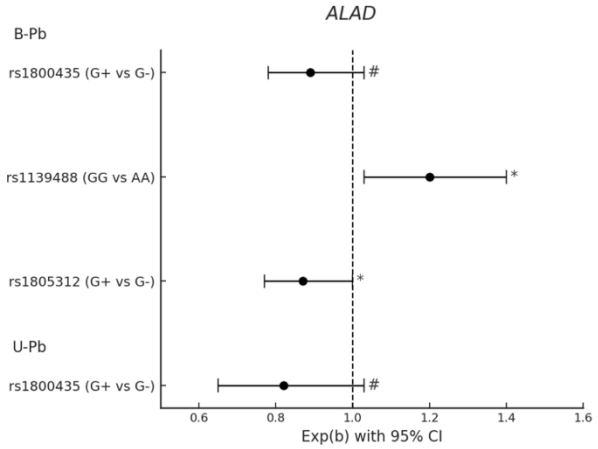


Figure 4.2: Summarized influences of significant SNPs on B-Pb and U-Pb based on the multiple linear regression models in the Slovenian men population (HBM). Presented are estimation coefficients (Exp(b)) based on genotype or allele stratification; # p <0.1, *p< 0.05; **p < 0.01; ***p < 0.001). Models were adjusted for: age, height, current smoking, alcohol consumption, source of drinking water, living in Pb-contaminated UMV area, B-Zn, Hb (for B-Pb only) and SG (for U-Pb only). B-blood; exp(b)-exponentiation of the B coefficient.

Individual SNPs in the model explained only around 1 additional percentage point of B-Pb variability beyond the baseline model without genetic factors. However, combination analyses identified six SNPs pairs that significantly influenced B-Pb levels, explaining 3–20 additional percentage points of variability.

Among the most studied SNPs besides rs1800435 are rs1805313 and rs1139488. The latter, located near rs1800435, was consistently associated with higher B-Pb levels in our studies and corroborated by other research (Pawlas et al., 2012; Shaik, 2018; Szymańska-Chabowska et al., 2015). Experimental studies suggest rs1805313 influences ALAD expression in blood cells (Warrington et al., 2015), and we confirmed a negative association between the rs1805313 variant allele and B-Pb levels, consistent with results from previous genome-wide association study (Warrington et al., 2015).

However, individual SNPs explain only a small portion of Pb variability in blood, particularly at non-occupational exposure levels (Broberg et al., 2015). This was also observed in our work, specifically in Article 3, where each SNPs explained at most an additional 1 % of Pb variability beyond the baseline model without genetic factors. Recognizing this limitation, we investigated the haplotypes and the combined effects of different SNPs, irrespective of their LD. The most significant impact was seen with the rs1139488-rs1805313 and rs1139488-rs1800435 combinations. Similarly, in the Italian population of pregnant women (PHIME), a combination of three *ALAD* SNPs (rs1800435, rs1805313, rs1139488) provided a much better R2 than individual SNPs alone.

4.2.2 Five *VDR* SNPs

In the study involving Italian pregnant women and their newborns, no associations were found between (cord)blood Pb levels and four *VDR* SNPs (*FokI*, *BsmI*, *ApaI*, *TaqI*) (Article 1). Although LD analysis revealed haplotypes involving *FokI*, *BsmI*, and *ApaI*, they were not associated with (cord)blood Pb levels.

Similarly, in a study on Slovenian men of reproductive age (Article 3) no associations were found between these SNPs, including additional SNP, BglI, and Pb concentrations in blood or urine.

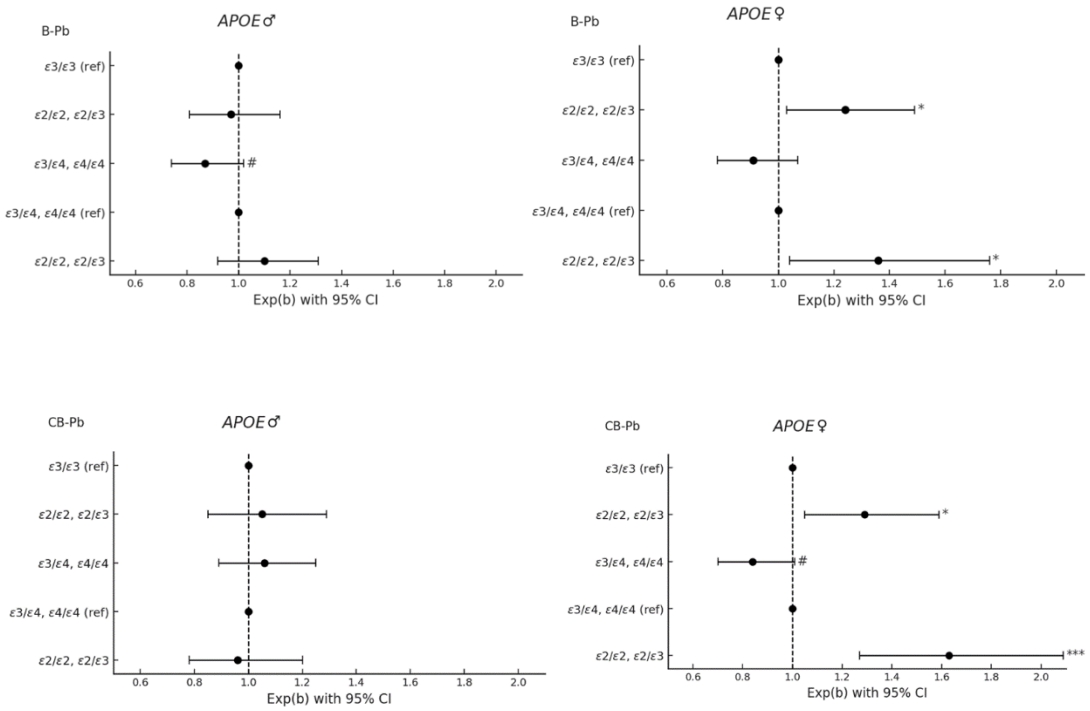
The physiological roles of vitamin D which exists in many forms (with two main forms being D2-ergocalciferol and D3-cholecalciferol), their metabolism and affinity towards vitamin D receptors are extremely complex. Both are metabolized in the liver to form (er)calcidiol, and then further converted to active form (er)calcitriol in the kidneys, however by different enzymes. Nevertheless, calcitriol is the most potent form; it binds to vitamin D receptors, activating genes that regulate Ca absorption in the intestines, which supports skeletal health and Ca balance (Janoušek et al., 2022). These processes may also influence lead (Pb) toxicokinetics. This complexity may help explain the inconsistent results in studies examining solely *VDR* SNPs and Pb blood levels (Broberg et al., 2015; Bergdahl & Skerfving, 2022), particularly in populations with low Pb exposure. Combined with the highly polymorphic nature of the *VDR* gene, these factors could account for the variability seen across studies. While some studies (Broberg et al., 2015), including ours, found no significant influence at low Pb levels, some have reported an effect, however mostly at higher Pb exposures (Broberg, 2015; Mani et al., 2019; ATSDR, 2020).

In Article 1, our research population consists of pregnant women. It has been suggested that the increase in calcitriol during pregnancy is an independent programmed response that ensures fetal mineral metabolism and skeletal development are protected from maternal vitamin D deficiency (Ryan & Kovacs, 2020). This indicates that maternal adaptations in mineral and bone metabolism during pregnancy may occur independently of sun exposure or dietary intake of vitamin D3 and its transformation into calcitriol. Nonetheless, *VDR* SNPs may still influence the formation of Ca-binding proteins and represent an important genetic factor for bone density in non-pregnant populations (ATSDR, 2020).

4.3 *APOE* Genotypes

In a study of 873 pregnant women and their 619 newborns from Italy (B-Pb GM: 11.0 ng/g, range: 3.09-60.5 ng/g; B-Hg GM: 2.16 ng/g, range: 0.05-39.6 ng/g; CB-Pb GM: 10.4 ng/g, range: 2.63-47.0 ng/g; CB-Hg: 3.88 ng/g, range: 0.12-54.8 ng/g), we investigated the influence of *APOE* genetic variations (Article 1). Firstly, we compared ε4 carriers to non-carriers, finding that the ε4 allele was associated with lower maternal B-Hg as well as CB-Hg levels. Secondly, we compared ε2 carriers to non-carriers, noting that ε2 allele was associated with higher CB-Pb levels, but only in newborn girls.

Building on that findings, we expanded the study to include participants from Croatia and Slovenia, excluding smokers (pregnant women N=817; newborns N=772; B-Pb GM: 11.1 ng/g, range: 3.58–87.6 ng/g; B-Hg GM: 2.17 ng/g, range: 0.11-39.6 ng/g; CB-Pb GM: 9.31 ng/g, range: 1.82–47.0 ng/g; CB-Hg GM: 3.05 ng/g, range: 0.12-32.8 ng/g) (Article 2). We analyzed the effects of the ε2 allele, ε4 allele, and ε3/ε3 genotype separately, and included the importance of fetal sex and parity by stratifying participants accordingly. In cases with female fetuses, the maternal ε2 allele was associated with higher, while the ε4 allele was linked to lower maternal B-Pb and B-Hg and CB-Pb levels. The maternal ε4 allele was linked to lower B-Pb levels regardless of the fetal sex. The observed associations were stronger when only including *nulliparous* women (Fig 4.3), which are generally more vulnerable due to less effective placental detoxification functioning (Prior et al., 2014). Including *ALAD* SNPs in the multiple linear regression model, despite ALAD's well-established role in Pb toxicokinetics, did not influence the associations.



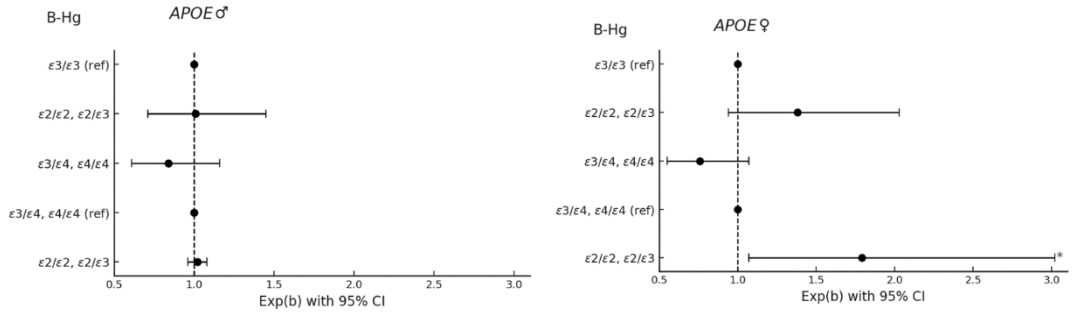


Figure 4.3: Summarized influences of *APOE* genotypes on B-Pb, CB-Pb, and B-Hg based on the multiple linear regression models in the Italian, Croatian, and Slovenian population of pregnant women and their newborns (PHIME). Firstly, comparing ε2/ε2, ε2/ε3 and ε3/ε4, ε4/ε4 genotype to ε3/ε3 (reference genotype) and secondly, comparing ε2/ε2, ε2/ε3 genotype to ε3/ε4, ε4/ε4 (reference genotype). Presented are estimation coefficients (Exp(b)) based on genotype or allele stratification; # p <0.1, *p< 0.05; **p < 0.01; ***p < 0.001). Models were adjusted in the case of B-Pb and B-Hg for: mAge, mBMI, mEducation, mSeafood consumption, mEGW, mCountry (ITA/CRO), mB-Zn-log; included only non-smoking *nulliparous* women; and in the case of CB-Pb for: mAge, mBMI, mEducation, mSeafood consumption, mCountry (ITA/CRO/SLO), CB-Zn-log, cEGA, cWeight, cLength; included only children born to *nulliparous* women. ♂-fetal/newborn sex is male; ♀-fetal/newborn sex is female; m – maternal; c-child; B – blood; CB – cord blood; exp(b) – exponentiation of the B coefficient.

In our populations of pregnant women, the ε4 allele showed a protective effect against (cord)blood Hg levels, consistent with some previously studies, including one that linked ε4 to higher Se levels in a pregnant Croatian population (PHIME study) (Trdin et al., 2020). Additionally, ε4 has been associated with higher Ca in vitamin D levels in targeted replace mice and humans (Huebbe et al., 2011). The relative mRNA levels *Lrp2* and *Gc* genes were higher in the kidneys of *APOE4* mice compared to those with *APOE3* and *APOE2* (Huebbe et al., 2011). These factors could as well explain the protective effect of ε4 on (cord)blood Pb levels observed in Article 1 and Article 2, along with the proposed influence of different *APOE* alleles on maintaining bone mass.

In the literature, the ε4 allele has often been identified as a risk factor for lower bone mineral density (BMD), but much of this research has focused on menopausal women, who experience declining estrogen levels (Niemeier et al., 2012), a key regulator of bone metabolism (Khosla & Monroe, 2018). Salamone et al. (2000) reported that the presence of at least one ε4 allele was associated with a significantly greater loss of spinal BMD only in peri- and post-menopausal women, but not in pre-menopausal women, suggesting that estrogen modifies the effect of APOE on bone. This implies that ε4 may negatively affect bone only when estrogen levels decline. In contrast, our study involves pregnant women, who are known to have increased concentrations of sex hormones (estrogen, testosterone, etc) in comparison with pre-menopausal women.

In most studies focusing on the effect of the ε4 allele versus the combined effect of the alleles (ε3+ε2), the influence of the ε2 allele may have been overlooked, potentially missing its specific effects. The ε2 allele was proposed to have the least protective effect on maintaining bone mass (Dieckmann et al., 2014; Niemeier et al., 2012). The effects of the ε2 allele on bone metabolism may be further amplified during pregnancy, a period characterized by unique and intensified Ca dynamics and fluctuations in sex hormones. As we identified the ε2 allele as a possible risk factor for higher (cord)blood Pb levels, which

follow Ca kinetics we speculate that among Pb source can also be bones. However, in our population with low Pb exposure, this effect was observed only in cases with a female fetus. This may be attributed to differences in sex hormones, as study (Kuijper et al., 2013) showed that mothers carrying female fetuses had higher levels of estrogen, progesterone, and testosterone—all of which are known to influence bone protection. Additionally, the sex differences might relate to lipid metabolism, which is more active in female fetuses. It has been shown that female newborns have higher leptin levels than males (Al Atawi et al., 2005), likely due to differences in amount of fat tissue and the heavier placental weight associated with female fetuses as the placenta produces leptin, which the majority is being released into maternal circulation (Alexe et al., 2006). During pregnancy, leptin mainly acts via the peripheral pathway, as it has been suggested that there is central leptin resistance during this period (Gustafson et al., 2019). Peripheral leptin directly acts on bone metabolism by increasing bone mass (Xu & Tianfu, 2015), which could subsequently affect Ca and Pb dynamics. However, many other metabolic pathways could influence the effect of APOE on Pb toxicokinetics.

4.4 Challenges

Studies like ours, which aimed to determine the genetic influence on the TEs levels, present unique challenges. Numerous physiological processes—such as pregnancy, vitamin D activation, sex hormones, and the amount of adipose tissue—can affect Pb kinetics, potentially obscuring or disguising genetic influences, particularly when Pb levels are low. Additionally, at lower concentrations, methodological errors or mistakes during sampling and measurement inaccuracies have a more significant impact on the precision of the results compared to when concentrations are higher. Despite these difficulties, low Pb concentrations reflect the reality of developed countries over recent decades. Moreover, external and/or internal sources of Pb exposure of participants could not be optimally estimated, so the observed associations should be interpreted cautiously.

Our studies were part of an HBM program, which involved single-time sampling. While experimental and clinical studies with more controlled conditions and multiple-time sampling, especially important during the course of pregnancy, would be more suited for researching the role of genetics in TEs kinetics, one of the major advantages of HBM studies is the large population size. This is crucial when analyzing SNPs with low MAF, such as *ALAD* rs1800435 and *APOE* (rs429358, rs7412).

Furthermore, mixed cord blood was used for TE measurements. Ideally, we should have measured hemoglobin levels, as these differ depending on the arterial/venous blood ratio. Measuring hemoglobin would help account for variations in red blood cell concentrations, where potentially toxic TEs like Pb and Hg accumulate. Such approach we applied in a recently recruited EXHES population from Celje (WR1), we have collected venous cord blood and have hemoglobin data as well, which should provide more accurate insights into exposure to potentially toxic TEs.

4.5 Future Work

We should consider that B-Pb levels in our populations may also be influenced by other SNPs, such as variations in the SLC4A7 gene. SLC4A7, an ion transporter gene highly expressed in erythrocytes, may affect Pb transport into cells. At low Pb exposure levels, various transporter proteins could play significant roles, but they are often

underrepresented in the Pb research. Addressing their impact should be accomplished in the future work.

To further validate *ALAD* SNP combinations as influential factors, it is essential to rule out the possibility of coincidental findings. These combinations could serve as more reliable susceptibility biomarkers for Pb exposure than single SNPs.

Our findings regarding the ε2 allele should be confirmed in other populations, preferably pregnant women, such as the recently recruited group in Celje, whose basic characteristics are detailed in this thesis under the JSI work report (WR1). The influence of the child's *APOE* genotype on Pb toxicokinetics should as well be analyzed. Further research on the influence of fetal sex on maternal metabolic and hormonal adaptations, including the handling of potential toxic substances, should be done.

Chapter 5

Conclusions

In our work, we successfully gathered important insight from vulnerable populations, particularly pregnant women and men of reproductive age, highlighting the genetic influence on blood TE levels, with emphasis on Pb (and Hg where applicable). The key findings include:

1. In both the pregnant populations and men of reproductive age, we confirmed the influence of the *ALAD2* allele on B-Pb levels, with carriers showing lower concentrations.
2. We confirmed that *ALAD* rs1800435 affects B-Pb levels differently depending on the level of Pb exposure (men living in historically Pb polluted environment vs non-polluted environment).
3. We identified *ALAD* SNP combinations that explained a significantly higher percentage of B-Pb variability than individual SNPs alone.
4. Our results confirm that even at low Pb exposure levels, *ALAD* SNPs—namely rs1800435, rs1805312, rs1805313 and rs1139488—and their combinations can significantly influence Pb levels.
5. We found no influence of *VDR* SNPs and their haplotypes on Pb levels in either pregnant women, newborns or male adults.
6. Maternal *APOE* ε2 allele was associated with higher, while the ε4 allele was linked to lower maternal blood Pb, blood Hg, and cord blood Pb levels, but only when the fetus/newborn was female.
7. We speculate that the significant source of increased Pb may originate form bone tissue. Our findings may support the theory that ε2 has the least protective effect on maintaining bone mass, while the ε4 exemplifies antagonistic pleiotropy—offering protective benefits in younger populations, but presumably having negative implications in later life (e.g., Alzheimer disease).
8. The important insight from our research is the significant role of fetal sex in prenatal cohorts, underscoring the need for sex-based stratification in future studies, including those investigating Pb toxicokinetics, as fetal sex also influences many maternal metabolic processes.
9. The observed effects are based on associations estimated through statistical models (adjusted linear regression analysis) and do not necessarily imply causation, which should be confirmed by mechanistic studies.

Appendix A

Working Report

A.1 Pregnant Women Characteristics and Selected Trace Element Levels (HEALS - EXHES Study; Celje Region, Slovenia, Sampling 2019 - 2023)

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IJS DP – 14630

Pregnant women Characteristics and Selected Trace Element Levels (HEALS - EXHES study; Celje region, Slovenia, sampling 2019 - 2023) - Part 1

Authors: Neža Palir, Anja Stajnko, Alja Špicar, Marta Jagodic Hudobivnik, Polona Klemenčič, Agneta Annika Runkel, Darja Mazej, Janja Snoj Tratnik, Jakob Koren, Milena Horvat, Ingrid Falnoga

Ljubljana, 28. 02. 2024

1. SLOVENIAN HEALS - EXHES STUDY POPULATION

The study conducted in Slovenia was part of the HEALS (Health and Environment-wide Associations based on Large population Surveys) project, more precisely the HEALS sub-study EXHES (Exposure and Health Examination Survey) covering ten EU states. The Slovenian EHXES study encompassed a cohort of 227 expecting mothers along with their newborn, sampled between May 2019 and May 2023. Collaborating with the Maternity and Gynecological Department at the General Hospital of Celje, we aimed to gain insights into various aspects of maternal and neonatal health associations with potential environmental/diet chemical exposure. The participants, aged between 18 and 46, were residents of Celje or its vicinity. Among them seven were expecting twins, while the remaining participants were carrying singleton babies.

2. DATA AND SAMPLE COLLECTION

Within the study, sampling was conducted at four intervals. Firstly, during the third trimester of pregnancy, a crucial period presumed to have important vulnerability for the observed outcome. Secondly, during labor; thirdly, a few days post-delivery; and finally, 1-23 weeks after childbirth. Comprehensive questionnaires were administered at these four time points, collecting information on maternal personal characteristics, socio-economic status (SES), lifestyle, frequency of food consumption, and general health status, and newborns' characteristics.

Specifically, samples presented in this report were collected during and after pregnancy. Maternal venous blood, plasma, and spot urine were obtained during the third trimester of pregnancy. Maternal blood was withdrawn using EDTA vacutainers, while urine was collected in PP containers. After aliquoting, sub-samples of whole blood, plasma, and urine were promptly stored at -80 °C. Hair samples were collected during home visits at 3-23 weeks postpartum. Using clean scissors, a hair strand of approximately 0,5 cm of diameter was cut as close as possible to the scalp, at the back of the head, close to the neck.

3. Hg, Pb, Cd, As, Zn, Se, AND Cu ANALYSIS IN MATERNAL BLOOD, PLASMA, URINE, AND HAIR

The following chemicals were used for the preparation of both the reference materials (RMs) and human samples: deionized water (18.2MΩ cm) obtained using a Milli-Q System (Merck, Millipore, Watertown, MA, USA), nitric acid (HNO3, 65% Suprapur® for trace analysis, Supelco®). For calibration, both multi-element and single-element standards were prepared. These included Hg NIST 3133 and Periodic table mix 1 for ICP (TraceCERT®, Sigma-Aldrich, 33 elements) for Pb, Cd, As, Zn, Se, and Cu.

Approximately 0.3 g of whole blood/ plasma sample or 0.5 mL of urine sample was transferred into pre-cleaned Teflon digestion vials. Samples were digested using 0.5 mL of 65% nitric acid (Suprapur) in a microwave system (ULTRAWAVE, Single Reaction Chamber Microwave Digestion System, MILESTONE, Italy) following this program: 1) 20 minutes temperature rise to 240°C, 2) kept for 15 minutes at 240°C and max 100 bar). Digested solutions were transferred into measuring tubes and diluted to 5 mL. For digestion of hair samples, approximately 0.02 g of the sample was taken and mixed with 1 mL of 65% HNO3. After digestion, hair samples were diluted to 10 mL. Blank samples and RMs were run in the same batch as the samples to ensure the quality of the results.

Determination of selected elements (Hg, Pb, Cd, As, Zn, Se, and Cu) was performed using an Agilent 8800 triple quadrupole inductively coupled plasma mass spectrometer (QQQ-ICP-MS Agilent, USA). The isotopes monitored in different modes were: $^{63->63}$Cu[He], $^{66->66}$Zn[He], $^{75->91}$AsO[O2], $^{78->94}$SeO[O2], $^{111->111}$Cd[He], $^{202->202}$Hg[He] and $^{208->208}$Pb[He]. Internal standards (Y, Rh, Sc, and Gd) were added online. External calibration was used for quantification.

The accuracy of results was checked using reference materials: Seronorm serum Level 1 and 2 (SERO AS); for plasma samples; Seronorm whole blood Level 1 and 2 (SERO AS) for whole blood samples; Seronorm urine Level 1 and 2 (SERO AS) and ClinChek Level 1 (RECIPE) for urine samples; IAEA 086 Human Hair and NIES No. 13 Human Hair for hair samples. Limits of detection (LOD) were calculated as three times the standard deviation of the blank samples. In order to ensure the quality of the results, our laboratory also successfully participated in interlaboratory testing scheme the G-EQUAS i (The German External Quality Assessment Scheme) for urine and whole blood matrices. Results obtained for reference materials and LODs are presented in **Tables 1-4**.

Table 1: LODs and results obtained for reference materials for plasma samples

	P-Zn	P-Se	P-Cu
LOD (ng/g)	25	0.2	0.6
Name	Seronorm Serum L-1; lot: 1801802		
Reference value (ng/g)	1460	95	1128
Reference range (ng/g)	1160-1750	76-114	901-1355
Average of measured values (N=93) (ng/g)	1680	101	1150
rsd (%) of measured values	4.03	0.36	1.99
Name	Seronorm Serum L-2; lot: 1801803		
Reference value (ng/g)	2090	139	2175
Reference range (ng/g)	1670-2510	111-167	1737-2613
Average of measured values (N=80) (ng/g)	2277	149	2139
rsd (%) of measured values	6.64	1.30	0.24

LOD – limit of detection.

Table 2: LODs and results obtained for reference materials for blood samples

	B-Hg	B-Pb	B-Cd
LOD (ng/g)	0.04	0.10	0.01
Name	Seronorm WB L1; Lot: 2011920		
Reference value (ng/g)	1.63	10.3	0.29
Reference range (ng/g)	1,30-1,96	8,2-12,4	0,23-0,35
Average of measured values (N=39) (ng/g)	1.6	9.9	0.3
rsd (%) of measured values	12	6	6
Name	Seronorm WB L2; Lot: 2011921		
Reference value (ng/g)	15.0	295.0	5.0
Reference range (ng/g)	12,0-18,0	264-325	4,0-6,0
Average of measured values (N=40) (ng/g)	15.1	268.4	5.3
rsd (%) of measured values	9	2	2

LOD – limit of detection.

Table 3: LODs and results obtained for reference materials for urine samples

	U-Hg	U-Cd	U-As
LOD (ng/g)	0.04	0.02	0.06
Name	Seronorm Urine L-1; lot: 1706877		
Reference value (ng/g)	1.110	0.062	97
Reference range (ng/g)	0.88-1.33	0.049-0.074	77-116
Average of measured values (N=16) (ng/g)	1.33	0.083	88
rsd (%) of measured values	13.8	20.8	12.1
Name	Seronorm Urine L-2; lot: 1706878		
Reference value (ng/g)	41.5	4.7	198
Reference range (ng/g)	35.2-52.9	4,1-6,1	209-314
Average of measured values (N=30) (ng/g)	38.3	5.28	191
rsd (%) of measured values	9.1	4.0	5.9
Name	Clincheck; lot: 8847		
Reference value (ng/g)	1.92	2.56	17
Reference range (ng/g)	1.41-3.28	1.98-2.96	34.8-52.2
Average of measured values (N=22) (ng/g)	2.01	2.64	15.9
rsd (%) of measured values	5.6	3.7	9.7

LOD – limit of detection.

Table 4: LODs and results obtained for reference materials for hair samples

	H-Hg
LOD (ng/g)	3
Name	IAEA - 086
Reference value (ng/g)	573
Reference range (ng/g)	534-612
Average of measured values (N=47) (ng/g)	497
rsd (%) of measured values	12.6
Name	NIES No. 13

Reference value (ng/g)	4420.0
Reference range (ng/g)	4220-4620
Average of measured values (N=29) (ng/g)	4000
rsd (%) of measured values	6.4

4. RESULTS

In this report, we present a basic overview of the study participants' characteristics, including both maternal and newborns data (**Table 5**). The maternal characteristics include age, pre-pregnancy BMI, weight gain during pregnancy, parity, education, current smoking status, number of amalgam fillings, folic acid usage, private water supply, employment status, and estimated gestation week (EGW) at blood and urine sampling. Newborn characteristics include estimated gestational age at birth (EGA), birth weight, length, head circumference, type of delivery, and sex.

Additionally, we present data on selected maternal trace element levels which were included into participants' first report. Namely: i) blood concentrations of lead (Pb), mercury (Hg), and cadmium (Cd); ii) maternal urine concentrations of Hg, Cd, and arsenic (As); iii) maternal hair concentrations of Hg; and iv) maternal plasma concentrations of zinc (Zn), selenium (Se), and copper (Cu). The dataset for singleton pregnancies is further stratified based on the fetus's sex (**Table 6**).

Due to the limited number of mothers pregnant with twins and the complexities in stratifying for sex in twin pregnancies with different sexes, their data are presented separately without fetus sex stratification (**Table 7and 8**).

Table 5: Singleton mothers and newborns general characteristics – all and stratified by fetal/newborn sex.

	AM ± SD (min-max)	**N (%)**	**AM ± SD (min-max) ♂**	**N (%) ♂**	**AM ± SD (min-max) ♀**	**N (%) ♀**	**p**
MOTHERS(m)		220					
mAge (years)	31.0 ± 4.9 (18-46)	220	31.8 ± 5.0 (21-43)	102	30.4 ± 4.7 (22-46)	112	**0.044**
mPre-pregnancy BMI (kg/m²)	24.7 ± 4.9 (17.4-45.3)	220 (100)	24.1 ± 4.4 (17.4-38.5)	102 (100)	25.0 ± 5.1 (17.9-45.3)	112 (100)	0.185
Underweight (< 18.5)		8 (3.64)		4 (3.92)		4 (3.57)	
Normal (18.5 – < 25)		125 (56.8)		64 (62.7)		61 (54.5)	
Overweight		60 (27.3)		25 (24.5)		35 (31.3)	

(25 – < 30)							
Obesity (30 – < 40)		25 (11.4)		9 (8.82)		16 (14.3)	
Severe obesity (≥ 40)		2 (0.91)		0 (0)		2 (1.79)	
mWeight gain	13.8 ± 6.67 (-25-35)	212	15.1 ± 5.95 (0-35)	101	12.6 ± 7.08 (-25-27)	111	**0.033**
mParity	0.45 ± 0.68 (0-4)	220 (100)	0.53 ± 0.74 (0-3)	102 (100)	0.41 ± 0.64 (0-4)	112 (100)	0.332
nulliparous		140 (63.6)		62 (60.8)		72 (64.3)	
primiparous-1		63 (28.6)		27 (26.5)		36 (32.1)	
multiparous-2		15 (6.82)		12 (11.8)		3 (2.68)	
multiparous-3		1 (0.45)		1 (0.98)		0 (0)	
multiparous-4		1 (0.45)		0 (0)		1 (0.89)	
mEducation		212 (100)		100 (100)		112 (100)	0.647
primary school		2 (0.94)		1 (1.00)		1 (0.89)	
secondary vocational education		15 (7.08)		10 (10.0)		5 (4.46)	
high school		37 (17.5)		16 (16.0)		21 (18.8)	
vocational college degree		24 (11.3)		9 (9.00)		15 (13.4)	
university degree		73 (34.4)		31 (31.0)		42 (37.5)	
masters or doctorate		61 (28.8)		33 (33.0)		28 (25.0)	
mCurrent Smoking (>5 cigarettes)		220 (100)		102 (100)		112 (100)	0.346
yes		4 (1.82)		2 (1.96)		2 (1.79)	
no		216 (98.2)		100 (98.0)		110 (98.2)	
mAmalgam fillings		213 (100)		101 (100)		112 (100)	0.140
0		91 (42.7)		49 (48.5)		42 (37.5)	
1-3		73 (34.3)		33 (32.7)		40 (35.7)	
4-7		41 (19.2)		16 (15.8)		25 (22.3)	
8-11		8 (3.74)		3 (2.97)		5 (4.46)	
mFolic acid		220 (100)		102 (100)		112 (100)	0.442
yes		208 (94.6)		98 (96.1)		105 (93.8)	
no		12 (5.45)		4 (3.92)		7 (6.25)	
mUsage of private water supply		213 (100)		101 (100)		112 (100)	0.471
yes		18 (8.45)		10 (9.90)		8 (7.14)	
no		195 (91.6)		91 (90.1)		104 (92.9)	
mEmployment status		212 (100)		101 (100)		111 (100)	0.574
employed full-time		184 (86.8)		89 (88.1)		95 (85.6)	
employed reduced time		3 (1.42)		0 (0)		3 (2.70)	
unemployed		19 (8.96)		11 (10.9)		8 (7.21)	
student		6 (2.83)		1 (0.99)		5 (4.50)	
mEGW (weeks)	35.1 ± 1.92 (30-40)	220	35.5 ± 1.99 (30-40)	102	34.7 ± 1.84 (30-39)	112	**0.004**
cEGA (weeks)	39.2 ± 1.17 (35-41)	215 (100)	39.2 ± 1.16 (36-41)	102 (100)	39.2 ± 1.18 (35-41)	112 (100)	0.770
Pre-term (< 37)		5 (2.33)		3 (2.94)		2 (1.79)	
Full term (37–42)		210 (97.7)		99 97.1)		110 (98.2)	
Post-term (> 42)		0 (0)		0 (0)		0 (0)	
cBirth weight (g)	3450 ± 413 (2240-4620)	213	3538 ± 453 (2240-4620)	102	3369 ± 356 (2560-4270)	111	**0.000**
cBirth length (cm)	51.0 ± 1.95 (45-57)	213	51.5 ± 1.88 (46-57)	102	50.5 ± 1.89 (45-55)	111	**0.000**
cBirth head circumference (cm)	34.9 ± 1.26 (31.5-39)	208	35.2 ± 1.42 (31.5-39)	100	34.5 ± 0.97 (32-36.5)	108	**0.000**
cType of delivery		212 (100)		101 (100)		111 (100)	0.659
Vaginal		150 (70.8)		70 (69.3)		80 (72.1)	
Cesarean		62 (29.3)		31 (30.7)		31 (27.9)	

AM – arithmetic mean; SD – standard deviation; MIN – minimum; MAX – maximum; N – number of observations; m – maternal; c – child; ♂ – male fetal sex; ♀ – female fetal sex; p – values indicate statistically significant difference between the fetal sexes; EGW – estimated gestation week of pregnancy at maternal blood sampling; EGA – estimated gestational age at delivery.

Table 6: Singleton mothers trace element concentrations stratified by fetal/newborn sex.

MOTHERS	GM (95%CI) (MIN-MAX)	N	GM (95%CI) (MIN-MAX) ♂	N ♂	GM (95%CI) (MIN-MAX) ♀	N ♀	p
B-Pb (ng/g)	7.28 (6.88, 7.71) (2.81, 47.5)	219	7.56 (6.89, 8.29) (2.81, 47.8)	101	7.03 (6.53, 7.57) (3.30, 20.0)	112	0.231
B-Hg (ng/g)	1.00 (0.91, 1.10) (0.10, 12.6)	219	1.00 (0.85, 1.17) (0.10, 12.6)	101	1.01 (0.90, 1.12) (0.10, 3.89)	112	0.926
B-Cd (ng/g)	0.29 (0.27, 0.31) (0.09, 2.00)	219	0.29 (0.26, 0.33) (0.09, 1.47)	101	0.29 (0.26, 0.32) (0.10, 2.00)	112	0.833
U-Hg (ng/g)	0.53 (0.48, 0.57) (0.10, 4.16)	219	0.48 (0.42, 0.55) (0.10, 4.16)	101	0.57 (0.51, 0.64) (0.13, 3.50)	112	**0.031**
U-Cd (ng/g)	0.19 (0.17, 0.20) (0.03, 1.71)	219	0.19 (0.17, 0.21) (0.05, 1.71)	101	0.19 (0.17, 0.21) (0.03, 0.54)	112	0.833
U-As (ng/g)	4.92 (4.30, 5.62) (0.62, 170)	219	4.60 (3.81, 5.54) (0.62, 51.8)	101	5.26 (4.31, 6.43) (0.81, 171)	112	0.456
H-Hg (ng/g)	190 (171, 212) (19.3, 1835)	199	186 (158, 220) (19.3, 1835)	98	195 (167, 114) (24.8, 1051)	101	0.533
P-Zn (µg/g)	0.71 (0.70, 0.73) (0.37, 1.71)	219	0.71 (0.69, 0.74) (0.37, 1.16)	101	0.71 (0.69, 0.73) (0.42, 1.46)	112	0.838
P-Se (ng/g)	77.7 (76.0, 79.5) (52.3, 136)	219	80.0 (77.4, 82.6) (54.7, 126)	101	76.5 (74.2, 79.0) (52.3, 136)	112	**0.046**
P-Cu (µg/g)	2.10 (2.06, 2.14) (1.44, 2.99)	219	2.07 (2.01, 2.13) (1.44, 2.86)	101	2.13 (2.08, 2.19) (1.62, 2.99)	112	0.270

GM – geometric mean; CI – confidence interval; MIN – minimum; MAX – maximum; N – number of observations; ♂ – male fetal sex; ♀ – female fetal sex; p – values indicate statistically significant difference between the fetal sexes; B – blood; U – urine; H – hair; P – plasma.

Table 7: Twin mothers and newborns general characteristics.

	AM ± SD (min-max) (Twin1)	**N (%)**
MOTHERS(m)		7
mAge (years)	32.6 ± 2.23 (29-35)	7
mPre-pregnancy BMI (kg/m^2)	22.4 ± 2.19 (19.7-25.6)	7 (100)
Underweight (< 18.5)		0 (0)
Normal (18.5 – < 25)		6 (85.7)
Overweight (25 – < 30)		1 (14.3)
Obesity (30 – < 40)		0 (0)
Severe obesity (≥ 40)		0 (0)
mWeight gain	17.0 ± 9.61 (5-34)	7
mParity	0.57 ± 0.79 (0-2)	7 (100)
nulliparous		4 (57.1)
primiparous-1		2 (28.6)
multiparous-2		1 (14.3)
multiparous-3		0 (0)
multiparous-4		0 (0)
mEducation		5 (100)
primary school		0 (0)
secondary vocational education		0 (0)
high school		3 (60)

vocational college degree		1 (20)
university degree		0 (0)
masters or doctorate		1 (20)
mCurrent Smoking (>5 cigarettes)		7 (0)
yes		0 (0)
no		7 (100)
mAmalgam fillings		5 (100)
0		2 (40)
1-3		1 (20)
4-7		2 (40)
8-11		0 (0)
mFolic acid		7 (100)
yes		7 (100)
no		0 (0)
mUsage of private water supply		5 (100)
yes		0 (0)
no		5 (100)
mEmployment status		5 (100)
employed full-time		4 (80)
employed reduced time		0 (0)
unemployed		1 (20)
student		0 (0)
mEGW (weeks)	34.6 ± 1.62 (21-36)	7
cEGA (weeks)	37.0 ± 1.15 (35-38)	14 (100)
Pre-term (< 37)		4 (28.6)
Full term (37–42)		10 (71.4)
Post-term (> 42)		0 (0)
cSex		14 (100)
Boys		5 (35.7)
Girls		9 (64.3)
cBirth weight (g)	2651 ± 238 (2230-3060)	12
cBirth length (cm)	48.0 ± 1.60 (45-50)	12
cBirth head circumference (cm)	33.8 ± 1.25 (32-36)	12
cType of delivery		12 (100)
Vaginal		2 (16.7)
Cesarean		10 (83.3)

AM – arithmetic mean; SD – standard deviation; MIN – minimum; MAX – maximum; N – number of observations; m – maternal; c – child; EGW – estimated gestation week of pregnancy at maternal blood sampling; EGA – estimated gestational age at delivery.

Table 8: Twin mothers trace element concentrations.

MOTHERS	GM (95%CI) (MIN-MAX)	N
B-Pb (ng/g)	9.38 (7.03, 12.5) (5.02-13.3)	7
B-Hg (ng/g)	0.57 (0.46, 0.71) (0.40- 0.76)	7
B-Cd (ng/g)	0.51 (0.31, 0.84) (0.33-1.57)	7
U-Hg (ng/g)	0.49 (0.21, 1.13) (0.18-3.05)	7
U-Cd (ng/g)	0.43 (0.31, 0.60) (0.28-0.83)	7
U-As (ng/g)	6.07 (3.48, 10.6) (2.72-15.8)	7

H-Hg (ng/g)	104 (47.7, 228) (39.9-189)	5
P-Zn (µg/g)	0.76 (0.63, 0.90) (0.65-1.11)	7
P-Se (ng/g)	58.9 (45.2, 76.8) (35.9-86.7)	7
P-Cu (µg/g)	2.10 (1.77, 2.51) (1.68-2.82)	7

GM – geometric mean; CI – confidence interval; MIN – minimum; MAX – maximum; N – number of observations; B – blood; U – urine; H – hair; P – plasma.

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Working Report

Palir, Neža, Stajnko, Anja, Špicar, Alja, Jagodic Hudobivnik, Marta, Klemenčič, Polona, Runkel, Agneta Annika, Mazej, Darja, Snoj Tratnik, Janja, Koren, Jakob, Horvat, Milena, Falnoga, Ingrid. Pregnant women characteristics and selected trace element levels (HEALS - EXHES study; Celje region, Slovenia, sampling 2019 - 2023): part 1. Ljubljana: Institut "Jožef Stefan", 2024. 1 online resource (1 PDF file (9 pages)), illustrated. IJS Working Report, 14630. http://library.ijs.si/. [COBISS.SI-ID 187210755]

Interview

Hudopisk, Neda (interviewee), Horvat, Milena (interviewee), Palir, Neža (interviewee), Snoj Tratnik, Janja (interviewee), Kosjek, Tina (interviewee), White, Iain R. (interviewee). Kako kemikalije v našem okolju vplivajo na naše zdravje? MMC RTV SLO: Prvi multimedijski portal. 4 Jan. 2024. Online source. ISSN 1581-372X. https://www.rtvslo.si/znanost-in-tehnologija/kako-kemikalije-v-nasem-okolju-vplivajo-na-nase-zdravje/693637. [COBISS.SI-ID 180795395]

Biography

The author of this thesis, Neža Palir, was born on December 27th, 1989 in Celje, Slovenia. She finished primary school in Hruševec, Slovenia, and high school at Gymnasium Celje-Center, Celje, Slovenia. In 2008, she enrolled in the undergraduate studies "Laboratory Biomedicine" at the Faculty of Pharmacy, University of Ljubljana and received a Bachelor's degree in 2011. She continued her master's studies in the same field. She completed her final year at the Faculty of Pharmacy, University of Lisbon, where she conducted research for her master's thesis entitled *Characteristics of Newly Discovered Mutations Related to Medium-Chain Acylcoenzyme A Dehydrogenase Deficiency.* Before graduating with a Master's degree in Laboratory Biomedicine in 2015, she completed an Erasmus internship at the Academic Medical Center Amsterdam in the field of metabolism and genetics.

In 2019, she enrolled in the PhD program "Ecotechnologies," at the Jožef Stefan Postgraduate School, Ljubljana, Slovenia. She worked on projects related to human exposure to environmental chemicals. Her work also included project presentations, volunteer recruitment, close collaboration with medical staff, human sample collection, sample preparation for analysis, DNA extraction, genotyping, statistical analysis, and result presentation. She also completed a three-month MerFish secondment at the University of São Paulo in Ribeirão Preto, Brazil, which included a one-month boat journey to the Amazon region.