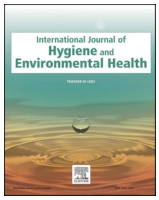
International Journal of Hygiene and Environmental Health 256 (2024) 114315

Contents lists available at ScienceDirect

International Journal of Hygiene and Environmental Health

journal homepage: www.elsevier.com/locate/ijheh



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

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ARTICLE INFO

Keywords:
Lead
Single nucleotide polymorphism
ALAD
Haplotype
Men
Blood

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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https://doi.org/10.1016/j.ijheh.2023.114315
Received 30 August 2023; Received in revised form 20 December 2023; Accepted 21 December 2023
Available online 1 January 2024

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

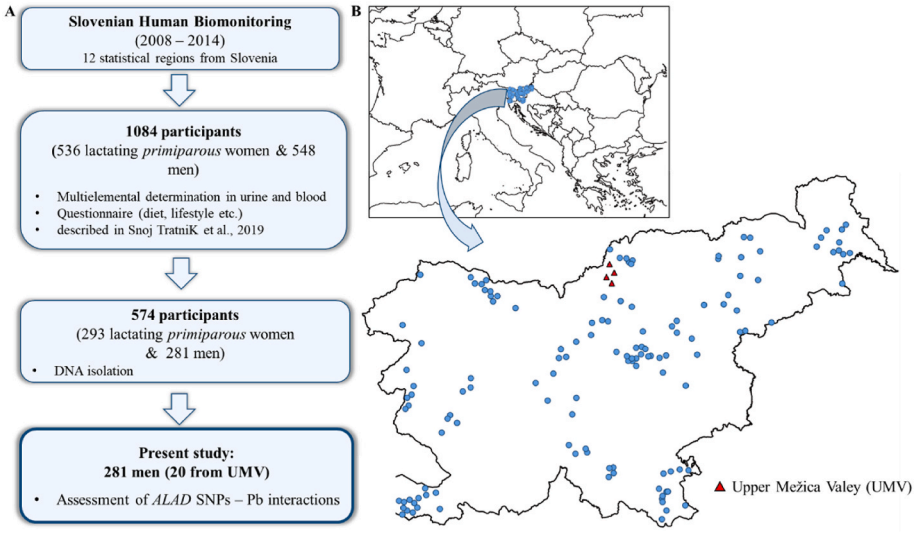


Fig. 1. Study population selection (A) and sampling areas (settlements of study participants' residencies) including Pb polluted areas (marked with a red triangle) (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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).

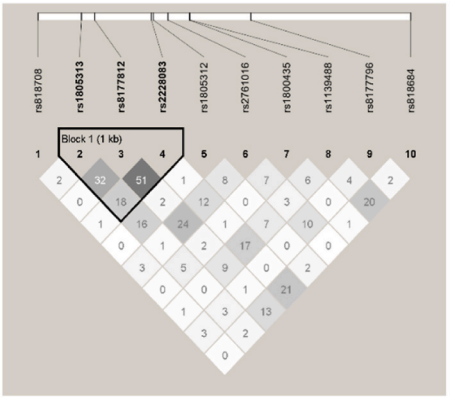
In contrast, variant homozygotes (GG) of rs1139488 exhibited significantly higher BPb concentrations compared to heterozygotes (GC) or common homozygous (AA). This was evident from the group comparisons (GM: 24.1, 18.7, and 19.0 μg/L, respectively; $p = 0.009$; Fig. 3) and after the adjustment for possible 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.)

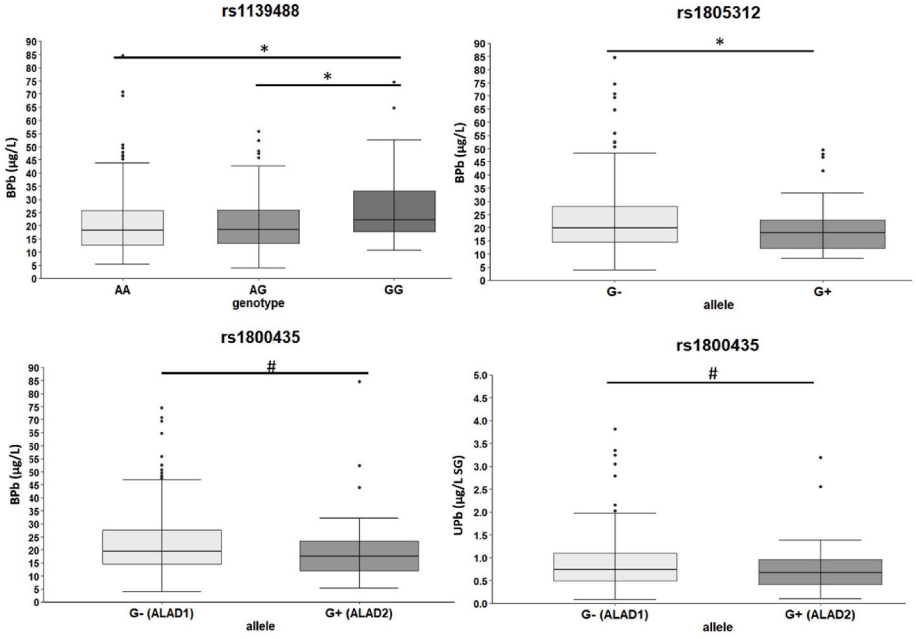


Fig. 3. Distribution of BPb (µg/L) based on the rs1139488 (A > G) genotypes, and presence or absence of variant alleles of rs1800435 (C > G, ALAD1>ALAD2), rs1805312 (C > G)and of UPb (µg/L SG) based on the presence or absence of variant alleles of rs1800435 (C > G, ALAD1>ALAD2) (*p < 0.05 and #p < 0.1).

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; ΔR^2 = 20) and for the rs1800435-rs1139488 combination (G+(ALAD2)-AA vs CC(ALAD1/1)-GG; ΔR^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.

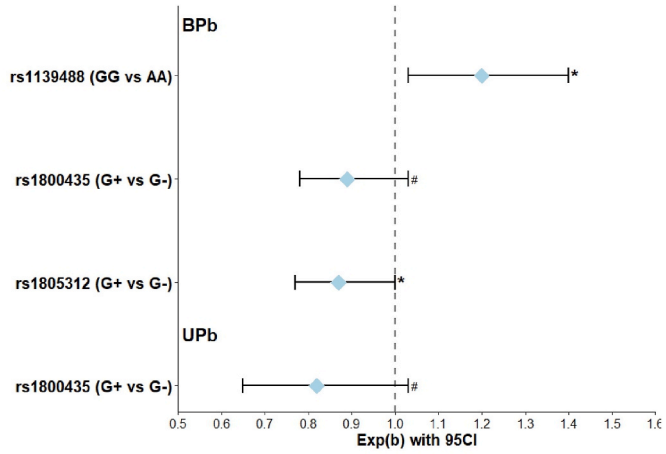


Fig. 4. Summarized influences of significant SNPs on BPb (μg/L) and UPb (μg/L) based on the linear regression models. Presented are estimation coefficients (Exp(b)) based on genotype stratification (variant homozygotes vs. common homozygotes; for rs1139488) or allele stratification (presence of variant 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.

Funding

The work was supported by the National Human Biomonitoring program financed by the Chemicals Office of the Republic of Slovenia (Contracts numbers C2715–07Y000042, C2715-11-634801, C2715-14-634801, C2715-11- 000005, C2715-18-634803, and C2715-19-634801), and the programme funded by the Slovenian Research Agency (research programme P1-0143).

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.

Acknowledgements

The authors would like to acknowledge men who participated in this study and the personnel of the Regional Health Centres, who collaborated in recruiting participants and collecting biological samples. We also acknowledge Prof. Dr. Joško Osredkar, Dr. Marija Prezelj, and the late Mladen Kersnik from the University Medical Centre Ljubljana, as well as Dr. Marta Jagodic Hudobivnik and Dr. David Kocman from the Jozef Stefan Institute, Ljubljana, for their contributions to the study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114315.

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