CONCENTRAÇÕES DE METAIS NO SANGUE HUMANO: ASSOCIAÇÕES COM PADRÕES ALIMENTARES, FATORES SOCIOAMBIENTAIS E DE RISCO

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UNIVERSIDADE ESTADUAL DO NORTE FLUMINENSE DARCY RIBEIRO - UENF

CAMPOS DOS GOYTACAZES – RJ
JULHO 2022

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Tese apresentada ao Centro de Biociências e Biotecnologia da Universidade Estadual do Norte Fluminense Darcy Ribeiro como parte das exigências para a obtenção do título de Doutor em Ecologia e Recursos Naturais.

Orientador: Prof. Dr. Carlos Eduardo de Rezende

Coorientadora: Profª. Drª. Cristiane dos Santos Vergilio

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Governo do Estado do Rio de Janeiro

Universidade Estadual do Norte Fluminense Darcy Ribeiro

Pró-Reitoria de Pesquisa e Pós-Graduação

DECLARAÇÃO

Eu, Marina Satika Suzuki, coordenadora do Programa de Pós-Graduação em Ecologia e Recursos Naturais (PPG-ERN) da Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), seguindo a Resolução CPPG nº2 de 2021, declaro validadas as assinaturas constantes da Folha de Assinaturas da Tese intitulada "**Concentrações de Metais no Sangue Humano: Associações com Padrões Alimentares, Fatores Socioambientais e de Risco"** de autoria de Diego Lacerda de Souza, defendida no dia 22 de julho de 2022.

Campos dos Goytacazes, 03 de novembro de 2022

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DEDICATÓRIA

Dedico aos meus amigos, mestres e familiares.

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LISTA DE ABREVIATURAS

Abreviatura		Descrição
Al	-	Alumínio/Aluminum
ANCOVA	-	Análise de covariância/Analysis of covariance
ANOVA	-	Análise de variância/Analysis of variance
BMI	-	Índice de massa corporal/Body mass index
C	-	Carbono/Carbon
Ca	-	Cálcio/Calcium
CAPES	-	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
Cd	-	Cádmio/Cadmium
Ce	-	Cério/Cerium
Co	-	Cobalto/Cobalt
Cu	-	Cobre/Copper
Dy	-	Disprósio/Dysprosium
EDTA	-	Ácido etilenodiamino tetra-acético/Ethylenediaminetetraacetic acid
Er	-	Érbio/Erbium
Eu	-	Európio/Europium
FAPERJ	-	A Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro
FAPES	-	Fundação de Amparo à Pesquisa e Inovação do Espírito Santo
Fe	-	Ferro/Iron
Gd	-	Gadolínio/Gadolinium
GOT	-	Transaminase glutâmico oxalacética/Glutamic-oxaloacetic transaminase
GPT	-	Transaminase glutâmico pirúvica/Glutamate-pyruvate transaminase
HDL-C	-	Lipoproteínas de alta densidade/High-density lipoprotein cholesterol
Hg	-	Mercúrio/Mercury
Ho	-	Hólmio/Holmio
IDA	-	Iron deficiency anemia
IFCC	-	Federação Internacional de Química Clínica e Medicina Laboratorial/International Federation of Clinical Chemistry and Laboratory Medicine
IUPAC	-	União Internacional de Química Pura e Aplicada/International of Pure and Applied Chemistry
La	-	Lantânio/Lanthanum
LDL-C	-	Lipoproteínas de baixa densidade/Low-density lipoprotein cholesterol
Lu	-	Lutécio/Lutetium
Mg	-	Magnésio/Magnesium
N	-	Nitrogênio/Nitrogen

Abreviatura		Descrição
Nd	-	Neodímio/Neodymium
Ni	-	Níquel/Nickel
NO	-	Óxidos de nitrogênio/Nitrogen oxides
P	-	Fósforo/Phosphorus
Pb	-	Chumbo/Lead
Pr	-	Praseodímio/Praseodymium
PUC-Rio	-	Pontifícia Universidade Católica do Rio de Janeiro
ROS	-	Espécies reativas de oxigênio/Reactive oxygen species
RV	-	Valores de referência/Reference values
S	-	Enxofre/Sulfur
Sc	-	Escândio/Scandium
Se	-	Selênio/Selenium
Sm	-	Samário/Samarium
Tb	-	Térbio/Terbium
Tm	-	Túlio/Thulium
UENF	-	Universidade Estadual do Norte Fluminense Darcy Ribeiro
UFES	-	Universidade Federal do Espírito Santo
USA	-	Estados Unidos da América/United States of America
US-ATSDR	-	Agência de Substâncias Tóxicas e Registro de Doenças/Agency for Toxic Substances and Disease Registry
US-CDC	-	Centro de Prevenção e Controle de Doenças dos Estados Unidos/United States Centers for Disease Control and Prevention
US-EPA	-	Agência de Proteção Ambiental dos Estados Unidos/United States Environmental Protection Agency
WHO	-	Organização Mundial da Saúde/World Health Organization
Yb	-	Itérbio/Ytterbium
Zn	-	Zinco/Zinc

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RESUMO

A avaliação da exposição humana aos metais e sua toxicidade representa um desafio visto a grande complexidade do organismo, dos metais em si, e da dificuldade em se obter relações de causa e efeito, uma vez que a exposição a diferentes elementos é concomitante de maneira geral. Assim, o monitoramento dos níveis de exposição destas substâncias, a avaliação dos fatores que aumentam essa exposição e os possíveis efeitos à saúde é fundamental para a compreensão da dinâmica desses elementos. Desta forma, esta tese teve como objetivo avaliar a variação nas concentrações de quatro elementos, são eles Ni, Co, Cd e Pb em uma população de agricultores residentes na microrregião do Caparaó Capixaba, sudoeste do Espírito Santo. Como a alimentação é a principal fonte de elementos químicos para o organismo, o primeiro capítulo avalia a aplicabilidade dos isótopos estáveis de carbono e nitrogênio no sangue dos agricultores como ferramenta para a avaliação de padrões alimentares. Os dados mostraram que a composição isotópica de ambos os elementos representa a frequência de consumo de diferentes fontes de proteínas, podendo ser utilizados como traçadores de preferências alimentares desta população. A partir daí, no segundo capítulo, as concentrações dos metais foram associadas com as assinaturas isotópicas, com variáveis sócio geográficas, com fatores de risco, como o consumo de álcool e de tabaco, além da relação com indicadores de saúde (antropométricos e do sangue). Os quatro elementos avaliados foram positivamente associados com os valores de $\delta^{13}C$ e $\delta^{15}N$ refletindo o efeito do maior consumo de carne na concentração destes metais. Também foi observado o efeito da idade no aumento das concentrações de Pb na população geral, enquanto o índice de massa corporal foi associado negativamente com Ni, Co e Cd em mulheres. Maiores concentrações para todos os elementos em fumantes foram observadas, os níveis de Cd foram estatisticamente maiores, apresentando um aumento mediano de cerca de 60% neste grupo. A frequência do consumo de álcool provocou aumento apenas das concentrações de Pb. Diferenças entre os gêneros também foram observadas para o Pb, sendo que os homens apresentaram concentrações medianas 46% maiores. Foram observadas ainda, diferentes associações de metais com parâmetros sanguíneos, incluindo associações com variáveis do hemograma, indicadores de funcionalidade hepática, glicose, vitamina D e pressão arterial, que apontam possíveis efeitos tóxicos e outros fatores que influenciam a dinâmica dos metais. A população avaliada apresentou concentrações elevadas para todos os elementos, com níveis elevados variando de 2 a 31 vezes maiores em comparação com valores de referência de outros estudos para Pb e Cd, respectivamente. Por fim, no terceiro capítulo, foi conduzida uma revisão sistemática sobre as concentrações de Pb no sangue humano em uma escala global. Os dados mostraram uma tendência consistente de diminuição exponencial das concentrações de Pb no sangue em todos os continentes em função da remoção da utilização do Pb tetraetila como aditivo da gasolina, resultando em uma tendência global ao longo dos anos. Além disso, os resultados também mostraram que, apesar de a remoção do aditivo proporcionar menores níveis de exposição, a população em geral continua exposta ao elemento através de fontes ambientais, ocupacionais e através de hábitos de vida como o tabagismo e o consumo de álcool.

Palavras chave: Isótopos estáveis, metais, exposição humana, chumbo.

ABSTRACT

The assessment of human exposure to metals and their toxicity remains a challenge, given the great complexity of the organism, the metals themselves, and the difficulty in obtaining cause and effect relationships since the exposure to different elements is generally concomitant. Thus, monitoring the levels of these substances and evaluating the factors that increase this exposure and the possible health effects is essential for understanding their dynamics. Thus, this thesis aimed to evaluate the concentrations of four elements, Ni, Co, Cd, and Pb, in a farmers' population residing in the micro-region of Caparaó Capixaba, southwest of Espírito Santo. As food is the organism's main source of chemical elements, the first chapter evaluates the applicability of stable isotopes of carbon and nitrogen in farmers' blood as a tool for assessing dietary patterns. The data showed that the isotopic values of both elements represent the frequency of consumption of different protein sources and can be used as tracers of food preferences in this population. In the second chapter, metals were associated with isotopic composition, socio-environmental variables, and risk factors, such as alcohol consumption and smoking, and the relationship with the health indicators (anthropometrics and blood markers). The four elements evaluated were positively associated with the values of $\delta^{13}C$ and $\delta^{15}N$, reflecting the higher meat consumption in the concentration of these metals. The effect of age on increasing Pb concentrations in the general population was also observed, while body mass index was negatively associated with Ni, Co, and Cd in women. Higher concentrations were also observed for all elements in smokers, Cd levels were statistically higher, showing a median increase of about 60% in this group. The frequency of alcohol consumption affected only Pb concentrations. Differences between gender were also observed for Pb. Men had 46% higher median concentrations. Different associations of metals with blood parameters were observed, including associations with blood count variables and indicators of liver functionality, glucose, vitamin D, and blood pressure, which point to possible toxic effects. The population evaluated presented high concentrations for all elements, with levels ranging from 2 to 31 times higher than reference values from other studies for Pb and Cd, respectively. Finally, in the third chapter, a systematic review was conducted on human blood Pb concentrations on a global scale. The data showed a consistent trend of exponential decrease in blood Pb concentrations across all continents due to the removal of the tetraethyllead as a gasoline additive, resulting in a global trend over the years. In addition, the results also showed that, despite the removal of the additive providing lower levels of exposure, the general population remains exposed to the element through environmental and occupational sources and lifestyle habits such as smoking and alcohol consumption.

Keywords: Stable isotopes, metals, human exposure, lead.

ESTRUTURA DA TESE

Esta teste foi escrita no formato de capítulos, e é composta de:

1. Uma introdução geral, onde é abordada de maneira sintética a problemática envolvendo a exposição humana aos metais tóxicos, incluindo seus principais aspectos como a bioacumulação, a biomagnificação, os fatores de risco e efeitos à saúde. Também é abordado o uso e a aplicação de isótopos estáveis de carbono e nitrogênio, bem como os fatores que regulam o fracionamento isotópico nos organismos promovendo o enriquecimento isotópico, e como a utilização dessa ferramenta pode contribuir para a avaliação da dinâmica de elementos químicos e de padrões alimentares contemporâneos.

2. O primeiro capítulo intitulado: **"Do isotopic composition of carbon and nitrogen in human whole blood reflect dietary patterns?"** Neste capítulo é avaliada a variação das concentrações elementares e dos valores da composição isotópica de carbono e nitrogênio no sangue humano, bem como, os efeitos da frequência do consumo de diferentes fontes de proteína nos valores de $\delta^{13}C$ e $\delta^{15}N$. Também é avaliada a influência de outras variáveis socioambientais e econômicas tais como, idade, altitude, etnia e renda mensal familiar. Por fim, é avaliada a associação dos valores de $\delta^{13}C$ e $\delta^{15}N$ com a composição corporal dos indivíduos (porcentagem de gordura corporal) e com marcadores do sangue.

3. O segundo capítulo intitulado: **"Associations between blood metal concentrations with carbon and nitrogen stable isotopes, risk factors, and health markers in Brazilian farmers"**. Neste capítulo foram avaliadas as variações nas concentrações de Ni, Co, Cd e Pb no sangue de uma população de agricultores do sudoeste do Espírito Santo, residentes na microrregião do Caparaó Capixaba. Além disso, foi avaliado se as concentrações destes elementos refletem os valores da composição

isotópica de carbono e nitrogênio, e outras variáveis socioambientais tais como idade, índice de massa corporal, altitude, consumo de tabaco e álcool e gênero. Buscando observar possíveis problemas à saúde dos indivíduos relacionados com a exposição a estes elementos foram avaliadas as associações das concentrações dos metais com parâmetros bioquímicos de funcionalidade hepática, pressão sanguínea e com variáveis de hemograma. Por fim, foram estabelecidos valores de referência para as concentrações de cada elemento na população avaliada.

4. O terceiro capítulo intitulado: **"Global decrease in blood lead concentrations due to the removal of leaded gasoline"**. Neste capítulo, é apresentada uma síntese de tendências temporais das concentrações de Pb no sangue de populações em escala global e regional (dos diferentes continentes) além da avaliação de fatores de risco de exposição ambiental, ocupacional, de hábitos de vida e diferenças entre os gêneros. Além disso, são apontadas lacunas na forma que os estudos reportam os dados que limitam a interpretação dos resultados e são feitas sugestões para estudos futuros.

5. As considerações finais: Onde são ressaltadas as contribuições deste estudo e lacunas que ainda devem ser preenchidas por futuros estudos relacionados à dinâmica da composição isotópica de carbono e nitrogênio no sangue humano e sobre a necessidade de esclarecimentos sobre fatores relacionados à exposição humana aos metais e sua dinâmica.

6. As referências bibliográficas.

1. INTRODUÇÃO GERAL

O biomonitoramento de substâncias tóxicas é uma crescente preocupação para as sociedades atuais, uma vez que, a ampliação da atividade industrial, mineradora e agrícola, tem aumentado a exposição humana a uma gama de contaminantes (Jaishankar *et al*., 2014). Dentre os diversos grupos de poluentes, os metais pesados, termo utilizado para definir elementos químicos (não necessariamente metálicos), que em geral, possuem elevada densidade e conhecida toxicidade se destacam, pois, possuem capacidade de acumulação no ambiente e são amplamente utilizados em diversos setores da sociedade (Smith e Huyck, 1999; Wong, 2003; Moors, Mulder e Vergragt, 2005; Mudgal *et al*., 2010; Jaishankar *et al*., 2014).

A dinâmica dos metais no ambiente perpassa por todos os elmentos do planeta, biosfera, litosfera, hidrosfera, e a atmosfera. Os metais são constituintes naturais das rochas, e de maneira natural, se tornam disponíveis aos organismos após o processo natural de intemperismo. Ocorre que, com o aumento das atividades antrópicas, também há o aumento da mobilização destes elementos, especialmente a partir da mineração. Os minérios metálicos são utilizados na indústria, e em último caso contaminam corpos aquáticos, solos e a atmosfera (Stoeppler, 1992; Garret, 2000; Sarkar, 2002; Ufelle e Barchowsky, 2019).

A principal forma de exposição humana aos metais é através da alimentação e do consumo de água contaminada, contudo, especialmente para alguns elementos (*e.g.* mercúrio e chumbo) a contaminação atmosférica também pode contribuir significativamente (Nriagu, 1990; Counter e Buchanan, 2004; Mudgal *et al*., 2010; Tchounwou *et al*., 2012; Zoroddu *et al*., 2019; Lacerda *et al*., 2022, *in preparation*). Após a absorção, os metais circulam pelos tecidos onde podem ser retidos em um processo chamado de bioacumulação. Processo que ocorre quando a capacidade do organismo de eliminar uma substância é menor do que a taxa de absorção. A biomagnificação é outro mecanismo envolvido na dinâmica de metais em organismos, e é definida como o aumento progressivo de substâncias de um nível trófico inferior para um superior. Esse processo ocorre naturalmente nas cadeias tróficas, mas é de maior interesse para os

consumidores de topo de cadeia, que inclui os seres humanos (Goyer, 1995; Gray, 2002; Luoma e Rainbow, 2005; Jakimska *et al*., 2011).

A toxicidade de metais varia de acordo com inúmeros fatores, sobretudo em função da via de exposição, do nível da exposição, ou seja, quanto do elemento foi absorvido, por quanto tempo durou a exposição e do tipo de metal. Os principais mecanismos de toxicidade dos metais envolvem a competição por canais de absorção celular com micronutrientes e competição por sítios ativos de enzimas, podendo causar seu mal funcionamento ou até mesmo sua inibição. Outro mecanismo associado a alta toxicidade de alguns elementos químicos (metais de transição) é a indução da produção de espécies reativas de oxigênio, moléculas instáveis e extremamente reativas resultantes da redução do oxigênio molecular. Estas moléculas podem causar danos irreversíveis nas biomoléculas resultando em último caso na morte celular, mas também estão associadas a indução de doenças degenerativas, metabólicas e o desenvolvimento de tumores (Tchounwou *et al*., 2012; Jaishankar *et al*., 2014; Mahurpawar, 2015; Ufelle e Barchowsky, 2019; Zoroddu *et al*., 2019).

Medir a toxicidade de elementos químicos é um desafio, especialmente porque a exposição aos metais é concomitante para as populações humanas, com excessão de casos específicos onde haja uma fonte conhecida como na baía de Minamata no Japão, onde aconteceu uma contaminação de Hg em animais e humanos, ou em casos de exposição ocupacional como acontece frequentemente em trabalhadores ocupacionalmente expostos. Neste sentido, os estudos com animais e outros modelos auxiliam na compreensão de alguns processos, especialmente quando as condições experimentais são suficientemente controladas, contudo, devido a alta complexidade dos organismos e também dos metais, mutas vezes é difícil estabelecer causa e efeito. Assim, os padrões de toxicidade dos elementos vão sendo descritos lentamente ao longo do tempo a partir da união de informações de estudos de caso e de biomonitoramento (Kurland *et al*., 1960; Harada, 1995; Gottesfeld e Pokhrel, 2011; Kim *et al*., 2012; Ahmad *et al*., 2014; Saravanabhavan *et al*., 2017; Wang e Wei, 2018).

Alguns fatores sociais contribuem para o aumento da exposição aos metais, o uso de tabaco tem sido reportado há décadas na literatura como uma

fonte de metais para o organismo, especialmente para o cádmio. Outro fator de risco reportado é o consumo de álcool, que é associado ao aumento da exposição a alguns elementos, além de alterações na funcionalidade hepática, que podem contribuir para acumulação de metais. O consumo de alguns alimentos vegetais, especialmente provenientes de solos contaminados e de peixes também tem sido associado com o aumento nas concentrações de alguns elementos no organismo (Maddrey, 2000; Mazumder e Dasgupta, 2011; Massey e Arteel, 2012; Ufelle e Barchowsky, 2019).

A exposição através de atividades ocupacionais também é um fator de preocupação, estudos tem reportado a elevação das concentrações de diferentes elementos em tecidos humanos especialmente em mineradores, soldadores, trabalhadores da fabricação de baterias ácidas, incineradores de lixo, agricultores e fundidores. A exposição ambiental também é um problema, concentrações elevadas de metais também são reportadas em pessoas vivendo em áreas com contaminação no solo e na água, e nas proximidades de áreas de mineração ou industriais (Baker *et al*., 1977; Wasowicz, Gromadzinska e Rydzynski, 2001; Ferré-Huguet *et al*., 2002; Chan *et al*., 2010; Kim e Lee *et al*., 2010; Ghazali *et al*., 2012; Cao *et al*., 2015; Rocha *et al*., 2015; Skalny *et al*., 2018; Deng *et al*., 2020).

Tendo em vista a complexidade da avaliação das concentrações de metais e os efeitos prejudiciais que estes podem causar à saúde humana, muitos países têm realizado monitoramentos dos níveis de exposição de suas populações através de matrizes como o sangue e urina. Em geral, são estudos envolvendo um grande número de participantes onde são avaliadas não só as concentrações de elementos prioritários como Cd, Hg, e Pb, mas também uma série de indicadores de saúde. Neste cenário, destacam-se países como os Estados Unidos, Alemanha, Canadá, China e Coreia do Sul. A partir destes monitoramentos, são estabelecidos os valores de referência (RVs) para as populações. Além do estabelecimento destes valores, alguns padrões têm sido encontrados em estudos com populações muito representativas, corroborando estudos laboratoriais e contribuindo para o conhecimento sobre as dinâmicas de exposição e toxicidade dos metais (Padilla *et al*., 2010; Kim *et al*., 2012; Moon *et*

al., 2013; Rhee *et al*., 2013; Saravanabhavan *et al*., 2016; Awata *et al*., 2017; Jin *et al*., 2018).

Além da dificuldade de estabelecer causa e efeito, outro desafio em estudos de exposição a metais é a atribuição de fontes. Em geral, as populações são comparadas em subgrupos com dados levantados a partir de questionários, por exemplo, quando se compara indivíduos tabagistas e não tabagistas. Ocorre que, os questionários podem trazer informações imprecisas que não permitem a acurada avaliação da influência de um determinado fator sobre as concentrações dos elementos. Como mencionado anteriormente, a alimentação é a principal fonte de metais para o organismo, e os padrões de alimentação influenciam no nível de exposição de determinados elementos. Uma ferramenta que tem uma contribuição importante na avaliação de padrões alimentares, é análise da composição elementar e isotópica de carbono e nitrogênio. A sua aplicação tem sido utilizada em função da sua alta fidelidade, e se resume no enriquecimento isotópico na biomassa do consumidor. Este enriquecimento se dá em função de um processo chamado de fracionamento, que é definido como a acumulação do isótopo mais pesado de C e N (^{13}C e ^{15}N, respectivamente) devido à sua menor cinética química comparado ao seu isótopo mais leve (^{12}C e ^{14}N, respectivamente). Desta forma, é possível diferenciar preferências alimentares dentro de uma mesma população (Vanderklift e Ponsard 2003; Petzke *et al*., 2005; McMahon e McCarthy, 2016; O'Brien, 2015; Litwak, 2018; Goto *et al*., 2018).

Apesar de a aplicação da ferramenta isotópica ser bem definida na literatura, até mesmo com outros objetivos, como estudos de contaminação ambiental e de ecologia de populações e comunidades, pouco se sabe na sobre a sua capacidade de retratar padrões alimentares contemporâneos em populações humanas, especialmente utilizando o sangue como indicador. Assim, é importante a avaliação da aplicabilidade desta ferramenta em estudos com humanos, além disso, a união da avaliação isotópica com os níveis de exposição aos elementos químicos pode trazer novas informações sobre a influêcia dos padrões alimentares nas concentrações destes elementos.

Portanto, tendo em vista a importância do biomonitoramento da exposição humana aos metais, esta tese teve como objetivo avaliar as concentrações de

Ni, Co, Cd, Pb, elementos que possuem reconhecida toxicidade (mesmo sendo o Ni e Co micronutrientes necessários em concentrações normais) e as razões isotópicas de carbono e nitrogênio no sangue de uma população de agricultores residentes na microrregião do Caparaó Capixaba, sudoeste do Espírito Santo. Estas variáveis foram avaliadas através de suas associações com fatores socioambientais e econômicos no intuito de fornecer informações sobre esta população e sobre a variabilidade das concentrações de cada elemento e das razões isotópicas, além de contribuir com a literatura científica sobre os fatores que possivelmente governam a dinâmica dos metais e da composição isotópica de carbono e nitrogênio no sangue humano. Por fim, foi realizada uma revisão sistemática sobre as tendências temporais e os níveis de exposição humana ao Pb em uma escala global e regional (nos diferentes continentes) que além de ressaltar a importância de estudos de biomonitoramento, mostra a importância da atividade industrial e da poluição atmosférica sobre a exposição humana a este elemento, bem como a necessidade de estudos de monitoramento para outros elementos ainda pouco avaliados.

2. CAPÍTULO I - Do isotopic composition of carbon and nitrogen in human whole blood reflect dietary patterns?

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Abstract

In human studies, the blood is the main matrix. The most diverse variables related to human health, nutritional status, and exposure to toxicants can be measured from blood. However, in studies using stable isotopes of carbon and nitrogen to evaluate dietary patterns, the blood has not been used frequently. Most studies involving stable isotopes in humans have been conducted with other matrices, especially hair and nails. Therefore, in this study, we measured the $\delta^{13}C$ and $\delta^{15}N$ values of whole blood of farmers in southeast Brazil to address the following questions: 1- How variable are $\delta^{13}C$ and $\delta^{15}N$ values in whole blood? 2 – Do the isotopic composition in whole blood reflects the individual's dietary patterns, *in casu* the intake frequency of different protein sources such as chicken, pork, beef, and fish? 3 – Do other factors such as age, altitude, ethnicity, and income rate influence the blood isotopic composition? 4 – Are the isotopic values in human blood associated with the anthropometric measures (*in casu* the body fat), blood lipids concentrations, and other blood markers, such as liver functionality indicators and blood hemogram? Carbon ratios varied from -22.2 to -15.6 ‰ with a mean of -18.8 ‰, while nitrogen ratios varied from 6.7 to 9.5 ‰ with a mean of 7.8 ‰. Both isotopes reflected the feeding preferences; the carbon ratios were higher in the individuals with a higher intake frequency of pork, beef, and fish (fish was not statistically significant). For nitrogen, the individuals who eat beef and fish more frequently presented higher nitrogen ratios than those who reported no

consumption of such items. The carbon values were also positively associated with males' body fat percentage. Conversely, nitrogen ratios were negatively associated with females' body fat percentage. We also observed different associations with blood parameters between gender, such as blood lipids profile, liver functionality enzymes, and blood hemogram. The present study provides new information about stable isotopes to evaluate contemporary diets and confirms the whole blood as a matrix to conduct such evaluations.

Keywords: Stable isotopes; carbon, nitrogen, blood, contemporary diet

2.1. Introduction

The stable isotopes ratios of carbon and nitrogen have been widely used to evaluate food chains (Di Beneditto *et al*., 2020; Gatts *et al*., 2021) in environmental studies, primarily to assess the sources of organic matter in aquatic systems and their association with pollutants (Cavalcante *et al*., 2021; Sobrinho *et al*., 2021; Gama *et al*., 2022), to evaluate changes produced by the removal of natural cover in the landscape (Vågen, Walsh and Sheperd, 2006) and in studies involving drought effects in plants (Linares and Camarero, 2012; Walker *et al*., 2015; Dulamsuren and Hauck, 2021).

In humans, the carbon and nitrogen stable isotopes have been used in studies evaluating historical and paleodiets diets, using bone and collagen (Van Der Merwe and Vogel, 1978; Richards *et al*., 2005; Fischer *et al*., 2007; Lee-Thorp, 2008) and as proxies of contemporary dietary patterns using predominantly non-invasive matrices such as nails and hair (Nakamura *et al*., 1982; Schoeller *et al*., 1986; Minagawa, 1992; O'Connell and Hedges, 1999; Nardoto *et al*., 2006). These studies have reported different carbon and nitrogen isotopic composition among vegans, vegetarians, and omnivores. They also observed regional effects, such as people living in lower and high-urbanized areas (Nardoto *et al*., 2006) and increased intake of market products (Wilkinson, Yai, and O'Brien, 2007).

The isotopic ratios of carbon and nitrogen in organisms are controlled by two processes: enrichment and fractionation. The $\delta^{13}C$ and $\delta^{15}N$ values observed in consumer biomass are higher than those of its diet (enrichment), and the magnitude of the difference is relatively constant among organisms. The fractionation is defined as accumulating the heavier isotope because of its lower chemical kinetics than the lighter isotope promoting its enrichment. Therefore, at

each trophic level, the isotopic compositions are more enriched (Gaebler *et al*., 1966; DeNiro and Epstein, 1978; DeNiro and Epstein, 1981; Minagawa and Wada, 1984). Due to this fractioning of isotopes, their ratios, heaver/lighter (for carbon, $\delta^{13}C$, for nitrogen, $\delta^{15}N$) isotopes, can indicate dietary patterns in the consumer's biomass.

The $\delta^{13}C$ values in animals generally fall within the range of $\delta^{13}C$ values of plants in their diet. Because of differences in C_3 and C_4 photosynthetic pathways, variations of about 20 ‰ can be found in different plants. When animals consume it, the carbon is incorporated into their tissues. On average, the fractionating of carbon is modest, at approximately 1 ‰, and occurs through respiration (Craig, 1953; Smith and Epstein, 1970; Farquhar, Ehleringer and Hubick, 1989; McCutchan *et al*., 2003; Nash *et al*., 2012). According to DeNiro and Epstein (1978), the respired CO_2 is depleted in ^{13}C, making the consumer enriched in ^{13}C compared to its diet. Since the fractionation of carbon in tissues of heterotroph organisms will reflect the tissues' metabolic rate. Then, as more metabolically active the tissue is, the higher its renovation rate, which directly influences its isotopic composition. The biochemical fractions which compose the tissues, such as lipids and amino acids, suffer different fractionation processes, and the concentration of such fractions in tissues also influences their isotopic composition. Because of these differences, different tissues or the whole body should be used to conduct the most accurate evaluation of dietary patterns using carbon isotopes in a heterotroph organism (DeNiro and Epstein, 1978; Tieszen, 1983; Fry and Sherr, 1988; Kennedy and Krouse, 1989; Nardoto *et al*., 2006).

For nitrogen, the higher $\delta^{15}N$ values in the consumer biomass protein occur when many amino acids are deaminated. Deamination is the removal of an amine group from a molecule. In this process, the amino acids are broken down to liberate ammonia and occur when there is an excess of amino acids. The deamination process can promote the fractioning of nitrogen through the preferential elimination of ^{14}N, leaving behind the ^{15}N amino group to form a new biomass protein. Another process that influences nitrogen fractionation is transamination. This chemical reaction transfers an amino group to a ketoacid to form new amino acids, especially when there is a lower protein intake. The body

can resort to catabolizing its tissues to build new amino acids. Transamination also occurs when there is energy demand. In this case, the amino acids are transported from the muscle to the liver, especially alanine. The alanine is transaminated, producing urea and energy in the last case. In the same way as deamination, transamination promotes the body tissues more enriched in ^{15}N. As excretion is the main process governing nitrogen fractionating, factors affecting nitrogen excretion such as growth, starvation, and differences in protein quality and amount, have the potential to alter the magnitude of nitrogen fractionation (Vanderklift and Ponsard, 2003; Mayes *et al*., 2003; Petzke *et al*., 2005; McMahon and McCarthy, 2016; O'Brien, 2015; Litwak, 2018; Goto *et al*., 2018). From this perspective, the higher $\delta^{15}N$ values appoint to higher protein intake due to the deamination process. On the other hand, if the individuals are in a malnutrition status, increasing the deamination and transamination, the higher $\delta^{15}N$ values could promote biased interpretations.

In human studies, the blood is the main matrix. The most diverse variables related to human health, nutritional status, and exposure to toxicants can be measured from blood. However, isotopic studies evaluating dietary patterns have not frequently used whole blood. We found in the literature only a few reports using red blood cells to evaluate dietary patterns (Wilkinson, Yai, and O'Brien, 2007, Nash *et al*., 2012; Nash *et al*., 2013) or blood serum (Yeung *et al*., 2010). Most studies involving stable isotopes to evaluate contemporary dietary patterns in humans have been conducted with other matrices, especially hair and nails. Even in studies with other animal species, the blood has been little used.

The blood is composed mainly of plasma (≅ 55% of the total volume) which is the fluid part and comprises ions, molecules, hormones, vitamins, and proteins. The remaining amount comprises the red cells, ≅ 45% of the total volume, leukocytes, and platelets (< 1%). The red cells are the main storage compartment of carbon and nitrogen in the blood and have a life span of ≅ 110-120 days (Klinken, 2002; Higgins, 2015). As blood is widely used in human studies, the evaluation of its feasibility to indicate dietary patterns should be of great interest since the association of carbon and nitrogen isotopes could be paired compared with other variables (e. g., toxicants, blood marks, etc.) and bring new information about the effects of the dietary patterns in humans.

In this study, we measured the $\delta^{13}C$ and $\delta^{15}N$ values of human blood of farmers in southeast Brazil to address the following questions: 1 – How variable are $\delta^{13}C$ and $\delta^{15}N$ values in whole human blood? 2 – Do the isotopic ratios in whole blood reflect the individual's dietary patterns, *in casu* the intake frequency of different protein sources such as chicken, pork, beef, and fish? 3 – Do other factors such as age, altitude, ethnicity, and income rate influence the blood isotopic compositions? 4 – Are the isotopic values in human blood associated with the anthropometric measures (*in casu* the body fat), blood lipids concentrations, and other markers, such as liver functionality indicators and blood hemogram?

2.2. Material and methods

The survey was conducted through personal interviews with 287 individuals between 18 and 84 old who lived in nineteen rural communities in ten municipalities located southwest of the Espírito Santo state in Brazil (Figure 1). A questionnaire was used to provide information on the sociodemographic characterization of farmers in 2015. The questionnaire applications and sample collection were conducted in health or community centers of each locality (Figure 1).

The following criteria were used to select subjects in the present study: (1) individuals living in rural areas of the cities selected, and (2) in a full mental decision-making capacity. The respondents were selected by adherence, and the individuals who volunteered signed an informed consent term following the resolution nº. 466/12, of the Brazilian National Health Council (Brasil, 2012). Qualified professionals performed anthropometric assessments (height, abdominal circumference, and weight) following the Brazilian Food and Nutritional Surveillance System technical report (Brasil, 2011). Stature was evaluated using an Alturexata® stadiometer, with a maximum capacity of 2.10 m and an accuracy of 0.5 cm. The weight was measured on a Tanita® bipolar bioimpedance balance with a BC6011® branded body fat monitor (with 100 g division and a maximum capacity of 150 kg). The body mass index (BMI) was calculated and classified according to the World health organization (WHO) reference for adults (WHO, 2000). The blood collection was performed following

the resolution nº. 466/12, of the Brazilian National Health Council (Brasil, 2012). Samples of 2 mL of peripheral blood from the participating individuals were collected into 5 mL tubes containing ethylenediaminetetraacetic acid (EDTA) for stable isotopes and hemogram analysis. The blood was also collected into 5 mL tubes without anticoagulant for biochemical determinations. Both anthropometric assessments and blood collection were conducted after an 8-hour fast. After the collection, the samples were placed in styrofoam boxes with ice, and one portion was sent to the Laboratório de Biotecnologia of the Centro de Ciências Exatas, Naturais e da Saúde at the Universidade Federal do Espírito Santo for biochemical analysis, and another portion was sent to the Laboratório de Ciências Ambientais of the Centro de Biociências e Biotecnologia at Universidade Estadual do Norte Fluminense Darcy Ribeiro where they were freeze-dried and stored at -20º C until the isotopic analysis was performed. To evaluate altitude effects on isotopic values, we assumed the altitude of the collection locations. This measurement was performed using the Google Earth Pro software (Google LLC©).

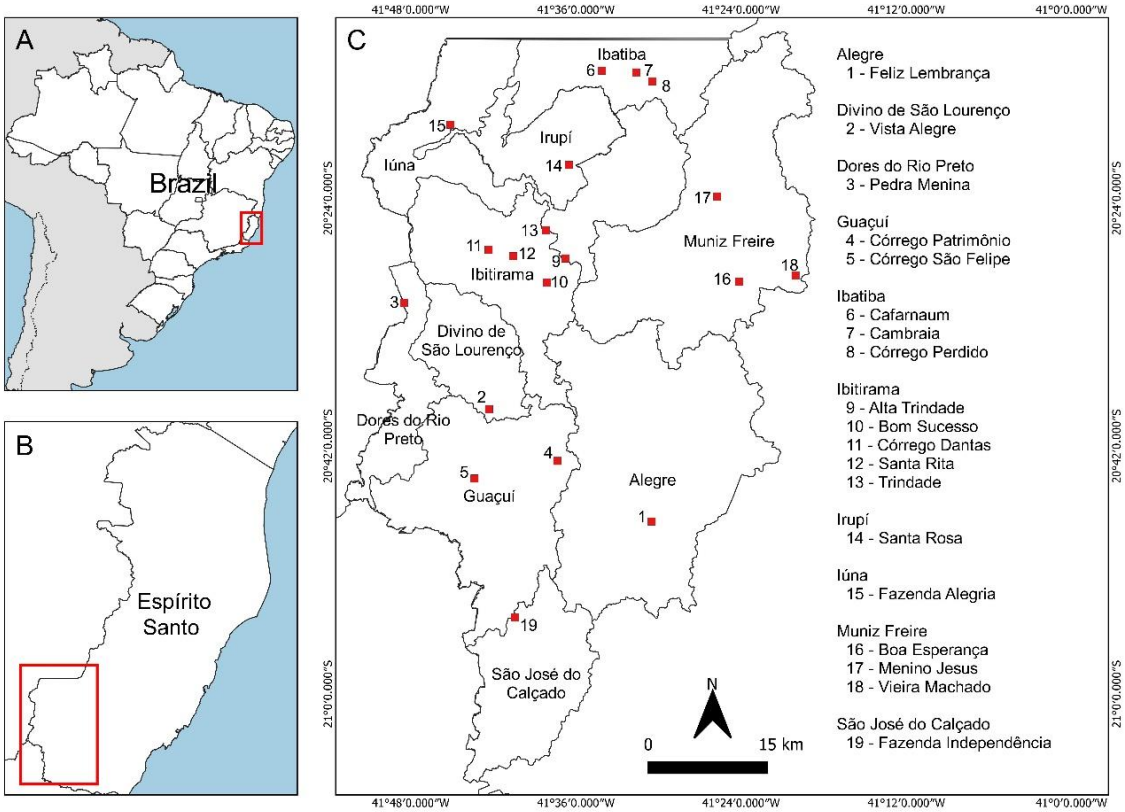

Figure 1. Study area. A - Brazil; B - Espírito Santo state, C - Map of the municipalities in the southwest region of Espírito Santo state with the locations of the 19 communities (red squares).

Blood parameters analysis

The hemogram profile (erythrocytes and leukocytes count, hemoglobin concentration, hematocrit, and platelets) was determined in an automatic hematologic analyzer (BC 5380 - Shenzhen Mindray Bio-Medical Electronics, China). Total cholesterol, high-density lipoprotein (HDL-C), triglycerides, total proteins, total bilirubin, creatinine, albumin, glutamic-oxaloacetic transaminase (GOT), the glutamate-pyruvate transaminase (GPT) and urea were determined with enzymatic colorimetric methods using an automatic biochemical analyzer, according to the manufacturer's recommendations (BS 120 - Quibasa-Bioclin, Brazil).

Stable isotopes analysis

For the carbon and nitrogen stable isotopes determination, approximately 0.40 ± 0.03 mg aliquots of the freeze-dried blood samples were weighed in tin capsules. The elemental and isotopic composition were determined by using an Elemental Analyzer (Flash 2000 - Thermo Scientific, Germany) with interface CONFLO IV coupled to an Isotope Ratio Mass Spectrometer (Delta V Advantage – Thermo Scientific, Germany) at Laboratório de Ciências Ambientais at the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF). The samples were analyzed with analytical blanks and urea analytical standards (IVA Analyzentechnik-330802174; CH_4N_2O M_w = 60, C = 20 %, N = 46 %), using certified isotopic compositions ($\delta^{13}C$ = −39.89 ‰ and $\delta^{15}N$ = −0.73 ‰). Analytical control was done for every 10 samples using certified isotopic standard (Elemental Microanalysis Protein Standard OAS: 46.5 ± 0.78 % for C; 13.32 ± 0.40 % for N; −26.98 ± 0.13 ‰ for $\delta^{13}C$; + 5.94 ± 0.08 ‰ for $\delta^{15}N$). Carbon and nitrogen contents were expressed as percent element (%), and the detection limits were 0.05 % and 0.02 %, respectively. Carbon and nitrogen isotope ratios were expressed in δ notation as ‰ relative to Pee Dee Belemnite and atmospheric nitrogen, respectively. Analytical reproducibility was based on triplicates for every ten samples: ± 0.3 ‰ for $\delta^{15}N$ and ± 0.2 ‰ for $\delta^{13}C$. Accuracy tests were carried out using the certified standard, with precision obtained through triplicates with recovery between 90 % and 110 %.

To evaluate the possible interference of EDTA in the collection tubes on carbon and nitrogen ratios and elemental concentrations, we conducted a comparison between ten blood samples collected in EDTA-containing tubes and polyethylene conic tubes (Table 1). The comparison showed no influence of EDTA in both isotopic ratios and elemental concentrations of carbon and nitrogen. Wilkinson, Yai, and O'Brien (2007) also reported the lack of EDTA influence in such results.

Table 1. Comparison of elemental concentrations and isotopic ratios of carbon and nitrogen between samples collected in EDTA-containing tubes (n=10) and polyethylene cone tubes (n=10). The letters show no statistical difference among the treatments according to the one-way analysis of variance (p>0.05). The values were expressed as mean ± standard deviation

	Total carbon (%)	δ^{13}C (‰)	Total nitrogen (%)	δ^{15}N (‰)	(C/N)a
With EDTA	48.0 ± 1.2a	-18.5 ± 0.1a	14.5 ± 0.3a	7.6 ± 0.2a	3.8 ± 0.1a
Without EDTA	47.5 ± 0.9a	-18.5 ± 0.1a	14.5 ± 0.5a	7.6 ± 0.1a	3.8 ± 0.1a

Various studies have demonstrated the need for lipid removal to produce reliable interpretations of δ^{13}C composition. According to Post *et al*. (2007), if the (C:N)a ratios are higher than 4 or the percentage of lipids is higher than 10 %, the lipid content negatively influences δ^{13}C values in terrestrial animals. In the present study, the mean (C:N)a ratio was 3.9 ± 0.1. Thus, we assume that the blood's lipid content did not affect their δ^{13}C values.

2.3. Statistics

The association between the elemental concentration and isotopic composition with the numeric predictor variables (anthropometrics and environmental variables) was assessed using the analysis of covariance (ANCOVA), using the gender as a covariable (lm, base package, R Core Team, 2022). The differences of the elemental concentration and isotopic composition among the levels of categorical variables (ethnicity, familiar income, and the ingestion frequency of chicken, pork, beef, and fish) were accessed using a one-way analysis of variance (ANOVA) (aov, base package, R Core Team, 2022), followed by Tukey's multiple comparison test (TukeyHSD, base package, R Core Team, 2022) and reported using the compact letter display (HSD.test, agricolae package, Mendiburu, 2021). The correlation analysis (cor.test, base package, R Core Team, 2022) was conducted to evaluate the association between the

isotopic composition and blood parameters. The assumptions of the ANCOVA, ANOVA and Pearson's correlation were evaluated through diagnostic plots (Altman & Krzywinski, 2016) and transformed, when necessary, to meet the linear model's assumptions (normality, linearity, and homoscedasticity of residuals), using a maximum likelihood function (boxcox, MASS package, Venables and Ripley, 2002). In all applicable cases, an *a priori* type I error of 5% ($\alpha = 0.05$) was assumed.

2.4. Results

Initially, to elucidate de dynamics of the carbon and nitrogen stable isotope ratios in human blood, we evaluated their elemental concentration associations with the measured variables. Carbon concentrations varied from 43 to 50 %, with a mean concentration of 47 %, while nitrogen concentrations varied from 12 to 16 %, with a mean concentration of 14 %. The elemental concentrations of both elements were positively associated with each other, and their relationship was relatively high ($r = 0.56$, $p < 0.05$; data not shown).

We conducted a univariate analysis of carbon and nitrogen concentrations with all possible predictors, including the different frequencies of protein intake, mensal familiar income, ethnicity, and age. However, it was only observed a statistically significant positive association between the elemental concentrations of both elements, carbon, and nitrogen, with the mean altitude of each locality sampled. The mean carbon and nitrogen concentrations increase was 0.23 % and 0.07 %, respectively, per 100 meters above sea level elevation (Figure 2). Furthermore, there was no significant relationship between carbon and nitrogen concentrations and their isotopic ratios (data not shown).

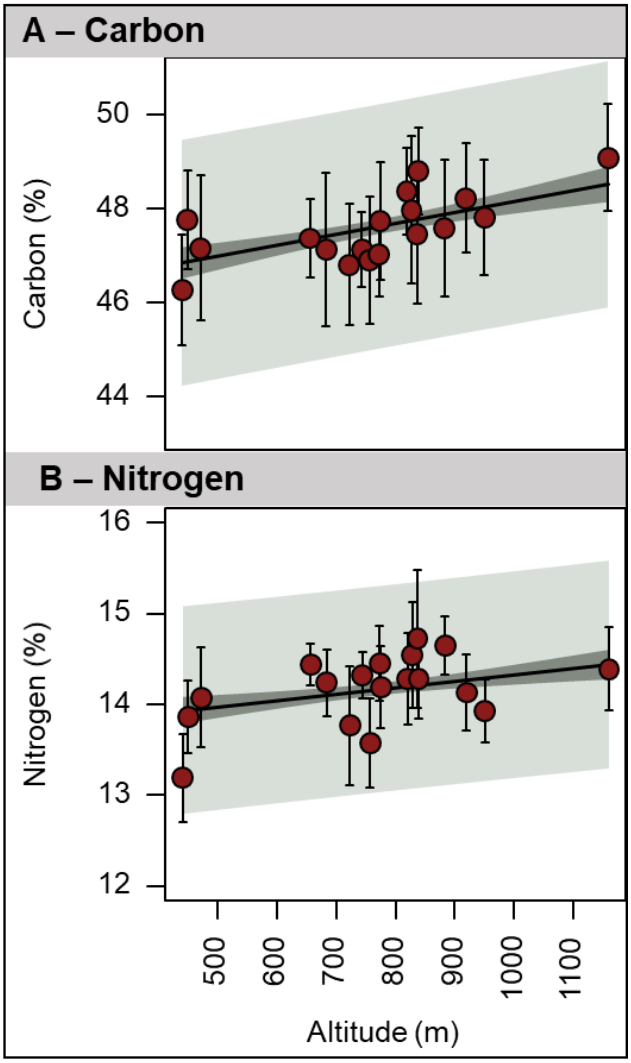

Figure 2. Association between carbon and nitrogen elemental concentrations with the mean altitude. A – Carbon. B – Nitrogen. Statistics: A - Y = 0.0023X + 45.83, $p < 0.001$, $R^2 = 0.08$. B - Y = 0.0007X + 13.63, $p < 0.001$, $R^2 = 0.04$. The light and the dark shadings identify the 95% prediction and confidence intervals of the regression models, respectively.

To our knowledge, this study first reports carbon and nitrogen stable isotope ratios in whole human blood. Carbon ratios varied from -22.2 to -15.6 ‰ with a mean of -18.8 ‰, while nitrogen ratios varied from 6.7 to 9.5 ‰ with a mean of 7.8 ‰. The carbon and nitrogen ratios were positively associated; the higher ^{13}C-enriched, the more ^{15}N-enriched, is the individual (Figure 3). However, the correlation coefficient was only 0.37 and varied among genders: in males, the correlation coefficient was r = 0.41, while in females was r = 0.18. Thus, besides this positive relationship, the isotopic ratios of carbon and nitrogen showed different associations with the evaluated variables, as presented below.

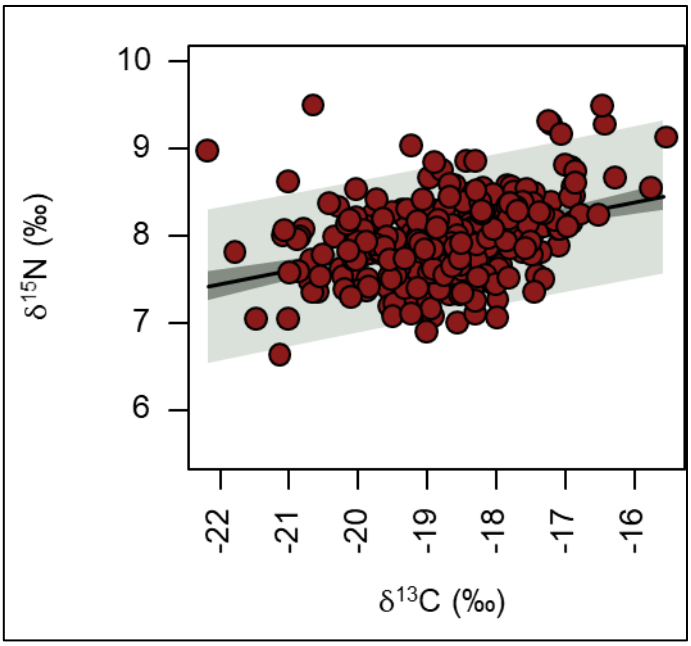

Figure 3. Association between carbon and nitrogen isotopic ratios. Statistics: Y = 0.1554X + 10.88, $p < 0.001$, $R^2 = 0.13$, $r = 0.37$. The light and the dark shadings identify the 95% prediction and confidence intervals of the regression model, respectively.

We performed univariate linear models with the other measured variables to evaluate the possible factors influencing the carbon isotopic ratios (Figure 4). Such analysis showed no association of carbon ratios with age. Although, there is a tendency in the statistical significance threshold ($p = 0.11$) that older individuals present 13C-enriched ratios (Figure 4 A). The statistical association between carbon isotopic ratios with altitude was not observed ($p = 0.07$), although a tendency of slightly higher $\delta^{13}C$ values at higher altitudes can be observed (Figure 4 B).

The comparison of the means between the genders was statistically significant according to the Tukey test, but the variation occurs in the decimal range. Females presented ^{13}C-enriched values compared to males, -18.4 and -18.8 ‰, respectively (data not shown). The ethnicity evaluation also revealed different carbon isotope ratios (Figure 4 C). The studied population was composed of three self-declared indigenous, seven self-declared yellow, fourteen self-declared afro descendants, eighty self-declared brown, and one hundred seventy-nine self-declared white. The results showed indigenous people with depleted $\delta^{13}C$ ratios, mean -20.7 ‰, followed by the afro descendants, -19.3 ‰,

yellow, -19.3 ‰, brown, -18.8 ‰, and white, -18.6 ‰, which were not statistically significant among them.

Because the income rate can affect the feeding patterns, we compared the isotopic ratios among different income rates classified according to the minimum salary in Brazil (R$ 788.00 in 2015) when the samples were collected, which corresponded to ≅ US$ 237.00 in that year. There were no statistically significant differences in isotopic ratios among the different income classes (Figure 4 D).

To evaluate the potential of stable carbon isotopes in blood to reflect the feeding patterns, we compared the ratios between different ingestion frequencies of protein sources such as chicken, pork, beef, and fish (Figure 4 E - H). The intake frequencies of such items were classified as no intake, once a month or less, twice a week or less, and more than two times per week. Such analysis showed that the carbon ratios are higher in the individuals with a higher consumption frequency of pork, beef, and fish (fish was not statistically significant) than in those who reported no consumption of such items. The increase in ^{13}C between the individuals with higher frequency intake of such protein sources and those who reported no consumption was 1.0, 0.9, and 0.9 ‰, respectively (Figure 4 E - H).

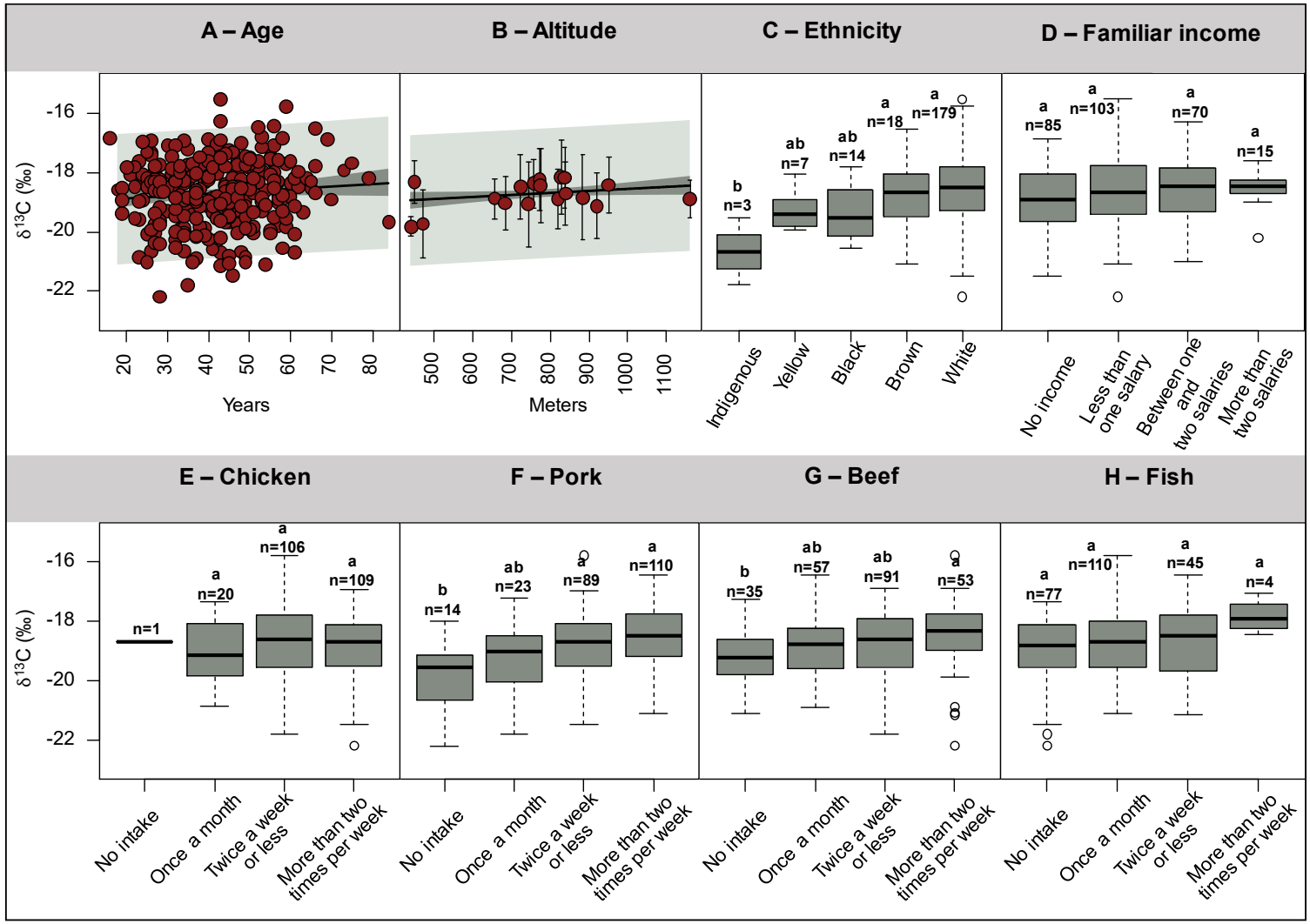

Figure 4. Associations of the carbon isotopic ratios with the measured variables. A – age, B – altitude, C – ethnicity, D – familiar monthly income, and the ingestion frequency (E – H) of chicken, pork, beef, and fish, respectively. Statistics: A - Y = 0.0086X -19.08, p = 0.11, R^2 = 0.00, B - Y = 0.0007X -19.27, p = 0.07, R^2 = 0.00. The light and the dark shadings in figures A and D identify the regression models' 95% prediction and confidence intervals, respectively. The letters represent statistically different values according to the Tukey test ($p < 0.05$).

The nitrogen ratios also showed no statistically significant tendency of $\delta^{15}N$ values with age (Figure 5 A). Also, the nitrogen ratios were slightly higher in women with a mean of 8.1 ‰ compared to 7.9 ‰ observed in men (data not shown) and do not present different means or tendencies between ethnic groups or the different income rates (Figure 5 C and E). Inversely to the carbon ratios, the association with altitude was negative, besides not statistically significant. The individuals living at higher altitudes presented lower nitrogen isotopic ratios. According to the linear model adjusted, the mean decrease of $\delta^{15}N$ ratios is 0.03 ‰ per 100 meters above sea level (Figure 5 D).

The protein feeding frequencies are also reflected in nitrogen isotopic ratios. Individuals who eat beef and fish more frequently presented higher nitrogen ratios than those who reported no consumption of such items. The increase in $\delta^{15}N$ values between the individuals with higher frequency intake of such protein sources and those who reported no consumption was 0.3 and 0.8 ‰, respectively (Figure 5 E – H).

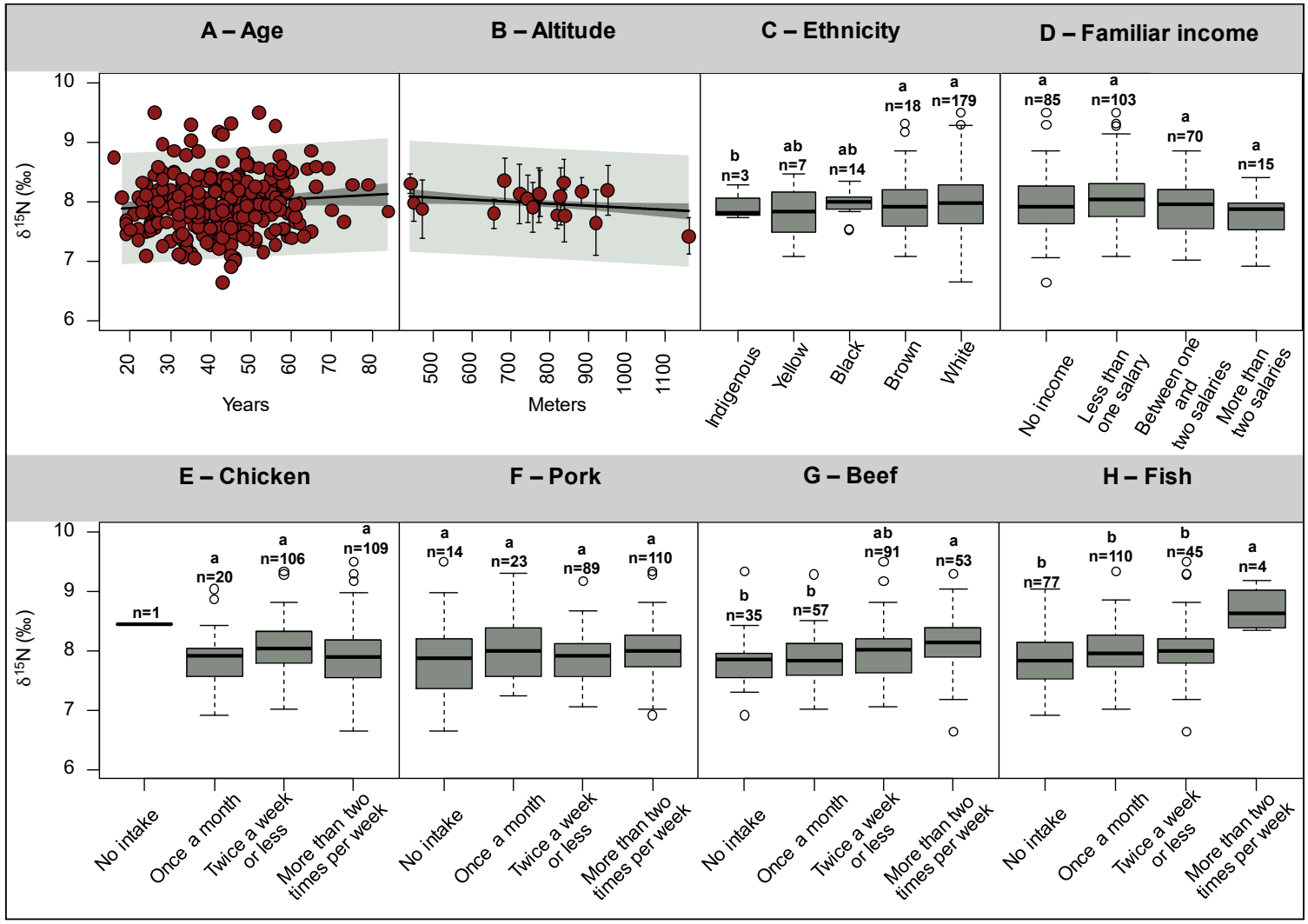

Figure 5. Associations of the nitrogen isotopic composition with the measured variables. A – age, B – altitude, C – ethnicity, D – monthly income, and the ingestion frequency (E – H) of chicken, pork, beef, and fish, respectively. Statistics: A - Y = 0.0036X +7.81, p = 0.11, R^2 = 0.00, B - Y = -0.0003X +8.24, p = 0.03, R^2 = 0.01. The light and dark shadings in figures A and D identify the regression models' 95% prediction and confidence intervals, respectively. The letters represent statistically different values according to the Tukey test ($p < 0.05$).

Since the carbon and nitrogen stable isotopes were associated with feeding patterns, we conducted a regression analysis to evaluate their association with body fat (Figure 6). A statistically significant association of carbon ratios with the body fat composition in males was observed. The increase in body fat percentage was 1.5 % per 1 ‰ increase in $\delta^{13}C$ values. Conversely, there was no observed relationship in females (Figures 6 A and B). On the other hand, the nitrogen ratios were not associated with the percentage of body fat in males and were statistically negatively associated in females. The higher the $\delta^{15}N$ values, the lower the body fat in females. The decrease in body fat percentage was 3.0 % per 1 ‰ increase in $\delta^{15}N$ values (Figures 6 C and D).

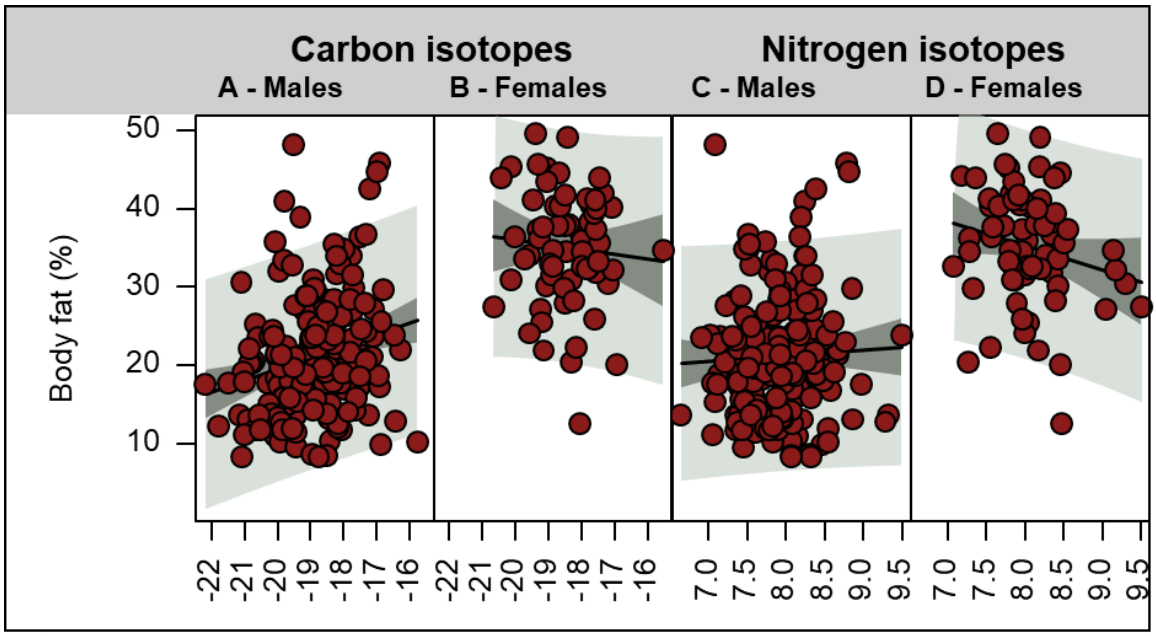

Figure 6. Association between carbon and nitrogen isotopic ratios with body fat and cholesterol. A – body fat x $\delta^{13}C$ in males, B – body fat x $\delta^{13}C$ in females, C – body fat x $\delta^{15}N$ in males, D – body fat x $\delta^{15}N$ in females. Statistics: A - Y = 1.4732X + 49.01, p = 0.001, R^2 = 0.05, B - Y = -0.6212X + 23.71, p = 0.52, R^2 = -0.01, C - Y = 0.7368X + 15.42, p = 0.52, R^2 = -0.00, D - Y = -3.0442x + 59.67, p = 0.09, R^2 = 0.03. The light and the dark shadings identify the 95% prediction and confidence intervals of the regression models, respectively.

Finally, we evaluated the relationship of the isotopic blood ratios with the measured parameters in the blood through a correlation analysis (Figure 7). The correlations were conducted to each sex separately since the blood composition varies between the genders. For the description of blood parameters ranges see Lacerda *et al*. (2022b, in preparation - segundo capítulo). In general, the correlation coefficients were low, varying from -0.24 to 0.41 for both isotopes, because of the

reduced sample size of females, some correlations with the same magnitude as those observed in males were not significant since, for the description of the results, we will consider correlation coefficients: -0.15 ≤ r ≥ 0.15. We observed a positive relationship between $\delta^{13}C$ values and total cholesterol in males and HDL – C in females (Figures 7 A and B). We also observed a positive correlation between leukocytes in males (Figure 7 A) and urea in females (Figure 7 B). In females, a negative correlation was observed between carbon composition with erythrocytes and hematocrit (Figure 7 B).

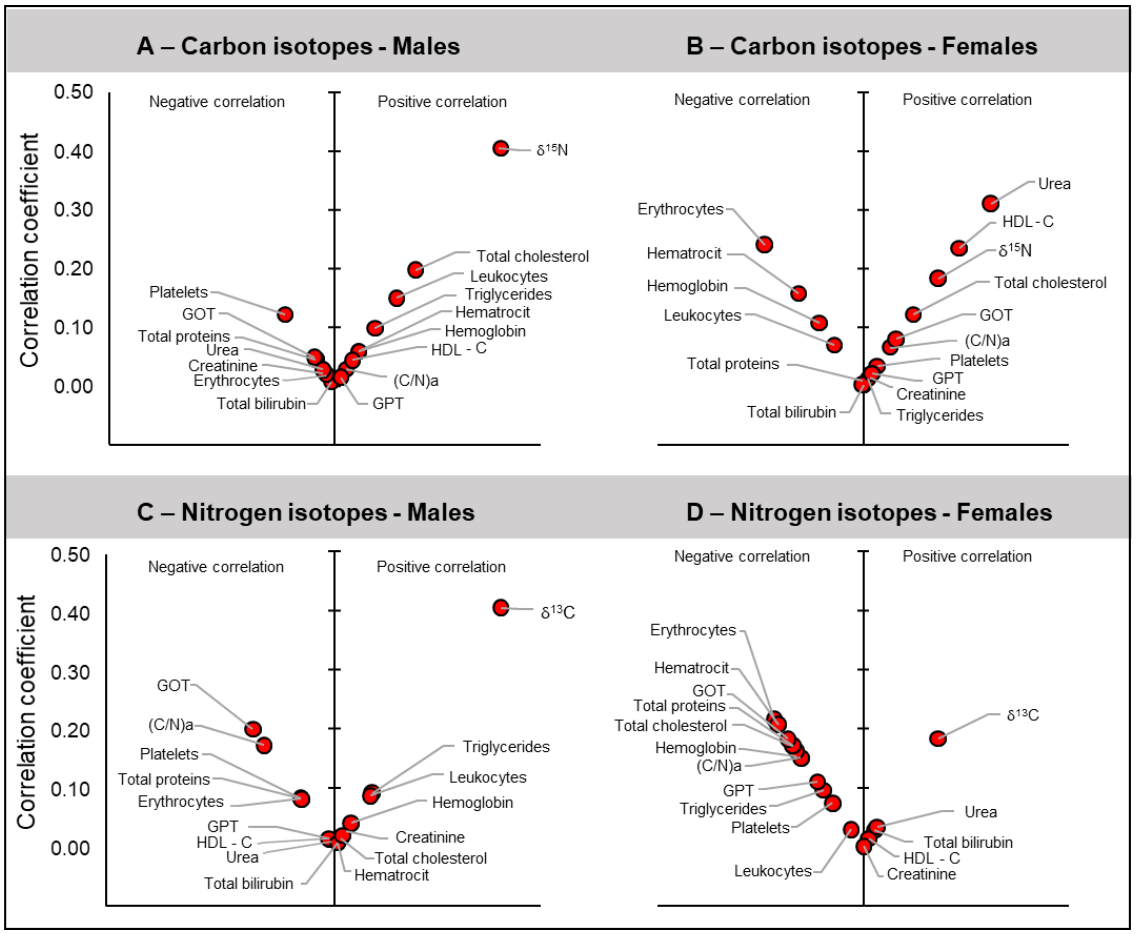

Figure 7. Pearson's correlation between carbon and nitrogen isotopic ratios with blood markers. A – correlations with $\delta^{13}C$ in males, B – correlations with $\delta^{13}C$ in females, C – correlations with $\delta^{15}N$ in males, and D – correlations with $\delta^{15}N$ in females.

The nitrogen ratios were not associated positively with any measured parameter (except for $\delta^{13}C$ ratios) and were negatively correlated with the (C/N)a ratio (r = -0.17) and the glutamic-oxaloacetic transaminase (GOT) (r = -0.20) in males (Figure 7 C). In females, the nitrogen ratios were negatively associated with the (C/N)a ratio (r = -0.15), hemoglobin (r = -0.16), total proteins (r = -0.17), total cholesterol (r =

-0.17), the glutamic-oxaloacetic transaminase (GOT) (r = -0.18), hematocrit (r = -0.21), and erythrocytes (r = -0.22) and (Figure 7 D).

2.5. Discussion

Diet evaluations remain challenging to measure because of errors and bias caused by self-reported methods. Therefore, the development of accurate markers is a requirement. In this perspective, stable isotope ratios are a promising candidate because their natural variability among foods is reflected in consumers' tissues (O'Brien, 2015). Although the isotopic ratios showed lower associations with the other evaluated variables, some patterns were observed in the present study and are discussed below.

As mentioned in the results section, the only association of the elemental carbon and nitrogen concentrations was with the altitude. The individuals living at high altitudes presented increased concentrations. This relationship might be a result of adaptation to hypoxia: in high altitudes, the barometric pressure falls, which makes necessary biochemistry adaptations to increase oxygen circulation. In our sampling population, the altitude varied from ≅ 440 to almost 1200 meters above sea level. According to Levett *et al*. (2011), the increasing altitudes promote an increase in the hematocrit profile (percentage of red cells), and this process could explain the higher concentrations of carbon and nitrogen in the human blood of people from higher altitudes. In fact, there is a positive association of carbon and nitrogen with the hematocrit profile (r = 0.16 and 0.14, respectively) and hemoglobin (r = 0,14 and 0.13, respectively), data not shown.

The present study showed no statistical association of the isotopic ratios with age. However, Wilkinson, Yai and O'Brien (2007) published a previous study that reported differences in isotopic ratios in native individuals from Alaska (United States of America - USA) between teenagers and elders. They observed enriched nitrogen and depleted carbon ratios in the red blood cells of the elders. The results were attributed to differences in feeding patterns. According to the authors, the high intake of marked products among teenagers might enrich ^{13}C, because of the increased intake of corn and cane products. On the other hand, the increased $\delta^{15}N$ values reported in elders were associated with increased intake of marine subsistence feeding. In our study, there were no statistically

significant tendencies. Therefore, these data indicate homogeneity in the feeding patterns among all individuals. Thus, the association of isotopic ratios with age might be observed only in specific cases, such as Wilkinson, Yai, and O'Brien's (2007) population study, which presented differences in feeding preferences among the different age groups.

The different associations of carbon and nitrogen isotopic ratios with altitude (increasing $\delta^{13}C$ and decreasing $\delta^{15}N$ values), although not statistically significant, are in accordance with a previous study that reported the same behavior on grassland and grazers in the European Alps (Männel, Auerswald, and Schnyder, 2007). Altitude affects soil and climatic conditions and, consequently, the carbon and nitrogen fractioning in vegetal tissues (Raich *et al*., 1997; Austin and Vitousek, 1998; Sah and Brumme, 2003; Luo *et al*., 2004; Drewnik, 2006). In higher altitudes, the plants increase their water use efficiency, which favors the assimilation of ^{13}C (Farquhar *et al*., 1989; Hultine and Marshall, 2000). Conversely, decreasing $\delta^{15}N$ values are associated with decreased volatile nitrogen losses caused by lower temperatures, lower pH, and increasing precipitation (Handley *et al*., 1999; Amundson *et al*., 2003). We hypothesize that this effect was only observed because this study's population is composed of farmers. The consumption of foods from their farming and the production for subsistence is common in our study population. Furthermore, part of their protein sources also come from livestock farming. The lack of statistical significance might result from our methodology for assessing the altitude effect. We assumed the altitude of the collection locations, which may not accurately reflect the altitude in which the individuals live.

Another factor that influenced the carbon isotopic ratios was ethnicity. Besides not being statistically representative, the indigenous (n=3) individuals presented depleted ^{13}C values. The lower $\delta^{13}C$ ratios may indicate a higher intake of plants from the forest and the cultivation of some vegetables, such as cassava, an important staple food to indigenous individuals (Araújo and Kubo, 2017; Maciel *et al*., 2021). Nardoto *et al.* (2006) also reported lower carbon ratios in the fingernails of residents of isolated communities in the Brazilian Amazon.

The familiar monthly income also did not affect the blood isotopic ratios. This variable was evaluated since the different income rates might affect the feeding patterns, mainly the frequency of protein intake. However, this was not observed since most individuals in the present study reported having a frequent protein intake independent of their income.

The increased frequency of intake of beef and fish increased the ratios of carbon and nitrogen. People who eat beef more than two times per week presented a median increase of 0.8 ‰ and 0.3 ‰, while the higher fish intake frequency increased by 1.1 ‰ (not statistically significant) and 0.9 ‰ in $\delta^{13}C$ and $\delta^{15}N$ values, respectively, compared to those who reported no intake of such items. The pork consumption frequency did not affect nitrogen ratios. Also, no differences in the isotopic values were observed according to the frequency of chicken intake. A probable reason might be the accuracy of the respondents about both items. The questionaries are the most accessible tool to evaluate feeding patterns in random populations. However, their data may not reflect reality, especially when evaluating feeding habits, which vary among individuals and over time. However, the results clearly showed protein intake's influence on the isotopes ratios.

The association of the isotopic composition of blood with the body fat mass was very interesting. At the same time that $\delta^{13}C$ values were positively associated with higher body fat in males and not in females, the $\delta^{15}N$ was not associated with body fat in males and was negatively associated in females. Fat accumulation in the body differs between the genders, which is well-documented. Generally, females have higher fat accumulation than males (Gallagher *et al*., 1996; Blaak, 2001; Manolopoulos, Karpe and Frayn, 2010). In our population, the mean body fat in males varied from 8.3 to 48.2 %, with a mean of 21.3 %. In females, the body fat varied from 12.6 to 49.6 %, with a mean of 35.2 % (data not shown). These results indicate the $\delta^{13}C$ correlation with C_3 and C_4 plants consumption. Indeed, the higher carbon ratios may be, in part, associated with increased intake of products derived from C_4 plants causing increased body fat gain in men. This might be especially due to the consumption of refined carbohydrates, as the added sugar promotes weight gain and, consequently,

accumulation of body fat (Vermunt *et al*., 2003; Jahren *et al*., 2006; Yeung *et al*., 2010); this pattern was not observed in women. If we assume that the higher $\delta^{13}C$ values in the blood are correlated with the higher intake of C_4-derived products, we can suppose that the lack of association with $\delta^{13}C$ with the percentage of body fat in women might be related to their higher capacity for fat accumulation independently of their source of carbohydrates.

On the other hand, the $\delta^{15}N$ values were not associated with body fat percentage in men and were negatively associated in women. The observed association is in accordance with previous studies, which show that higher protein intake is associated with weight loss and preserved lean body mass in women. This is attributed to the reduced hunger and increased satiety caused by the increased protein intake (Piatti *et al*., 1994; Farnsworth *et al*., 2003; Layman *et al*., 2005; Leidy *et al*., 2007). However, higher protein intake is associated with lean mass preservation and a lower percentage of body mass in both genders (Halton and Hu, 2004; Green *et al*., 2010; Pesta and Samuel, 2014; Sahni *et al*., 2015). Farnsworth *et al*. (2003) argue that the non-observed reduction of body fat in men in controlled studies might be a result of non-sufficient dietary protein. This could explain the lack of association of $\delta^{15}N$ with body fat in men. Even presenting mean $\delta^{15}N$ values close to women, their protein needs are higher; therefore, no fat gain is observed as a result.

The relationship between the stable isotopes and blood markers was evaluated to assess possible mechanisms affecting the fractionating of carbon and nitrogen, such as impaired liver function and the blood composition itself. The results showed different associations of isotopic composition with blood parameters in males and females, illustrating the different metabolisms inherent to each genre.

The positive association between the total cholesterol and HDL-C with $\delta^{13}C$ values also indicates the whole blood as a proxy of diet using stable isotopes. The association of lower total cholesterol levels in males and HDL-C in females with lower $\delta^{13}C$ values might result from increased ingestion of complex carbohydrates, especially legumes such as beans, soy, lentil, and pea. The

increased intake of complex carbohydrates is associated with decreased serum lipids levels. Additionally, the main sources of complex carbohydrates in the human diet are C_3 plants, which are in line with the results (Hodges and Krehl, 1965; Knuiman *et al*., 1987; Mensink and Katan, 1987; Winham and Hutchin 2007; Siri-Tarino, 2011; Angeles *et al*., 2021). Studies have also associated decreased blood lipids in higher protein diets, especially plant protein and some selected animal-based proteins. However, in the present study, the $\delta^{15}N$ values were not associated with blood lipids (Farnsworth *et al*., 2003; Appel *et al*., 2005; Li *et al*., 2017; Chalvon-Demersay *et al*., 2017; Zhao *et al*., 2020; Zhubi-Bakija *et al*., 2021). Based on such information and our results, showing higher $\delta^{15}N$ values in individuals are associated with increased frequency of animal protein intake, a positive association of nitrogen isotopes ratios with the serum lipids should be expected. The discussion about this lack of association is limited since the dynamics of blood isotopic composition need to be clarified more.

The negative association between nitrogen ratios and the glutamic-oxaloacetic transaminase (GOT) was interesting. As mentioned, deamination is the main process controlling the fractionation of nitrogen isotopes. In this process, enzymes remove the 14N-amino group preferentially from the pool of amino acids from the diet to form ammonia and produce energy. Most of the amino acids from the diet are deaminated, and consequently, the remaining amino acids are ^{15}N enriched. To build new proteins, transamination takes place, being necessary to produce essential amino acids. This process transfers an amino group from glutamic acid to form aspartic acid. Because most of the amino acids are deaminated, the hypothesis is that transamination promotes ^{15}N enrichment by using the remaining pool of amino acids that were not deaminated, even though the enzyme discriminates against the heavier isotope (Macko *et al*., 1986; Miuta and Goto, 2012; Goto *et al*., 2018). In experimental studies conducted by Macko *et al*. (1986) and Miura and Goto (2012), it was observed the enrichment of ^{15}N in the glutamic acid and aspartic acid according to the rate of conversion, and the authors concluded that transamination was responsible for such results.

Therefore, assuming that transamination produces enriched biomass and that the concentrations of GOT in the blood may reflect the potential amount of transamination, we could expect a positive association between the enzyme and

the $\delta^{15}N$ values. However, this was not the case. We observed a negative association in males and females (r = -0.20 and -0.18, respectively), which suggests that transamination promotes the depletion of ^{15}N. This result is in accordance with the preferential translocation of ^{14}N conducted by the transaminases. This apparent inconsistency can be a result of different conditions of in vitro experiments and in vivo dynamics. During the experiments of Macko *et al.*, (1986) and Miura and Goto (2012), the substrate of the reaction was not replaced; therefore, the enrichment of both substrate and products occurred naturally. First, because of the discrimination against the heavier isotope, therefore, the remaining glutamic acid became enriched according to the conversion rate, and second because, in the course of the reaction, the enriched substrate produced enriched products. In vivo, the pool of amino acids under sufficient protein intake is constantly renewed, and the enzyme always promotes the fractionating of nitrogen, producing depleted amino acids.

The main factor resulting in increased GOT concentrations in blood is related to physical disturbances in hepatocytes with the leak of cytosolic content. The concentrations of this enzyme are usually measured to evaluate liver conditions. In the same way, the glutamate-pyruvate transaminase (GPT) concentrations in blood are linked to liver damage. GTP is an enzyme found mainly in the liver, whereas GOT can be found in the liver, heart, muscles, kidneys, pancreas, and erythrocytes (Amacher, 1998). In this study, both enzymes' concentrations were positively correlated (r = 0.68 in males and 0.65 in females, data not shown). This points out that, in fact, a portion of GOT concentrations in blood might come from the liver, but the association with $\delta^{15}N$ values points out that part of the GOT concentrations in blood are also related to the transamination rate.

Another interesting result was that we only observed an association between urea and carbon ratios in females, with a relatively high correlation coefficient r = 0.31. As urea levels are directly linked to increased protein intake, we should expect an association with both carbon and nitrogen composition (Baum, Dichoso, and Carlton, 1975). The hemogram variables were also distinctly correlated with isotopic composition: we observed a positive correlation of leukocytes with carbon ratios in males, whereas hemoglobin, hematocrit, and

erythrocytes were negatively correlated with carbon and nitrogen ratios in females. Total proteins were also negatively correlated with nitrogen ratios in females. These associations found between the genders might result from differences in blood composition and/or in the metabolism of amino acids. This is also evidenced by the differences in the strength of the association between $\delta^{13}C$ and $\delta^{15}N$ in males and females, r = 0.41 and 0.18, respectively. Assuming that transamination produces depletion of ^{15}N, this negative association with hemogram variables and total proteins in women can be linked with a higher transamination rate. However, we observed no association between GOT with such variables or higher GOT concentrations in women's blood.

This study has some limitations because of the smaller sample size of women. In addition, we did not carry out multiple linear models; therefore, we did not control for possible confounding factors. Besides these limitations, this study has great importance. To our knowledge, this is the first report to assess the association between blood carbon and nitrogen stable isotopes ratios with other socio-geographic, economic variables, and blood parameters. These results will contribute to future assays and data interpretation.

2.6. Conclusion

The present study provides new information about stable isotopes to evaluate contemporary diets and confirms the whole blood is a valuable matrix for such evaluations. Comparatively, carbon composition was more associated with a higher number of variables than nitrogen ratios, and its relationships and differences among the levels of categorical variables were more clearly observed. This is associated with the lower number of processes involved in fractionating carbon isotopes in animal tissues. The associations of carbon ratios with altitude, ethnicity, and blood parameters indicate that the observed composition reflects the different intakes of C_3 and C_4 plants derived foods affecting blood composition. These results will be helpful in further studies' interpretation.

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3. CAPÍTULO II - Associations between blood metal concentrations with carbon and nitrogen stable isotopes, risk factors, and health markers in Brazilian farmers

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Abstract

Metals exposure is of great concern because of their toxic effects. The rise of industrial, mining, and agricultural activities has increased human exposure to various elements causing harmful effects on human health; therefore, the knowledge of exposure levels and possible health effects is essential. Thus, in this study, we measured Ni, Co, Cd, and Pb concentrations in the whole blood of farmers in southeast Brazil to address the following questions: 1- How variable are metal concentrations in this population? 2 – Do the metal concentrations in whole blood reflect the carbon and nitrogen isotopic composition? 3 – Do other factors such as age, body mass index, altitude, smoking, alcohol consumption, and gender influence the blood concentrations of such elements? 4 – Are the metal concentrations associated with blood parameters, *in casu*, hemogram variables, liver markers, lipidic profile, blood proteins, glucose, vitamin D, and blood pressure? 5 – What are the metals' reference values for this population? The mean concentrations (μg · L^{-1}) observed were Ni 32 ± 51, Co 2.1 ± 1.5, Cd 9.3 ± 10.6, and Pb 95 ± 89. All elements were positively associated with carbon and nitrogen stable isotope ratios, showing the bioaccumulation process. The age effect on increased Pb concentrations in males and females was also observed, while the body mass index was negatively associated with Ni, Co, and Cd in females. We also observed higher concentrations for all elements in

smokers, Cd levels were statistically higher, presenting an increase of approximately 60 % in this group. Alcohol consumption only affected Pb concentrations, and an association was also observed with the frequency of alcohol ingestion. The differences between genres were observed only for Pb. Males presented 46% higher median Pb concentrations in blood. We observed different associations of metals with blood parameters, including associations with hemogram variables, liver functionality indicators, glucose, vitamin D, and blood pressure, which point out possible toxic effects or other factors influencing the dynamics of the metals. The evaluated population presented elevated concentrations for all elements, and consequently, the reference values ($\mu g \cdot L^{-1}$) were also high Ni 36, Co 3.3, Cd 15.7, and Pb 149. The reference values calculated are higher than the reported in studies of different countries, showing high exposure to the evaluated metals, probably due to point sources and occupational exposure during farming. These results emphasize variables witch increases metals exposure and the need for monitoring programs for toxic substances in Brazil, including evaluating possible health effects.

Keywords: Metals, stable isotopes, human exposure, reference values

3.1. Introduction

Humans are exposed to toxic elements, *i.e.,* heavy metals, mainly through food, drinking water, and air. After the absorption, these elements are distributed to soft tissues where they can bioaccumulate, causing damage. Metals' toxicity depends on the concentration and exposure pathway and can impair organs functionating, especially the brain, lungs, kidneys, and liver (Jarup, 2003). The organ's functionating is impaired because metals may compete for enzymatic sites with essential elements, *e.g.,* Ca, Fe, Zn, possibly causing enzymatic inhibition, impaired absorption of nutrients, and increased production of reactive oxygen species via Fenton reactions (Tchounwou *et al*., 2012; Jaishankar *et al*., 2014; Mahurpawar, 2015; Ufelle e Barchowsky, 2019; Zoroddu *et al*., 2019).

Because of the higher toxicity, exposure to toxic metals is a growing concern since the increasing human activities also increase the bioavailability and environmental release of such elements. Therefore, biomonitoring metal concentrations and their risk factors are essential, especially when evaluating markers that may indicate possible health effects of metal exposure (Meharg *et al*., 2009; Carey *et al*., 2012; Jaishankar *et al*., 2014; Koupaie and Eskicioglu, 2015).

Some socioenvironmental factors may increase human exposure to toxic metals. Occupational and environmental exposure to industrial and mining

activities are related to increased metal concentrations in human tissues and the development of diseases. The frequency of consumption of some foods may also increase metals exposure, especially fish and some vegetables, including rice, maize, potato, and some leafy vegetables. Social habits such as smoking and consuming alcoholic beverages are also known sources of metals for humans, especially Cd and Pb (Wang *et al*., 2007; Garrido *et al*., 2017; Ufelle e Barchowsky, 2019; Affone and Ifediba, 2020).

The concentrations of some elements may vary substantially in different regions, depending on food preferences, water, air, and soil contamination. Therefore, the biomonitoring of metals is challenging due to the lack of reference values for different populations. In addition, exposure to toxic metals may vary over time. Some countries, including the USA, Germany, Canada, China, Korea, and others, have frequently developed cohort studies to monitor metal concentrations in blood and urine. The evaluation of metals exposure is of great importance, not only to evaluate the contamination levels and the natural variability of the elements within a population but also to produce data for long-time biomonitoring programs and the establishment of threshold levels for human safety (Ewers *et al*., 1999; Padilla *et al*., 2010; Kim *et al*., 2012; Moon *et al*., 2013; Rhee *et al*., 2013; Saravanabhavan *et al*., 2016; Awata *et al*., 2017; Jin *et al*., 2018).

For example, lead is a known toxic element associated with neurotoxic effects. For decades, lead was used in gasoline as tetraethyllead, promoting rising atmospheric and blood levels worldwide (Lacerda *et al*., 2022b, *in preparation*). Initially, the Centers for Disease Control and Prevention of the United States (US-CDC) established the threshold level for blood lead concentration of 600 $\mu g \cdot L^{-1}$ in the 60s, which decreased to 300 $\mu g \cdot L^{-1}$ between 1970 and 1985, 250 $\mu g \cdot L^{-1}$ between 1985 and 1991, 100 $\mu g \cdot L^{-1}$ between 1991 and 2011, and since 2012 the recommended threshold is 50 $\mu g \cdot L^{-1}$. However, some studies have associated health effects of lead even with blood concentrations lower than the US-CDC's threshold (Needleman, 1999; US-ASTDR, 2020), which emphasizes the importance of biomonitoring evaluations and the frequent review of such values.

To improve the evaluation of human exposure to toxic substances, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) and the International of Pure and Applied Chemistry (IUPAC) proposed the concept of reference intervals which indicates the background exposure to a chemical in a reference population. There are some divergences in the application of such intervals. However, most articles have defined the reference values (RVs) as the 95th percentile of a pollutant of a reference population, *i.e.,* individuals without a known risk factor for metals exposure (Saravanabhavan *et al*., 2016).

In addition to establishing reference ranges, another challenge in metals exposure studies is determining the source of exposure. In general, the possible metal sources are evaluated through questionnaires; however, the questionnaires can bring inaccurate information. Because food is the main source of metals for the body and feeding patterns influence the level of exposure to certain elements, a more accurate measurement of its influence should be of great interest. A tool that has an important contribution to the assessment of dietary patterns, especially in ecological studies, is the elemental and isotopic composition analysis of carbon and nitrogen. Its application has been used due to the relatively constant enrichment of carbon and nitrogen isotopes in the consumer's biomass. This enrichment is due to a process called fractionation, defined as the accumulation of the heaviest C and N isotope (^{13}C and ^{15}N, respectively) due to their lower chemical kinetics than their lighter isotope (^{12}C and ^{14}N, respectively). In this way, it is possible to differentiate food preferences within the same population and consequently evaluate food preferences' influence on metal exposure (Vanderklift and Ponsard, 2003; Petzke *et al*., 2005; McMahon and McCarthy, 2016; O'Brien, 2015; Litwak, 2018; Goto *et al*., 2018, Lacerda *et al*., 2022a, in preparation).

In this study, we measured Ni, Co, Cd, and Pb concentrations in the whole blood of farmers in southeast Brazil to address the following questions: 1- How variable are metal concentrations in this population? 2 – Do the metal concentrations in whole blood reflect the carbon and nitrogen isotopic composition? 3 – Do other factors such as age, body mass index, altitude, smoking, alcohol consumption, and genre influence the blood concentrations of

such elements? 4 – Are the metal concentrations associated with blood parameters, *in casu*, hemogram variables, liver markers, lipidic profile, blood proteins, glucose, vitamin D, and blood pressure? 5 – What are the reference values for Ni, Co, Cd, and Pb in this population?

3.2. Material and methods

The survey was conducted through personal interviews among 18 and 84 years old who lived in nineteen rural communities in the ten municipalities located southwest of the Espírito Santo state in Brazil (Figure 1). A questionnaire was used to provide information on the sociodemographic characterization of farmers in 2015. The data and sample collection were performed in health or community centers of each locality (Figure 1).

The respondents were selected by adherence, and the individuals who volunteered signed an Informed consent term following the resolution nº. 466/12, of the Brazilian National Health Council (Brasil, 2011). Qualified professionals performed anthropometric assessments (height and weight) in the morning following the Brazilian Food and Nutritional Surveillance System technical report (Brasil, 2011). Stature was evaluated using an Alturexata® stadiometer, with a maximum capacity of 2.10 m and an accuracy of 0.5 cm. The weight was measured on a Tanita® bipolar bioimpedance balance with a BC6011® branded body fat monitor (with 100 g division and a maximum capacity of 150 kg). The Body Mass Index (BMI) was calculated and classified according to World Health Organization (WHO) reference for adults (WHO, 2000). The blood collection was performed following the resolution nº. 466/12, of the Brazilian National Health Council (Brasil, 2012). Samples of 2 mL of peripheral blood were collected into 5 mL tubes containing ethylenediaminetetraacetic acid (EDTA) for metals, stable isotopes, and hemogram analysis; in tubes with NaF for glucose determination, and in tubes without anticoagulant for biochemical determinations. Both anthropometric assessments and blood collection were conducted after an 8-hour fast. After the collection, the samples were placed in styrofoam boxes with ice, and one portion was sent to the Laboratório de Biotecnologia at Centro de Ciências Exatas, Naturais e da Saúde at the Universidade Federal do Espírito Santo for blood parameters analysis, and another portion was sent to the

Laboratório de Ciências Ambientais at Centro de Biociências e Biotecnologia at Universidade Estadual do Norte Fluminense Dary Ribeiro, where they were freeze-dried and stored at -20º C until the isotopic and metal analysis was performed. To evaluate altitude effects on metal concentrations, we assumed the altitude of the collection locations. This measurement was performed using the Google Earth Pro software (Google LLC©).

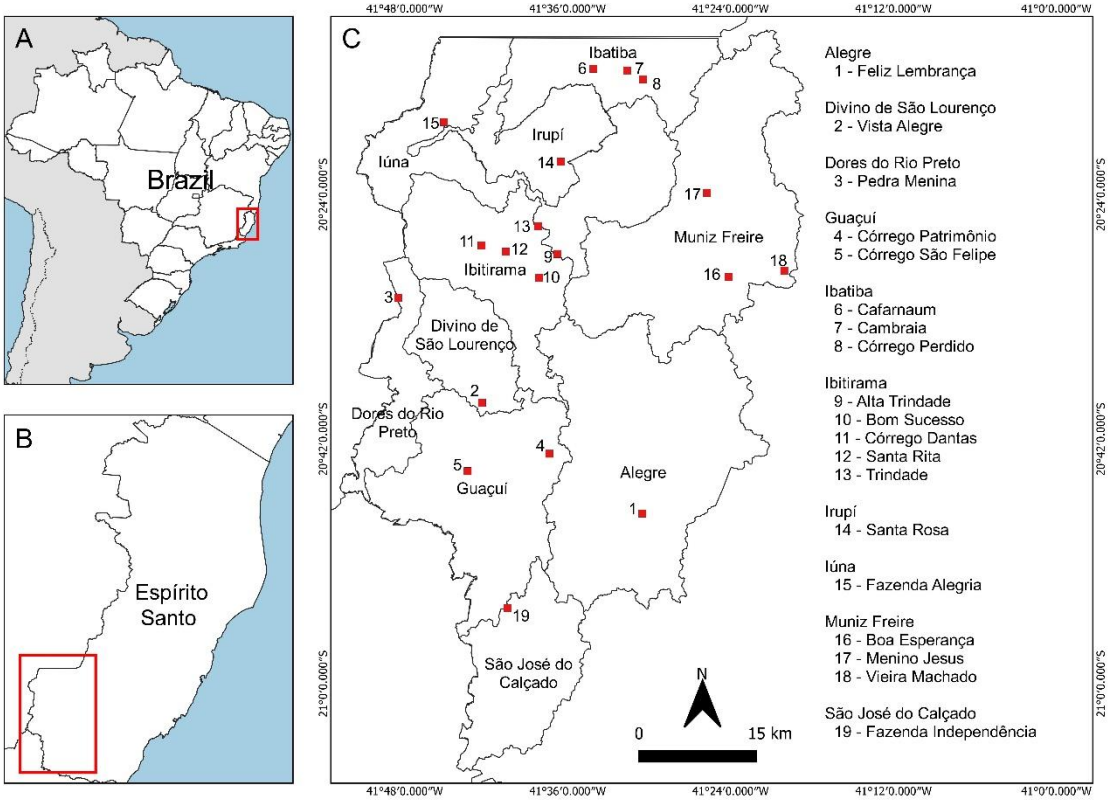

Figure 1. Study area. A - Brazil; B - Espírito Santo state, C - Map of the municipalities in the southwest region of Espírito Santo state with the locations of the nineteen communities (red squares).

Blood parameters analysis

An automatic hematologic analyzer determined the hemogram profile (erythrocytes and leukocyte count, hemoglobin concentration, hematocrit, and platelets) (BC 5380 - Shenzhen Mindray Bio-Medical Electronics, China). Total cholesterol, high-density lipoprotein (HDL-C), triglycerides, total proteins, total bilirubin, creatinine, albumin, glutamic-oxaloacetic transaminase (GOT), the glutamate-pyruvate transaminase (GPT), and glucose levels were determined with enzymatic colorimetric methods using an automatic biochemical analyzer, according to the manufacturer's recommendations (BS 120 - Quibasa-Bioclin,

Brazil). The serum 25-hydroxyvitamin D was determined using the Architect® 25-hydroxyvitamin D test (Abbott Diagnostics, USA), a chemiluminescent immunoassay by microparticles.

Metal determinations

Approximately 1 mL of blood was added in polyethylene tubes (15 mL), followed by 3 mL of HNO_3 (Suprapur® Merck). The samples were kept for 12 hours at room temperature for a pre-digestion, being subsequently heated at 95ºC for 10 hours, according to the adapted methodology from Bazzi, Nriagu, and Linder (2008). The obtained extracts were stored under refrigeration (-20 ºC) until the analysis. At the time of metal determinations of the samples, the extracts were raised to 15 mL with demineralized water. The accuracy of the method was ensured using certified blood samples (Seronorm Human Whole Blood - level 2 - Seronorm™), and the recovery results were Ni = 105 %, Co = 104%, Cd = 81%, and Pb = 92%, additionally, an internal standard of Rh was added to the samples. The determination of metals was performed in an Inductively Coupled Plasma Mass Spectrometer (7500ce ICP MS – Agilent Technologies) at Laboratório de Caracterização de Águas at Pontifícia Universidade Católica do Rio de Janeiro and the detection limits were calculated: Ni = 0.0446, Co = 0.0048, Cd = 0.0186, and Pb = 0.0914 $\mu g \cdot L^{-1}$.

Stable isotopes analysis

For the carbon and nitrogen stable isotopes determination, approximately 0.40 ± 0.03 mg aliquots of the freeze-dried blood samples were weighed in tin capsules for analysis. The elemental and isotopic composition was determined by using an Elemental Analyzer (Flash 2000 - Thermo Scientific, Germany) with interface CONFLO IV coupled to an Isotope Ratio Mass Spectrometer (Delta V Advantage – Thermo Scientific, Germany) at Laboratório de Ciências Ambientais at Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF). The samples were analyzed with analytical blanks and urea analytical standards (IVA Analyzentechnik-330802174; CH_4N_2O M_w = 60, C = 20 %, N = 46 %), using certified isotopic compositions ($\delta^{13}C$ = −39.89 ‰ and $\delta^{15}N$ = −0.73 ‰). Analytical control was done for every 10 samples using certified isotopic standard (Elemental Microanalysis Protein Standard OAS: 46.5 ± 0.78 % for C; 13.32 ±

0.40 % for N; −26.98 ± 0.13 ‰ for $\delta^{13}C$; + 5.94 ± 0.08 ‰ for $\delta^{15}N$). Carbon and nitrogen contents were expressed as percent element (%), and the detection limits were 0.05 % and 0.02 %, respectively. Carbon and nitrogen isotope ratios were expressed in δ notation as ‰ relative to Pee Dee Belemnite and atmospheric nitrogen, respectively. Analytical reproducibility was based on triplicates for every ten samples: ± 0.3 ‰ for $\delta^{15}N$ and ± 0.2 ‰ for $\delta^{13}C$. Accuracy tests were carried out using the certified standard materials, considering recoveries between 90 % and 110 %.

3.3. Statistics

The association between metal concentrations with the numeric possible predictor variables ($\delta^{13}C$, $\delta^{15}N$, age, body mass index, and altitude) was assessed using the analysis of covariance (ANCOVA) using the gender as a covariable (lm, base package, R Core Team, 2022). The differences in metal concentrations and biochemical parameters among the levels of categorical variables (smoking status, alcohol consumption, and genre) were accessed using a one-way analysis of variance (ANOVA) (aov, base package, R Core Team, 2022), followed by Tukey's multiple comparison test (TukeyHSD, base package, R Core Team, 2022). The assumptions of the ANCOVA and ANOVA were evaluated through qq-plot and transformed, when necessary, to meet the linear model's assumptions (normality, linearity, and homoscedasticity of residuals), using a maximum likelihood function (boxcox, MASSpackage, Venables and Ripley, 2002). The correlation analysis (cor.test, base package, R Core Team, 2022) was conducted to evaluate the association among the metals and between the metal concentrations and blood parameters. All variables were previously log-transformed to avoid o possible outliers' effects in correlations. In all applicable cases, an *a priori* type I error of 5% (α = 0.05) was assumed.

Lastly, to evaluate the farmers' current exposure, we calculated the reference values (RVs) for this population and compared them with other populations in different countries. According to the German Human Biomonitoring Commission, the RV comprises the 95^{th} percentile of the measured pollutant concentration levels of a reference population, indicating the upper margin of the current background exposure (Saravanabhavan *et al*., 2016). Since to calculate

the RVs for the present study, we excluded from the analysis all smokers, past smokers, and individuals who reported alcohol consumption.

3.4. Results

Metal associations

The evaluation of metal associations is important to infer the eventually shared source or indicate co-exposures. In this study, the metal associations were measured through Pearson's correlation (Figure 2). Because of the different levels of metals among the genres, the correlation analysis was performed for each gender separately. The elements Co, Ni, and Cd were statistically positively associated in males and females. However, a distinct behavior was observed for lead in males. The concentrations are lower correlated with the other metals, while in females, the correlations are moderately higher, which indicates different processes involving the dynamics of metal accumulation in both genres (Figure 2).

	Ni		Co		Cd		Pb	
Sex	Males	Females	Males	Females	Males	Females	Males	Females
Ni	-	-	**0.59**	**0.65**	**0.28**	**0.47**	*0.13*	0.20
Co	**0.59**	**0.65**	-	-	**0.28**	**0.57**	*0.18*	**0.52**
Cd	**0.28**	**0.47**	**0.28**	**0.57**	-	-	0.09	**0.32**
Pb	0.13	*0.20*	**0.18**	**0.52**	0.09	**0.32**	-	-

Figure 2. Metal correlations in whole blood. The bold and italic numbers represent statistically significant correlations, $p < 0.05$ and $p < 0.10$, respectively. The color pallet indicates the strength of the correlation coefficients.

Nickel

Ni concentrations in the whole population varied from 5.5 to 565 µg · L^{-1} with a mean concentration of 32 ± 51 µg · L^{-1} and were detected in 99,65 % of the samples. Some associations with the measured variables can be observed after removing some outliers (n = 33) (Figure 3).

The concentrations of this element were statistically positively associated with both isotopic ratios of carbon and nitrogen (Figures 3 A and B). There was a higher association of Ni concentrations with the nitrogen ratios between both isotopes, with a mean increase of 3.7 µg · L^{-1} per 1 ‰ increase of $\delta^{15}N$. According

to the carbon ratios, the mean increase of Ni concentrations was 0.9 µg · L^{-1} per 1 ‰ increase of $\delta^{13}C$.

An interaction between Ni concentrations and body mass index was observed between men and women. A negative association of Ni concentrations in blood with body mass index can be observed in women, as the higher the fat percentage, the lower the Ni concentrations. There is a decrease of 5.35 µg · L^{-1} of Ni concentrations at every ten units increase in body mass index. On the other hand, it was not observed in men (Figures 3 D and E). Besides not being statistically significant, the current smokers presented higher Ni concentrations. The median of this group was at least 23 % and 44 % higher compared to non-smokers or past smokers, respectively (Figure 3 G). There were no associations of age and altitude or the frequency of alcohol consumption and sex with the Ni concentrations (Figure 3 C and F, H and I).

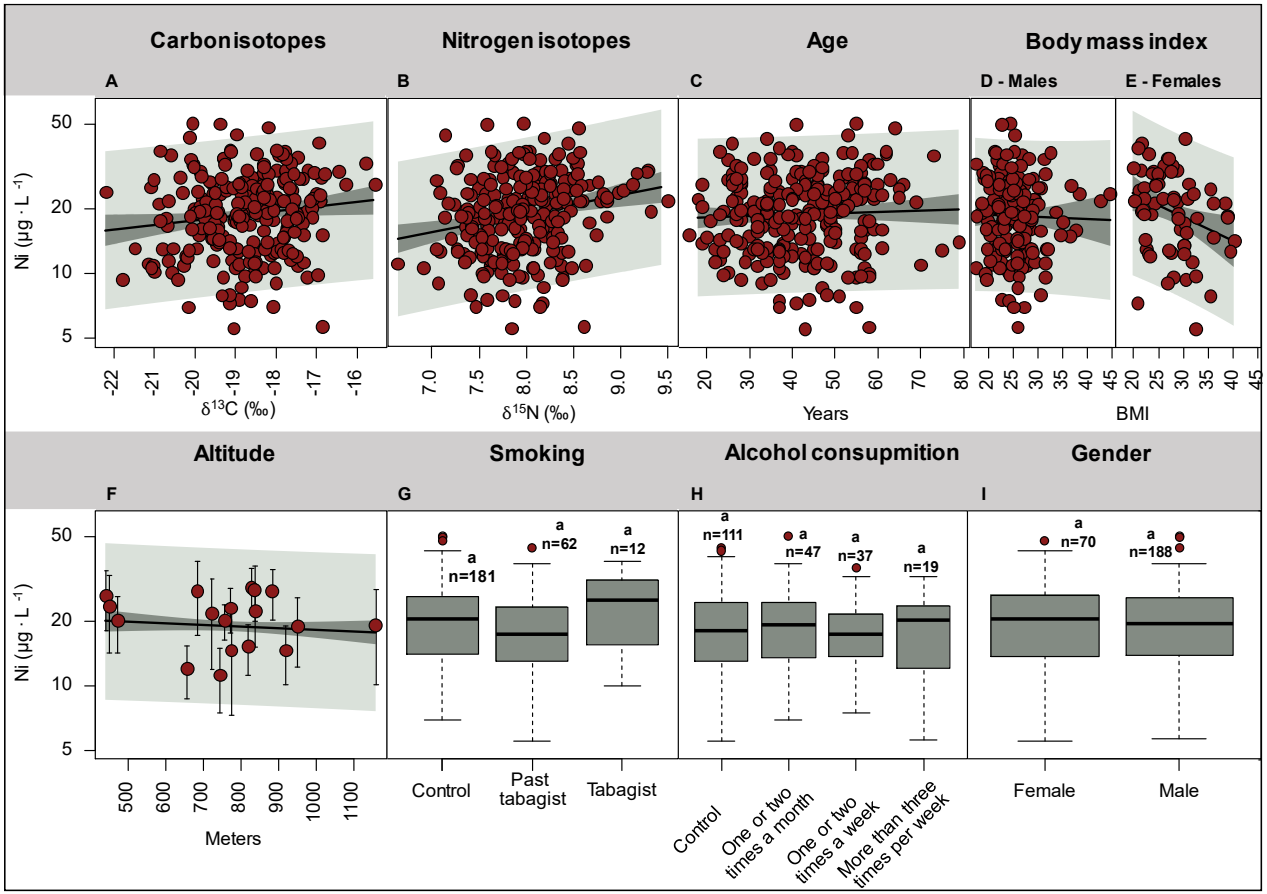

Figure 3. Association of Ni concentrations in blood samples with the measured variables. A – Carbon isotopes, B – Nitrogen isotopes, C – Age, D – Body fat – men, E – Body fat – women, F – Altitude, G – Smoking, H - Alcohol consumption, and I – Sex. Statistics: A - $Y = 0.856X + 36.544$, $p < 0.05$, $R^2 = 0.01$, B - $Y = 3.7166X - 9.127$, $p < 0.05$, $R^2 = 0.04$, C - $Y = 0.0495X + 18.562$, $p = 0.50$, $R^2 = -0.002$, D - $Y = -0.07438X + 21.990$, $p = 0.60$, $R^2 = -0.004$, E - $Y = -0.5353X + 36.149$, $p < 0.05$, $R^2 = 0.09$, F: $Y = -0.0028X + 22.843$, $p = 0.36$, $R^2 = -0.001$. The light and the dark shadings in figures A to F identify the 95% prediction and confidence intervals of the regression models, respectively. The bars in figure F represent the standard deviation. The letters represent statistically different values according to the Tukey test ($p < 0.05$). The distance between y-axis values was log-transformed to optimize data visualization due to the presence of outliers.

Cobalt

Co concentrations in the whole population varied from 0.3 to 14.5 μg · L^{-1} with a mean concentration of 2.1± 1.5 μg L^{-1}. After removing some outliers (n = 5), some associations with the measured variables were observed (Figure 4). The concentrations of this element were statistically positively associated with both nitrogen and carbon ratios (Figures 4 A and B). The Co concentrations increase in mean 0.44 μg · L^{-1} per 1 ‰ increase of $\delta^{15}N$ and 0.08 μg · L^{-1} per 1 ‰ increase of $\delta^{13}C$ values.

In the same way, as observed for Ni, the Co concentrations were negatively associated with the body mass index in women. There is a decrease of 0.40 μg · L^{-1} of Co concentrations at each ten units increase of body mass index in women. In men, no association between such variables was observed (Figures 4 D and E). Co concentrations were also associated with altitude. People living at higher altitudes presented a slight tendency to lower concentrations in the blood. The mean decrease was 0.07 μg · L^{-1} per 100 meters (Figure 4 F). The smoking status also was not statistically significant. However, current smokers presented higher Co concentrations; the median of this group was 28 and 40 % higher than non-smokers or past smokers, respectively (Figure 4 G). There were no associations of age, the frequency of alcohol consumption, and sex with the Co concentrations (Figure 4 C, G, and I).

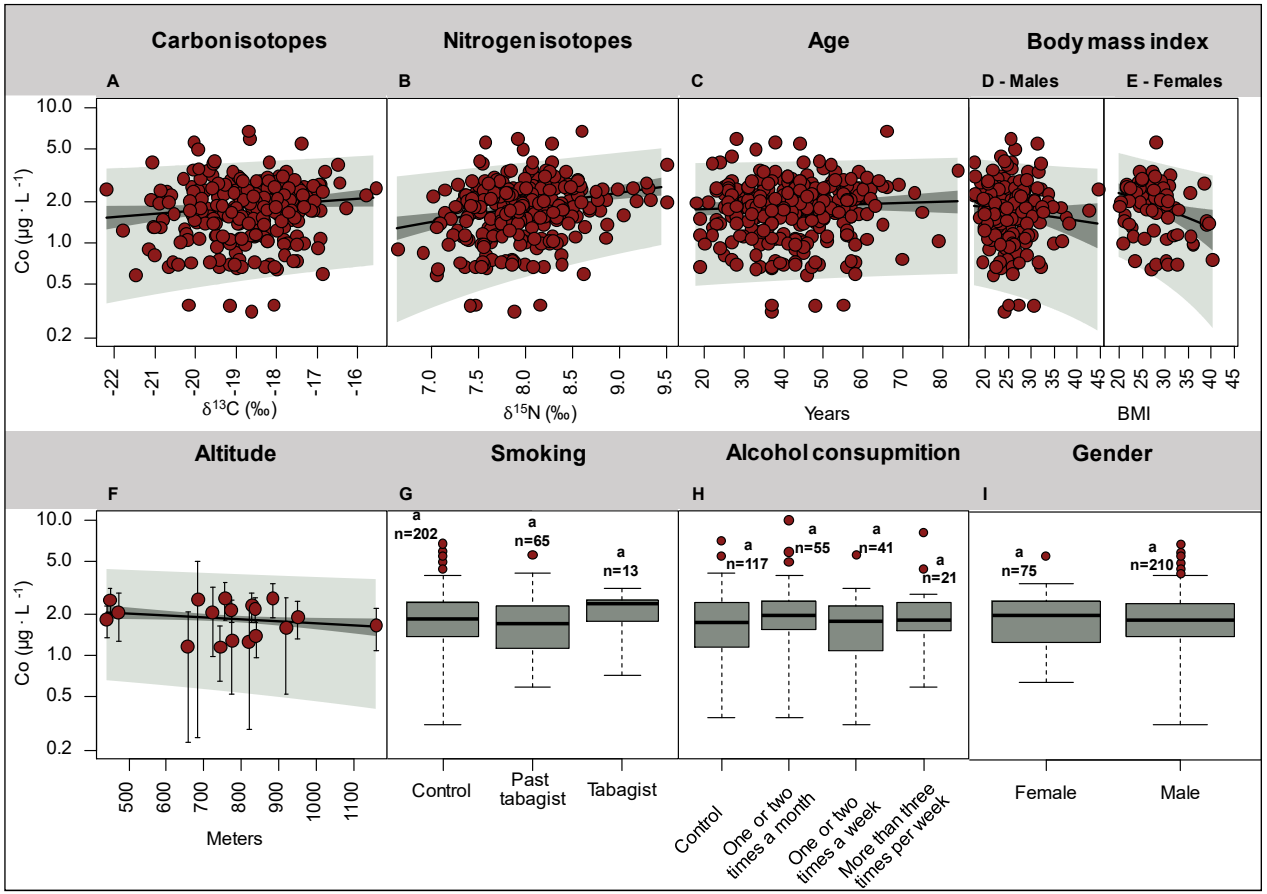

Figure 4. Association of Co concentrations in blood samples with the measured variables. A – Carbon isotopes, B – Nitrogen isotopes, C – Age, D – Body fat – men, E – Body fat – women, F – Altitude, G – Smoking, H - Alcohol consumption, and I – Sex. Statistics: A - $Y = 0.0844X + 3.53$, $p < 0.05$, $R^2 = 0.01$, B - $Y = 0.441X - 1.5712$, $p < 0.05$, $R^2 = 0.06$, C - $Y = 0.0039X + 1.7902$, $p = 0.42$, $R^2 = -0.001$, D - $Y = -0.006X + 2.0593$, $p = 0.22$, $R^2 = 0.002$, E - $Y = -0.0398X + 3.0773$, $p < 0.05$, $R^2 = 0.09$, F - $Y = -0.0007X + 2.4797$, $p < 0.05$, $R^2 = 0.01$. The light and the dark shadings in figures A to F identify the 95% prediction and confidence intervals of the regression models, respectively. The bars in figure F represent the standard deviation. The letters represent statistically different values according to the Tukey test ($p < 0.05$). The distance between y-axis values was log-transformed to optimize data visualization due to the presence of outliers.

Cadmium

Cd concentrations in the blood samples varied from 0.46 to 146 µg · L^{-1} with a mean concentration of 9.3 ± 10.6 µg · L^{-1}. After removing five outliers, some associations with the measured variables can be observed (Figure 5). The concentrations of this element were statistically positively associated with carbon and nitrogen ($p = 0.07$) ratios (Figures 5 A and B). For this element, there was a higher association with the carbon ratios, with a mean increase of 2.6 µg · L^{-1} per 1 ‰ increase of $\delta^{13}C$. The mean increase of Cd concentrations according to the nitrogen ratios was 1.5 µg · L^{-1} per 1 ‰ increase of $\delta^{15}N$ values.

As observed in Ni and Co, Cd concentrations also decrease with increased body mass index in females. There is a decrease of 1.6 µg · L^{-1} of Cd concentrations at every ten units increase of body mass index in women. In the same way, as observed in the other elements, there was no association between Cd and body mass index in males (Figures 5 D and E). The smoking status was statistically significant; the current smokers presented higher Cd concentrations; the median of this group was 59 and 90 % higher than non-smokers or past smokers, respectively (Figure 5 G). The frequency of alcohol consumption was not statistically associated with Cd concentrations (Figure 5 H). However, individuals who reported ingesting alcohol more than three times per week had median Cd concentrations 43 % higher than those who reported no alcohol ingesting. Age, altitude, and sex were not associated with the Cd concentrations (Figure 5 C, F, and I).

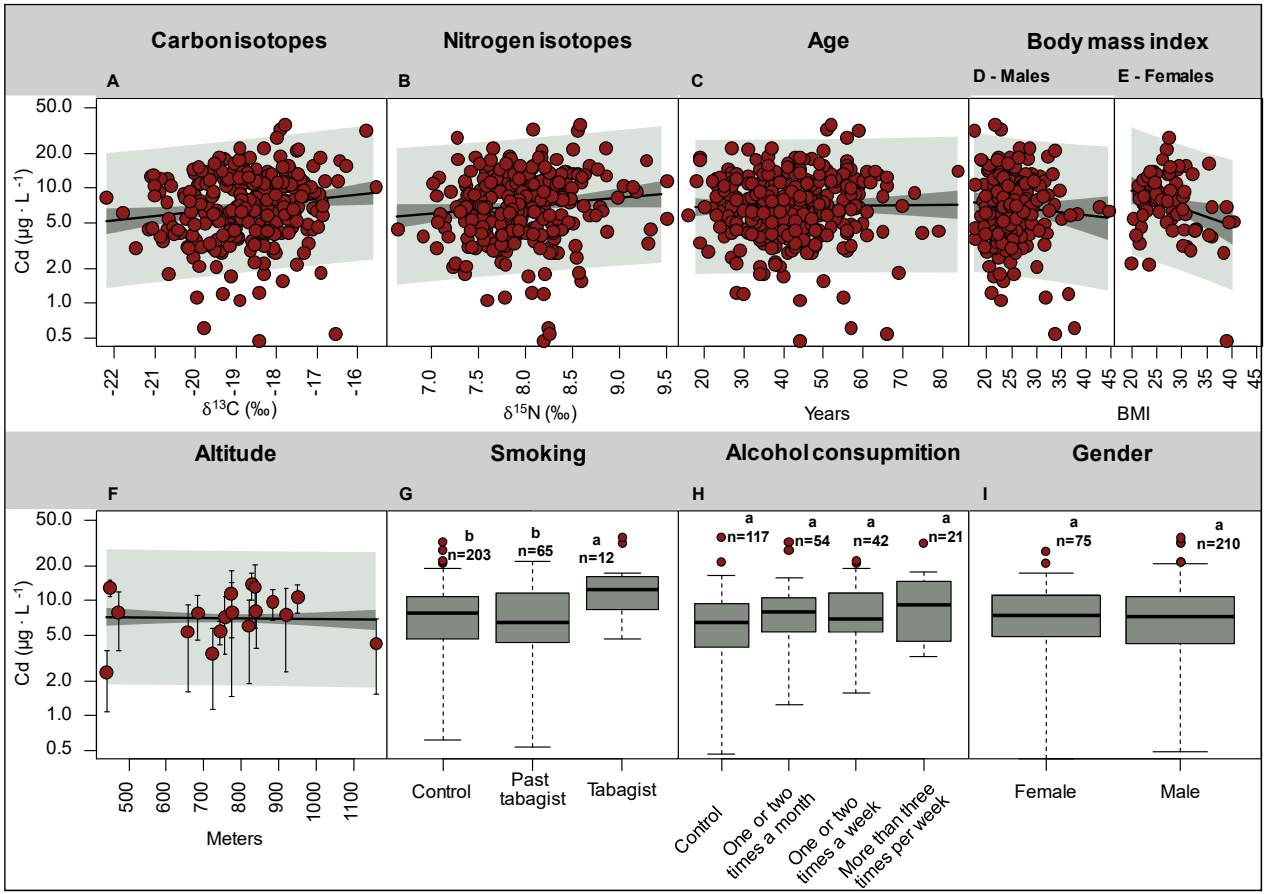

Figure 5. Association of Cd concentrations in blood samples with the measured variables. A – Carbon isotopes, B – Nitrogen isotopes, C – Age, D – Body fat – men, E – Body fat – women, F – altitude, G – Smoking, H – Alcohol consumption, and I – Sex. Statistics: A - $Y = 0.8702X + 24.737$, $p<0.05$, $R^2 = 0.02$, B - $Y = 1.5065X - 3.5742$, $p = 0.07$, $R^2 = 0.01$, C - $Y = 0.0255X + 7.3696$, $p = 0.82$, $R^2 = -0.003$, D - $Y = -0.0928X + 10.752$, $p = 0.28$, $R^2 = 0.001$, E - $Y = -0.1613X + 13.012$, $p <0.05$, $R^2 = 0.06$, F - $Y = -0.0007X + 9.0197$, $p <0.71$, $R^2 = 0.003$. The light and the dark shadings in figures A to F identify the 95% prediction and confidence intervals of the regression models, respectively. The bars in figure F represent the standard deviation. The letters represent statistically different values according to the Tukey test ($p < 0.05$). The distance between y-axis values was log-transformed to optimize data visualization due to the presence of outliers.

Lead

Pb concentrations in the whole population varied from 19 to 847µg · L^{-1} with a mean concentration of 95 ± 89 µg · L^{-1}. After removing eight outliers, it was observed associations with almost all the measured variables (Figure 6). The lead concentrations were statistically positively associated with both carbon and nitrogen ratios (Figures 6 A and B). The mean increase of Pb concentrations according to the nitrogen ratios was 12.4 µg · L^{-1} per 1 ‰ increase of $\delta^{15}N$. The association of Pb concentrations with the carbon ratios was represented by an increase of 8.5 µg · L^{-1} per 1 ‰ increase of $\delta^{13}C$ values.

Pb concentrations were also associated with age; older individuals presented increased lead concentrations in the blood (Figure 6 C). The mean increase according to the aging was 1.1 µg · L^{-1} per year. Lead also presented a marginal association with altitude ($p = 0.10$). People living at higher altitudes presented lower lead concentrations in the blood. However, the locality of Pedra Menina, which is at a higher mean altitude (≅ 1200 meters above sea level), also presented higher mean lead levels. Because of local effects that can strongly influence the concentrations, we also evaluated the association without this locality. Such analysis confirms the association of lead concentrations in blood with altitude. There was a statistically significant decreasing in lead concentrations in higher altitudes ($Y = -0.0532X + 122.25$, $p<0.05$, $R^2 = 0.03$). The mean decrease was 5.3 µg · L^{-1} per 100 meters (Figure 6 E).

The smoking status was not statistically significant; however, the current smokers presented higher Pb concentrations. The median of this group was 41 % higher compared to non-smokers and 17 % higher compared to past smokers (Figure 6 F). A statistically significant association was observed with the frequency of alcohol consumption (Figure 6 G). Individuals who reported ingesting alcohol more than four times per week had median Pb levels 161 % higher than those who reported no alcohol ingesting. Lastly, there was a sex effect. Males' lead concentrations were statistically higher than females. The median concentration in men was 80 µg · $L^{-1,}$ while women presented a median concentration of 55 µg · L^{-1} (Figure 6 I). There was no statistical association between the lead concentrations and the body mass index (Figure 6 D).

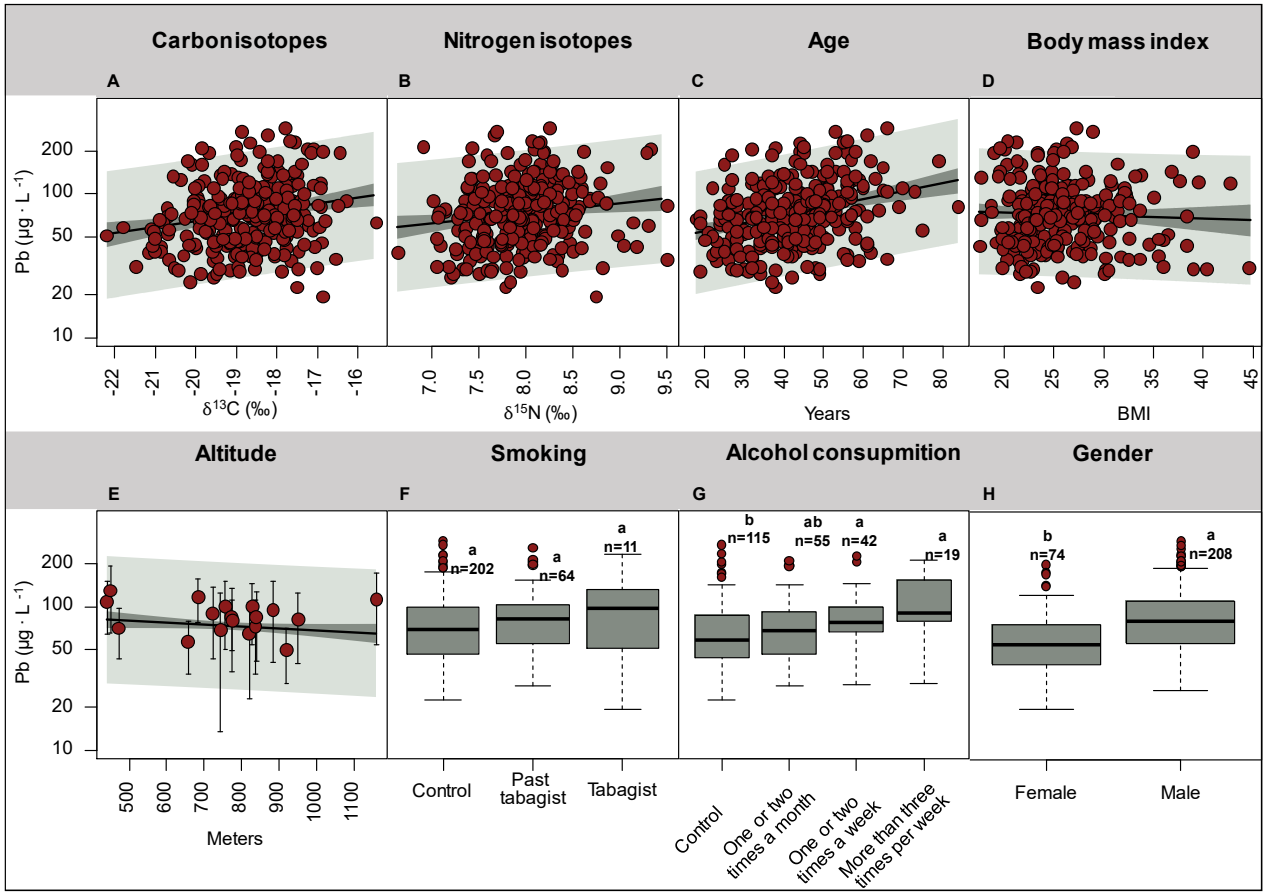

Figure 6. Association of Pb concentrations in blood samples with the measured variables. A – Carbon isotopes, B – Nitrogen isotopes, C – Age, D – Body fat, E – altitude, F – Smoking, G – Alcohol consumption, and H – Sex. Statistics: A - Y = 8.5068X + 242.21, $p < 0.05$, $R^2 = 0.04$, B - Y = 12.412X - 16.086, $p < 0.05$, $R^2 = 0.02$, C - Y = 1.122X + 35.98, $p < 0.05$, $R^2 = 0.09$, D - Y = = -0.4749X + 94.47, $p = 0.43$, $R^2 = -0.001$, E - Y = -0.0239X + 101.84, $p = 0.10$, $R^2 = 0.006$. The light and the dark shadings in figures A to E identify the 95% prediction and confidence intervals of the regression models, respectively. The bars in figure E represent the standard deviation. The letters represent statistically different values according to the Tukey test ($p < 0.05$). The distance between y-axis values was log-transformed to optimize data visualization due to the presence of outliers.

The blood parameters evaluated are presented in table 1. They include the hemogram profile (erythrocytes, hematocrit, hemoglobin, leukocytes, and platelets), liver markers (albumin, total bilirubin, blood creatinine, glutamic-oxaloacetic transaminase (GOT), and the glutamate-pyruvate transaminase (GPT), the lipidic profile (total cholesterol and triglycerides), other blood markers (total proteins, glucose, vitamin D) and blood pressure. The mean of most parameters is in the reference ranges (Table 1). Nevertheless, 24% of females and 22 % of males presented cholesterol levels higher than desirable (Table 1). Also, a considerable percentage (15%) of males presented liver markers (total bilirubin and aspartate aminotransferase) higher than the recommended (Table 1). In addition, a greater percentage of males and females had increased blood pressure (Table 1).

The metal association with blood parameters was evaluated through correlation analysis (Figure 7). The associations varied according to the element and also between the genders. The correlation coefficients between metal concentrations and blood parameters were generally low. Because of the reduced sample size of females, some correlations with the same magnitude as those observed in males were not significant since, for the description of the results, we will consider correlation coefficients: $-0.15 \leq r \geq 0.15$.

The erythrocytes count was negatively associated with Co in both genders and with Ni and Cd in females (Figure 7). On the other hand, the hematocrit profile and the hemoglobin concentration were only associated with Co; both males and females presented a negative association (Figure 7). The leukocytes were associated only with Cd in females, showing a positive correlation, while the platelets were also positively associated with Cd in males (Figure 7).

Liver markers also presented correlations with the metal concentrations. In males, albumin was negatively associated with Cd (Figure 7). The bilirubin concentrations were not associated with any element (Figure 7). Creatinine was negatively associated with Cd in both genders and with Co and Pb in females (Figure 7). GOT was positively correlated with Co and Pb in males and females, while GTP was positively correlated with Co in males and Pb in both genders (Figure 7).

The lipidic profile also showed associations with blood metal concentrations. A positive correlation of HDL with Ni, Co, and Cd in females, with Pb in males and females, and between total cholesterol and Cd in females was observed, while the triglycerides and total proteins were not associated with any element (Figure 7). The glucose levels correlated positively with Cd and Pb in females (Figure 7). Vitamin D correlated negatively with Co in males and females and Cd in females (Figure 7). Blood pressure presented negative associations with Ni, Co, and Cd, while with Pb, the association was positive in females (Figure 7). In contrast, no associations between blood pressure and metal concentrations were found males (Figure 7).

Table 1. Comparison of blood parameters with the reference ranges. RR – Reference range. SD – Standard deviation. The letters represent statistically different values between males and females according to the Tukey test ($p < 0.05$). The reference ranges were obtained from PNCQ, 2019; Rosenfeld *et al*., 2019; Kratz; Lewandrowski and Kent, 1998; Greenland and Peterson, 2017.

			Females					Males				
		Unit	Mean±SD		Reference range	<RR	>RR	Mean±SD		Reference range	<RR	>RR
Hemogram	Erythrocytes	x 10^{12}/L	4.7 ± 0.4	b	4.3 - 4.8	16%	34%	5.12 ± 0.39	a	4.5 - 5.5	4%	14%
	Hematocrit	%	41.5 ± 2.98	b	36 - 46	3%	7%	45.86 ± 4.27	a	40 - 50	3%	7%
	Hemoglobin	g/dL	13.69 ± 1.04	b	12 - 15	6%	10%	15.12 ± 1.43	a	13 - 17	3%	2%
	Leukocytes	x 10^{9}/L	6.76 ± 1.55	a	4 - 10	3%	5%	5.98 ± 2.11	b	4 - 10	4%	2%
	Platelets	x 10^{9}/L	258 ± 66	a	150 - 400	1%	1%	232 ± 55	b	150 - 400	3%	1%
Liver markes	Albumin	g/dL	4.04 ± 0.37	b	3.5 - 5.5	10%	0%	4.22 ± 0.39	a	3.5 - 5.5	4%	0%
	Total bilirubin	mg/dL	0.52 ± 0.25	b	0.3 - 1.0	13%	1%	0.69 ± 0.39	a	0.3 - 1.0	9%	15%
	Creatinine	mg/dL	0.71 ± 0.14	b	<1.5	100%	0%	0.85 ± 0.17	a	<1.5	100%	0%
	GOT	U/L	24.62 ± 5.82	b	0 - 35	0%	6%	29.47 ± 7.46	a	0 - 35	0%	15%
	GPT	U/L	16.57 ± 5.90	b	0 - 35	0%	1%	20.07 ± 7.27	a	0 - 35	0%	4%
Lipidic profile	HDL	mg/dL	54.2 ± 10.41	a	40 - 60	8%	29%	50.8 ± 11.28	a	40 - 60	16%	25%
	Triglycerides	mg/dL	112 ± 66	a	< 160	84%	16%	108 ± 64	a	< 160	80%	20%
	Total cholesterol	mg/dL	177 ± 32	a	< 200	75%	24%	175 ± 39	a	< 200	77%	22%
Other markers	Total proteins	g/dL	6.79 ± 0.54	a	5.5 - 8.0	4%	0%	6.63 ± 0.82	a	5.5 - 8.0	13%	2%
	Glucose	mg/dL	86 ± 12	a	75 - 115	16%	1%	91 ± 24	a	75 - 115	5%	4%
	25-hydroxyvitamin D	ng/mL	45.65 ± 14.60	b	10 - 68	0%	5%	50.42 ± 12.76	a	10 - 68	0%	7%
Blood pressure	Systolic pressure	mmHg	121 ± 19	b	120	41%	36%	128 ± 16	a	120	17%	49%
	Diastolic pressure	mmHg	81 ± 12	b	80	36%	33%	86 ± 11	a	80	14%	48%

		Ni		Co		Cd		Pb	
		M	*F*	*M*	*F*	*M*	*F*	*M*	*F*
Hemogram	Erythrocytes	-0.06	**-0.25**	**-0.31**	**-0.53**	-0.04	-0.18	-0.06	-0.11
	Hematocrit	-0.11	-0.08	**-0.23**	**-0.35**	-0.07	-0.09	-0.06	-0.05
	Hemoglobin	0.00	-0.10	-0.08	**-0.27**	0.02	-0.08	-0.02	-0.02
	Leukocytes	0.02	0.06	0.07	-0.06	0.09	0.12	0.03	-0.15
	Platelets	0.00	0.03	0.11	0.07	**0.21**	0.04	-0.01	-0.05
Liver markes	Albumin	-0.08	0.03	-0.04	-0.14	**-0.29**	-0.07	-0.14	0.02
	Total bilirubin	-0.05	0.00	-0.11	-0.05	-0.06	-0.12	-0.01	0.05
	Creatinine	0.10	-0.05	0.07	-0.05	**-0.28**	**-0.29**	-0.04	-0.20
	GOT	0.08	0.00	0.12	-0.15	-0.07	0.11	0.07	**0.23**
	GPT	0.13	0.02	**0.21**	0.17	0.08	0.06	**0.20**	0.15
Lipidic profile	Cholesterol	-0.11	0.10	-0.09	0.12	-0.12	**0.31**	0.12	0.14
	HDL	-0.08	0.21	-0.06	**0.39**	-0.05	0.25	**0.20**	**0.25**
	Triglycerides	0.04	-0.11	0.13	0.14	0.08	0.09	0.05	-0.04
Other markers	Total proteins	-0.01	0.09	-0.03	-0.12	0.13	0.13	0.10	0.14
	Glucose	-0.05	-0.12	-0.10	0.03	-0.02	0.16	-0.07	**0.25**
	25-hydroxyvitamin D	-0.05	-0.12	-0.06	**-0.33**	0.01	**-0.36**	0.11	-0.14
Blood pressure	Systolic pressure	0.02	-0.21	-0.13	-0.18	-0.13	-0.16	-0.06	0.16
	Diastolic pressure	0.00	-0.19	-0.13	-0.12	-0.08	-0.16	-0.11	0.17

Figure 7. Whole blood metal correlations with blood parameters. The color pallet indicates the strength of the correlation coefficients. The bold numbers represent statistically significant correlations, $p < 0.05$.

To evaluate the farmers' current exposure, we compared the reference values (RVs) calculated in this population. The RVs for this population was 36 µg · L^{-1} for Ni, 3.3 µg · L^{-1} for Co, 16 µg · L^{-1} for Cd, and 149 µg · L^{-1} for Pb (Table 2). A considerable proportion of the individuals presented metal concentrations higher than the calculated for their population. This proportion was classified as follows: Ni: 15.6 %, Pb: 11.4 %, Co: 8.3, and Cd: 8.0 % (Table 2).

The calculated RVs were also compared with published RVs for the general population of some countries (Table 2). It can be observed that the RVs of all elements were higher than all RVs compared. For example, the reference values of Ni were approximately 14 times higher than those observed in the general population of Italy (Table 2). The reference values of Co were almost 8 times higher than those observed in Canada by Saravanabhavan *et al*. (2017). For cadmium, the reference values varied from 5 to 31 times higher than those observed in Korea and another study in Brazil, respectively. The Pb reference values were calculated for all farmers, females, and males. These values are at least 1.7 times higher than those observed in other studies in different countries (Table 2).

Table 2. Comparison of reference values for metals in the blood. *Exclusion criteria: A – Smoking, B – Alcohol consumption. The reference values of the present study are expressed as 95th percentile and the 95 % confidence interval.

Element	Country	Period	Age	n	Exclusion criteria*	RV_{95} (µg · L^{-1})	Reference	> RV
Nickel	Italy	2008-2010	18-65	1422		2.62	Alimonti *et al*. (2011)	
	Brazil	2015	18-79	144	A and B	36.15 (34.32 - 42.89)	This study	15.6%
Cobalt	Canada	2009-2011	3-79	6009		0.38	Saravanabhavan *et al*. (2017)	
	Brazil	2015	18-84	152	A and B	3.34 (2.95 - 3.66)	This study	8.3%
Cadmium	Brazil	2010-2011	18-65	1059	A	0.65	Freire *et al*. (2015)	
	Brazil	2006	18-65	539	A	0.50	Kuno *et al*. (2013)	
	Czech Republic	2005-2009	18-58	896		1.00	Cerná *et al*. (2012)	
	Germany	1997-1999	18-69	3061		1.00	Wilhelm *et al*. (2004)	
	Italy	2008-2010	18-65	831	A	1.08	Alimonti *et al*. (2011)	
	Italy	2008-2010	18-65	617	B	1.41	Alimonti *et al*. (2011)	
	Korea	2005	>20	1997		2.98	Kim and Lee (2011)	
	Korea	2005	>20	1025	A	2.94	Kim and Lee (2011)	
	Korea	2005	>20	488	B	2.93	Kim and Lee (2011)	
	Canada	2012-2013	20-79	2507		0.83	Saravanabhavan *et al*. (2017)	
	Brazil	2015	18-84	158	A and B	15.73 (14.21 - 18.04)	This study	8.0%
Lead	Brazil	2006	18-65	539		56.2	Kuno *et al*. (2013)	
	Brazil	2006	18-65, females	223		44.2	Kuno *et al*. (2013)	
	Brazil	2006	18-65, males	316		65.9	Kuno *et al*. (2013)	
	Czech Republic	2001-2003	18-58	1188		75.0	Batáriová *et al*. (2006)	
	Germany	1997-1999	18-69, females	2303		70.0	Schulz *et al*. (2009)	
	Germany	1997-1999	18-69, males	2342		90.0	Schulz *et al*. (2009)	
	Italy	2008-2010	18-65	1423		51.7	Alimonti *et al*. (2011)	
	Korea	2005	>20	1997		56.1	Lee *et al*. (2011)	
	Korea	2005	>21	1025	A	51.1	Lee *et al*. (2011)	
	Korea	2005	>22	488	B	54.5	Lee *et al*. (2011)	
	Spain	2009	18-65	1880		56.8	Cañas *et al*. (2014)	
	Spain	2009	18-65, females	918		44.80	Cañas *et al*. (2014)	
	Spain	2009	18-65, males	962		64.00	Cañas *et al*. (2014)	
	Canada	2012-2013	20-79	3142		33.0	Saravanabhavan *et al*. (2017)	
	Brazil	2015	18-84	150	A and B	148.6 (136.3 - 169.0)	This study	11.4%
	Brazil	2015	22-84, females	48	A and B	140.9 (111.5 - 179.3)	This study	13.1%
	Brazil	2015	18-75, males	102	A and B	152.2 (135.5 - 168.7)	This study	11.4%

3.5. Discussion

The correlations observed among the metals evaluated indicate continuous co-exposure, except for the lead in males, which presented lower associations with the other elements. Especially Co and Ni presented stronger correlations, which is also an indicator of similar factors influencing the behavior of such elements in the study population (Padilla *et al*., 2010). As we observed in the results section, various variables were associated with the metal concentrations, and these associations varied among the elements and between the genres.

The isotopic composition of carbon and nitrogen in human blood is associated with the increased intake of animal-derived protein sources (Wilkinson, Yai, and O'Brien, 2007, Lacerda *et al*., 2022a, *in preparation*). Therefore the observed association between the isotopic values and the concentrations of Ni, Co, Cd, and Pb represents the effects of bioaccumulation of such elements in human blood. Among these elements, the most associated with the nitrogen isotopic composition according to the determination coefficient was Co > Ni ≅ Cd > Pb showing the meat consumption contribution to metal accumulation in the blood. On the other hand, the increase in $\delta^{13}C$ values was mainly associated with Pb concentrations. These differences in the strength of the associations between metals with carbon and nitrogen ratios need to be further investigated. This is the first report showing the association of carbon and nitrogen stable isotopes with metal concentrations in a human matrice. The dietary patterns are usually evaluated through questionaries, which can produce biased information since these data bring new information about the application of $\delta^{13}C$ and $\delta^{15}N$ values as predictors of human exposure to toxic metals.

The age was just associated with Pb, the mean increase in Pb concentrations was 1.1 $\mu g \cdot L^{-1}$ per year. The association between those variables is already documented. After the absorption, the Pb is accumulated, especially in bones, this bone accumulation increases with age, and the Pb is released into the blood according to the bones' turnover rate making the bone tissue act as an endogenous source of Pb. Indeed, according to Smith, Osterloh and Flegal (1996), about 40-70 % of the circulating blood Pb might be from the bone tissue. This evaluation is important because it shows that the exposure to this element is continuous over the years and evaluates the contribution of this endogenous source of lead.

The association between the body mass index with Ni, Co, and Cd in females was interesting. A previous report by Park and Lee (2013) observed a similar result for males in Korea. The study showed a negative association of Pb and Hg with the percentage of body fat. The dynamics of metals in each tissue depend partly on its turnover rate. As mentioned above, because adipose tissue turnover is slow compared to the skeletal muscle, the concentrations of metals in the blood of people who have more adipose tissue appear to be lower (Popovic *et al*., 2005; Park and Lee, 2013). The different associations between the genders and metal concentrations observed in the present study and by Park and Lee (2013) may be the different fat accumulation in both study populations. In the Korean population, 34.9 % of the male individuals presented a body mass index higher than 25 (obesity) against 26.1 % of females. However, in the present study population, most women (61.9 %) presented a BMI higher than 25, contrary to 43.2 % of males.

The air is not usually a major source of most metals in human blood, except for occupational exposure and people living in industrialized areas. However, for Pb, the use of leaded gasoline in the past decades contaminated the atmosphere worldwide and, consequently, humans. In urbanized areas, it is estimated that about 90 % of blood lead content in the 70 and 90s was due to leaded gasoline. The phase-out of leaded gasoline has decreased atmosphere emissions and blood concentrations. Most metals are transported in the air as particles and are removed by wet and dry deposition. Because the higher altitudes represent increased rain, higher particle deposition might occur, reducing airborne exposure. This process may explain the lower Co and Pb concentrations observed in higher altitudes. Those associations also indicate that in this population, airborne exposure to Co and Pb is also an important source since their concentrations varied according to the altitude, and this pattern was not observed in the other elements (US-ASTDR, 2020; US-ASTDR, 2004; Simonsen, Harbak and Bennekou, 2012; Lacerda *et al*., 2022b, *in preparation*).

Besides not being statistically significant for Ni, Co, and Pb, the median concentrations of all elements were higher in the current smokers. According to Bernhard, Rossmann and Wick (2005), tobacco leaves (*Nicotiana tabacum*) accumulate metals that are released during consumption. Comparing smokers to non-smokers, the increased concentrations followed the order Cd (59 %) > Pb (41 %) > Co (28 %) > Ni (23 %). On the other hand, the frequency of alcohol consumption increased

only Pb concentrations; this pattern has been known for a long time. The mechanisms involving increased Pb concentrations due to alcohol consumption are unclear. Some alcoholic beverages may have elevated Pb concentrations. Some studies also related liver dysfunction caused by increased alcohol intake, which may influence metals elimination (Magid and Hilden, 1975; Grandjean, Olsen and Hollnagel, 1981; Dally *et al*., 1989; Sharper *et al*., 1982; Dally *et al*., 1989; Ufelle and Barchowsky, 2019).

In the same way as the alcohol effect, the gender effect was only observed to Pb concentrations. Males presented higher Pb concentrations (the median was 46 % higher than women). This also is a known pattern (Kristiansen *et al*., 1997; Apostoli *et al*., 2002; Černá *et al*., 2012; Olsén, Lind and Lind, 2012; US-ASTDR, 2020; Lacerda *et al*., 2022c, in preparation). There is some discussion in the literature about the mechanisms involved in the higher Pb concentrations observed in males. These differences have been associated with sex hormones influencing differential bone reabsorption, and a higher number of erythrocytes in males since almost all Pb in the blood is present in the red cells. Additionally, males present higher bone mass, bone density, and bone Pb accumulation; these factors may altogether increase blood concentrations in males (Barry and Mossman, 1970; Barry, 1975; Yip, Johnson, and Dallman, 1984; Silbergeld *et al*., 1988; Rabinowitz, 1991; Smith, Osterloh, and Flegal, 1996; Bergdahl *et al*., 1997; Nieves *et al*., 2004; Barbosa *et al*., 2006; Lee and Kim, 2014; Lu *et al*., 2016, Lacerda *et al*., 2022c, in preparation).

Blood parameters provide information regarding human health and biochemical processes; these variables presented different associations with the measured metals in this study. Previous studies have reported differences in these associations and attributed them to genre differences in human metabolism (Vahter *et al*., 2007; Gade, Comfort, and Re, 2021). However, these associations can indicate possible health effects beyond these natural differences. Therefore, evaluating the relationship between metal concentrations and blood parameters can bring new insights into human exposure, toxic effects, and possible mechanisms controlling blood metal concentrations (Huang *et al*., 2022). Below we will discuss some associations previously reported in the literature and other new relationships observed in this study. The idea is to show these relationships to corroborate previous findings or stimulate further investigations contributing to the knowledge about metal dynamics.

The variables associated with red cells (hemogram variables), *i. e.* erythrocytes, hematocrit, and hemoglobin were all negatively associated with Co. The erythrocytes count was also negatively associated with Ni and Cd in females. These associations were surprising, mainly because of the role of Co in the stimulation of erythropoietin production, increasing erythropoiesis. In red cells, the uptake for Co is shared with Ca and is practically irreversible because Co in the cytosol is bound to the globin moiety of hemoglobin and is not extruded by the Ca-pump. Since we expected a positive association between Co concentrations with the hemogram variables, as many erythrocytes are present in the blood, the higher the Co concentrations should be (Simonsen *et al*., 2011; Simonsen, Harbak and Bennekou, 2012). A possible explanation for these associations might be the blood iron status, as the higher erythrocytes and hemoglobin concentrations, the higher the iron levels are. The influence of iron in the absorption of divalent metals such as Cd, Co, Mn, Cu, Zn, and Pb is well known. A study with Norwegian women showed that low iron stores were related to higher blood concentrations of Mn, Co, and Cd (Meltzer *et al*., 2010). The negative association of Ni and Cd with erythrocytes was only observed in women; this is consistent with their prevalence of erythrocytes and hemoglobin concentrations below the reference range in the present population.

Females presented a negative correlation between leucocytes and Pb. To our knowledge, this is the first report showing such an association. However, the association was within the threshold of the cutoff criteria ($-0{,}15 \leq r \geq 0{,}15$) since further investigation is needed to evaluate the associated factors possibly controlling this relationship. The platelet count was statistically positively associated with Cd in males. These cells are the smallest in human blood and play an essential role in various processes, including homeostasis and thrombosis, clot retraction, and vessel constriction and repair (Zeng *et al*., 2018). Previous studies have reported some effects of this element on animal and human hemostatic systems, including increased platelet aggregation, but the platelet count was not statistically changed. Therefore, this study brings new evidence of the possible effects of cadmium, which could impair the hemostatic system's function (Koçak and Akçil, 2006; Fahim *et al*., 2012; Nontarach *et al*., 2015).

Most liver markers were associated with the evaluated metals, except total bilirubin. Albumin, total bilirubin, and creatinine levels in the blood into the reference

ranges indicate good liver functionating. Albumin and creatinine were statistically negatively correlated with Cd in males and both sexes, respectively, while total bilirubin was not associated with any element. The liver is the main organ responsible for body detoxification since these negative associations might be related to higher liver metabolism, resulting in increased metal elimination and lower blood metal concentrations. On the other hand, increased GOT and GPT in the blood indicate physical disturbances in hepatocytes and the leak of cytosolic content, which also explains the statistically positive correlation of those markers with Co in males and Pb in both genres (Ufelle and Barchowsky, 2019).

The total cholesterol concentrations were statistically positively associated with Cd in females. Heavy metals, including Pb, Hg, and Cd, have been associated with raised serum lipids, possibly contributing to dyslipidemia (increased total cholesterol, LDL-C, and decreased HDL-C levels) (Cho, 2017; Sohn *et al*., 2020; Kim *et al*., 2022). Dyslipidemia is a risk factor for cardiovascular diseases, and its prevalence varies according to ethnicity and socioeconomic and cultural characteristics. The possible metal-induced dyslipidemia is attributed to the inhibition of enzymes related to lipid metabolism and enhanced production of reactive oxygen species (ROS), resulting in increased lipid peroxidation, but the mechanisms are not fully understood (Ding *et al*., 2016; Zhou *et al*., 2016). In a cross-sectional study evaluating the blood Cd concentrations and the lipidic profile of 3,900 adult Koreans, a positive association was observed between Cd concentrations and high-density lipoprotein cholesterol (HDL-C) and the triglyceride to HDL-C ratio. However, high blood cadmium was not associated with a risk of high total cholesterol, high low-density lipoprotein cholesterol, or high triglycerides (Kim, 2012). In another study evaluating an occupationally exposed population in China, the blood cadmium concentration of exposed workers with dyslipidemia was significantly higher than that of workers without dyslipidemia, 5.79 and 4.82 $\mu g \cdot L^{-1}$, respectively (Zhou *et al*., 2016). Tangvarasittichai *et al*. (2015) also reported high Cd exposure with increased triglycerides and reduced HDL-C compared to control individuals. Wang *et al*., 2021 reported a positive association between Co concentrations and HDL-C and argued that increased Co levels increase vitamin B12 and consequently decrease the risk of dyslipidemia. The present study showed a positive association between HDL-C with Ni, Co, and Cd in females and Pb in males and females. As we can observe, the findings are inconsistent, highlighting the need

for more research to investigate such associations and possible effects of metals on blood lipid profiles.

Glucose levels were positively correlated with Cd and Pb in females. Previous studies have reported this direct association and higher concentrations of such metals in diabetic individuals. Cd, Pb, and other metals are supposed to cause deleterious effects on the pancreatic islets through ROS production, promoting impaired pancreas function and the development of insulin resistance and diabetes (Cave *et al*., 2010; Bener *et al*., 2001; Kolachi *et al*., 2011; Leff *et al*., 2018).

Relatively strong correlations were found between females' vitamin D with Co and Cd concentrations. These associations were not expected since this vitamin D is associated with improved absorption of essential and non-essential elements, including Ca, Mg, Fe, P, Zn, Cu, Al, Cd, Co, and Pb (Moon, 1994; Schwalfenberg and Genuis, 2015). Many factors affect vitamin D status in general populations, including sunlight exposure, geographic latitude, skin pigmentation, genetic variation, and certain foods (Tsiaras and Weinstock, 2011). According to Schwalfenberg and Genuis (2015), sufficient minerals such as Ca, Mg, and Zn are essential in vitamin D metabolism. The authors point out that elevated exposure to toxic metals may decrease the absorption of such elements promoting impaired vitamin D metabolism. In addition, the accumulation of toxic elements such as Cd and Pb may disrupt the physiological functioning and the production of vitamin D, possibly due to reduced Ca absorption and toxic effects on kidneys (Moon, 1994; Chang *et al*., 2014). Another study with Korean women related depressed vitamin D with increased iron deficiency anemia (IDA) and that IDA increases Cd concentrations in blood (Suh *et al*., 2016).

Blood pressure was positively associated with Pb and negatively correlated with Ni, Co, and Cd in females. Pb has been associated with increased blood pressure in previous studies (Schwartz, 1991; Nash *et al*., 2003; Alissa and Ferns, 2011). This relationship has been attributed to inducing vasoconstriction, increased renin and angiotensin production due to impaired renal function, and disturbances in Ca metabolism (Bertel, Bühler and Ott, 1978; Jhaveri *et al*., 1979; Alissa and Ferns, 2011). Adverse associations of blood pressure with urinary concentrations of Ni were previously reported in the general population of the United States and were attributed to possible toxic effects on splanchnic sympathetic nerve activity, which controls

sympathetic tone (Miyawaki; Goodchild, and Pilowsky, 2003; Liu *et al*., 2022). This association of blood Ni concentrations and blood pressure was not evaluated yet, since further investigations are needed to elucidate the mechanisms involved in this relationship.

The negative association between Co and blood pressure was also previously reported in children with hypertension. The study was conducted by comparing the serum Co of 19 diagnosed patients and 18 controls in Bulgaria. It also found a negative correlation between Co concentrations with collagen and a positive correlation with elastin, the vascular wall's main proteins that control the rigidity, strength, and flexibility of the vessel's wall (Nicoloff *et al*., 2006). Conversely to our results, cadmium has been associated with raised blood pressure. Some studies report this association in general populations, while others report genre effects, especially in women, and risk factors such as obesity, smoking, and individuals with chronic kidney disease (Eum, Lee, and Paek, 2008; Wang and Wei, 2018; Garner and Levallois, 2017; Satarug, Versey, and Gobe, 2017, Martins *et al*., 2020). Wang and Wei (2018) evaluated the association of blood pressure and blood Cd of 32,791 USA adults from 1999 to 2014 and observed increased blood pressure associated with increased blood Cd in females and urinary Cd in all individuals. Eum, Lee, and Paek (2008) reported increased blood Cd concentrations in individuals with hypertension compared to controls, including a dose-response relationship in the general adult Korean population. Negative associations between cadmium exposure and blood pressure have been observed when evaluating urinary Cd, which is considered a better indicator of long-term exposure (Kurihara *et al*., 2010; Garner and Levallois, 2017; Vallée *et al*., 2020, Martins *et al*., 2020). The mechanisms linking blood pressure and Cd exposure were not fully elucidated (Wang and Wei, 2018) and are associated with effects on cell metabolism, which impairs vascular relaxation, and renal toxicity, individuals with reduced kidney function, have an increased risk of hypertension (Yoopan *et al*., 2008; Kazancioglu, 2013; Wang and Wei, 2018, Martins *et al*., 2020).

The RVs represent the upper values of the background exposure to a chemical in a given time and provide information allowing the identification of individuals with an increased exposure compared to the general population (Ewers *et al*., 1999). Because many factors can impact exposure to certain chemicals, the reference population will vary according to the chemical of interest. For example, it is well documented that

alcohol consumption (as mentioned above) is a risk factor for human exposure to Pb, but this relationship is uncertain for other metals. Thus, data from people who reported alcohol consumption should not be used to establish the reference values for Pb in a human matrice. Therefore, to calculate RVs for this study population, all smokers and the individuals who reported alcohol ingestion were removed from the dataset.

Human biomonitoring is defined as the measurement of the internal dose of chemicals resulting from all exposure routes and has been widely used to evaluate human public health, risk assessment, and risk management decisions (Saravanabhavan *et al*., 2017). Some countries, including Canada, the United States, Germany, France, Italy, the Republic of Korea, and others, have developed human biomonitoring programs, including thousands of participants to monitor the exposure of their populations to certain chemicals. However, most countries (including Brazil) have not developed such monitoring programs yet, and only local studies provide the available data. The comparison of RVs showed that the individuals in the present study presented elevated concentrations of all evaluated elements, even comparing other studies in Brazil. This can be due to their occupational exposure during farming or local sources through food or drinking water. Therefore, those results emphasize the need for monitoring programs for toxic substances in Brazil, including evaluating possible health effects.

This study has limitations because we did not conduct a case-control study, the results indicate only associations, and we cannot assume causality. Additionally, we did not realize multiple linear models because of the smaller sample size of females since we did not control possible confounding factors. Furthermore, the utilization of questionaries to evaluate sociodemographic factors such as drinking and smoking status can produce biased information and misclassification errors. Besides these limitations, this study has great importance. To our knowledge, this is the first report to measure the association between blood metal concentrations and the carbon and nitrogen stable isotope ratios. The study also shows associations between metal concentrations and blood parameters; besides some previously reported associations, our data emphasize such associations and corroborate further investigations. Likewise, this is the first report for blood metal concentrations in this population and will serve in future monitoring programs.

3.6. Conclusion

All elements, but especially Ni and Co, were positively associated in this assay, showing a possible co-exposition from the same source. This study first reports the associations of metal concentrations of carbon and nitrogen stable isotopes in human blood. We observed a bioaccumulation pattern for all evaluated elements since they were positively associated with the isotopic composition. Furthermore, the data also point to differences in metal dynamics between genres. In this study, the increased fat accumulation in women was associated with decreased blood Ni, Co, and Cd concentrations. Our data also supports the previous reports showing the air as an important source of exposure for Co and Pb. Besides not being statistically significant for Ni, Co, and Pb, the median concentrations of all elements were higher in the current smokers. In parallel, the frequency of alcohol consumption increased only Pb concentrations.

The associations observed between blood parameters and metal concentrations also point out the possible toxic effects of the evaluated elements, especially in women. Some metals were associated with decreased hemogram variables. Negative associations were also observed between metals and increased liver functionating markers. On the other hand, other liver markers (GOT and GTP), which indicate hepatocyte damage, were associated with increased concentrations of Pb in males and females and Co in males. Such associations indicate the liver's role in eliminating these elements or even possible toxic effects of such elements on hepatocytes. Other markers were also correlated with metal concentrations, glucose levels, vitamin D, and blood pressure. We cannot assume causality based only on these associations. However, these data will corroborate the development of case-control studies to elucidate these possible patterns.

As discussed, the population evaluated presented elevated concentrations of all evaluated elements, which might be associated with their occupational exposure or with local sources of such elements. These results emphasize the need for monitoring programs for toxic substances in Brazil, including evaluating possible health effects.

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4. CAPÍTULO III - Global decrease in blood lead concentrations due to the removal of leaded gasoline

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Abstract

Lead (Pb) is a chemical element with high toxicity that is classified as one of the ten chemicals of most significant concern to human health. The general population is exposed to Pb mainly through food, air, or occupational activity. The mechanisms of Pb toxicity are diverse, depend on the dose, duration, and route of exposure and are mainly associated with the nervous system. The main problem involving lead is its use as a fuel additive (tetraethyllead) at a global level, which raised the atmospheric Pb concentrations. It is estimated that between 80 and 90% of the atmospheric lead in large cities came from the use of tetraethyllead, and as a consequence, it was also the main responsible for human exposure to the element. Therefore, this work aimed to evaluate, through a systematic review, the blood concentrations of Pb in scientific articles published in the first two decades of the 2000s to compare the global and regional trends of each continent over time. Our data show the importance of removing tetraethyllead in decreasing human exposure to Pb worldwide. We observed exponentially decreasing blood Pb concentrations over the years after additive removal on all continents, resulting in a global trend. In addition, the results also showed that, despite the removal of the additive providing lower levels of human Pb exposure, the general population remains exposed to the element through environmental and occupational sources and lifestyle habits such as smoking and alcohol consumption.

Keywords: Lead, blood, gasoline, human exposure, tetraethyllead.

4.1. Introduction

Lead (Pb) was classified by the World Health Organization as one of the ten chemicals of greatest concern worldwide, and it received the first rank as the most dangerous substance on the United States Agency for Toxic Substances and Disease Registry (US-ATSDR) list between 1991 and 1997. Since then, it has occupied the second position, and each year, there are an estimated 600,000 new cases of children with intellectual disabilities caused by exposure to Pb, mainly in developing countries. The concern involving the element is due to its high toxicity, which mainly causes neurological problems in children and adults, and its widespread use in paints, electronic devices, batteries, and, until recently, as a fuel additive (WHO, 2020; US-ASTDR, 2020).

Human exposure to Pb occurs mainly through occupational activity, inhalation or ingestion of particles, and industrial workers are the most affected. The general population is exposed primarily by food ingestion, by household air inhalation due to Pb-particle emissions from paints, and by water consumption contaminated with Pb from old tubes used in water distribution (Silva, 1981; Needleman, 2004; Brown and Margolis, 2012). Adult individuals absorb between 5 and 15% of ingested lead and retain less than 5% of what is absorbed. After absorption, Pb is distributed through the soft tissues and can cause toxicity, and posteriorly is accumulated in the bones. Bone tissue reabsorption can be responsible for approximately 40 to 70% of the blood concentration of this element and is an important source of endogenous contamination. This process is of particular concern in people with accumulated exposure, in pregnant and lactating women, during menopause, or due to osteoporosis (Barry, 1975; Gulson *et al*., 2003; Silbergeld and Patrick, 2005).

The mechanisms of Pb toxicity are diverse and depend on the dose, duration, and route of exposure. Symptoms are mainly associated with the nervous system. In general, children are more sensitive to the effects on the central nervous system because, proportionally, children absorb more Pb than adults, reaching 41%, and retain approximately 30% of what is absorbed. As a result, several studies have shown the association between Pb exposure and cognitive deficits (Ziegler *et al*., 1978; Lanphear *et al*., 2000; Bellinger and Needleman, 2003;

Canfield *et al*., 2003; Lidsky and Schneider, 2003; Chiodo, Jacobson and Jacobson, 2004; Lanphear *et al*., 2005; Kordas *et al*., 2006; Téllez-Rojo *et al*., 2006). In adults, the main concerns are peripheral neurological disease and hypertension (Martin *et al*., 2006; Navas-Acien *et al*., 2007; Bellinger, 2011). Pb can affect the central nervous system by several mechanisms such as disrupting calcium homeostasis and acting as its substitute, modifying synaptic patterns, and compromising the functionality of the hepatocephalic barrier (Marchetti, 2003; Marchetti, 2014). The peripheral nervous system can be affected through demyelination and degeneration of axons (Goyer, 1993). Pb is also associated with diseases of the cardiovascular, immune, renal, and bone systems. In addition, some studies have identified inorganic Pb as probably carcinogenic to humans (Navas-Acien *et al*., 2007; WHO, 2020).

The main problem involving Pb is its use as a fuel additive at the global level. The manufacture of tetraethyllead for use in gasoline as an anti-knocking agent began in the United States in 1923. Afterward, the deaths of workers involved in the production of the additive and the development of neurological problems began to occur, leading to the creation of the first limits on the use of this compound of 1 mL per gallon (3.78 L) in 1926. The discussion regarding Pb toxicity persisted for years due to scientists who had conflicts of interest and insisted on denying the association between Pb and recorded occupational fatalities. Only in 1960 was the first limit of 600 $\mu g \cdot L^{-1}$ defined as the reference concentration of lead in human blood (Needleman, 1999). In 1986, the United States Environmental Protection Agency (US EPA) demonstrated an association between the amount of Pb used in gasoline and the element concentrations in the blood and the air (US EPA, 1986). The impact of using the additive on Pb concentrations in the atmosphere was significant. It was estimated that between 80 and 90% of the atmospheric Pb in large cities came from the use of tetraethyllead, and as a consequence, this compound was also mainly responsible for human exposure to the element (World Bank, 1999; WHO, 2020). Additive use occurred on a large scale. Only in the USA approximately 250,000 tons of lead were consumed per year between the late 1960s and early 1970s. The decline in additive use in the USA was progressive, starting in the late 1970s and continuing until the 1990s, when the total elimination of tetraethyllead was completed (OECD, 1994, Nriagu, 1990).

Due to the high toxicity and widespread use of tetraethyllead, several countries began the phasing-out. There was a gradual elimination of the additive use until the total phase-out, when each country reached 100% use of unleaded gasoline. The phase-out had Japan as a pioneer country in the 1980s, followed by almost every country until 2006, except for some countries in Africa and Asia (World Bank, 1997; Meyer, Brown and Falk, 2008). The use of leaded gasoline only stopped in all countries in 2021, when Algeria was the last country to remove tetraethyllead.

Thus, this review presents a synthesis of temporal trends in Pb concentrations in the blood of populations on a global and regional scale and the assessment of risk factors for environmental, occupational, gender and lifestyle exposure. In addition, we point out gaps in data reporting that limit the interpretation of the results. We also suggest paths for future studies.

4.2. Material and methods

4.2.1. Systematic search

We carried out a search for articles published through the ScienceDirect platform to obtain the data. The search strategy was organized into three steps. First, a search was performed for research articles from 2000 to 2019 that contained the terms heavy metal and blood in the title, abstract, or keywords, resulting in 2,298 articles. The choice of the term heavy metal was made because Pb in English is both a verb and a noun, which results in many papers unrelated to the objective of this review. The second step consisted of filtering the articles previously selected, in which only those that reported some measure of data centrality and sample size remained in the database. Due to the criteria of the second step, studies with Pb quantification in animals and other matrices, such as blood plasma or umbilical cord blood, and those involving pregnant or lactating women and people who had undergone implants of metallic prostheses were excluded. At the end of the second step, 132 studies were selected. The African continent and South American countries were underrepresented after this second stage, with only 13% and 9% of their total countries represented in the database, respectively. Thus, a third step was carried out, searching for articles in the

reference lists of the 132 selected studies containing data from these continents' countries. After the three steps, 163 studies containing data from 63 countries were selected for analysis, representing 22% of the countries in Africa, 38% in Asia, 42% in South America, 49% in Europe, and 100% in North America. Oceania was represented only by Australia; therefore, no statistical analyses were performed on this continent (Table 1).

To test the hypothesis that the blood Pb concentrations are associated with the removal of tetraethyllead from gasoline, a bibliographic survey of the year of removal of this additive in each country was carried out. The survey consisted of an active search for the date of removal of lead in gasoline in scientific articles and documents from international and national agencies in the countries in which it was possible to carry out temporal analyses. Since some documents, mainly from the World Bank, the United Nations and Organization for Economic Cooperation and Development, also reported data from other countries that were not analyzed in this work, it was possible to compile the year of elimination of the additive not only in the 34 countries that were used in the temporal analyses but also in another 131 countries (Supplementary Table 2).

4.2.2. Data extraction

The extracted data were grouped by continent (North America, South America [including Central America and Caribbean countries], Africa, Asia [including Australia], and Europe), country, year of sampling, sex, age (children and adults), presence or absence of comorbidities, type of exposure (occupational, environmental) and lifestyle habits such as smoking and alcohol consumption.

The exact year of blood sampling was reported in only 30% of the selected articles. To perform temporal analyses, studies that reported sampling periods, instead of sampling years, were used. When the study evaluated a population over a two-year period, the last year was considered the sampling year. When the study evaluated a population over a three-year period, the middle year was considered the sampling year. Studies that evaluated populations for periods greater than three years were not considered for temporal analyses.

For the statistical analyses, the normal distribution of the data reported by the authors was assumed. When the study reported more than one measure of centrality and dispersion, preference was given to measures in the following order: mean, median, geometric mean, accompanied by its respective measure of dispersion.

The different measures of dispersion were converted to standard deviations so that it was possible to simulate the original sample population of each study in the R environment (rnorm function, base package, R Core Team, 2020):

Confidence intervals and means were used to calculate the margin of error, which were later converted into standard deviations according to the equation:

$$s = \frac{(CIupper - m) * \sqrt{n}}{\Phi}$$

where *s* is the standard deviation, CI_{Upper} is the upper confidence interval, m is the mean, n is the sample number, and Φ is the upper percentile of the z-standard normal distribution.

Likewise, the interquartile ranges were converted into standard deviations according to the equation:

$$s = \frac{\mathrm{IQR}}{2\Phi^{-1}(\frac{0{,}75n - 0{,}125}{n + 0{,}25})}$$

where *s* is the standard deviation, IQR is the interquartile range, n is the sample number, and Φ is the upper percentile of the z-standard normal distribution.

When the study reported more than one percentile, all were converted to standard deviations, and the entire mean was used as the closest measure of the standard deviation of the original sample population (Wan *et al*., 2014).

4.2.3. Statistical analysis

After compiling the mean, standard deviation, and sample size of each variable, we simulated the original populations of each study (rnorm, basic package, R Core Team, 2020) and performed the statistical analyses. The original distribution of many

articles were asymmetric, generating measures of dispersion larger than the measure of centrality. Thus, when simulating these specific populations, negative values were generated. As negative values for the concentration of a chemical element are not possible, all values below zero were discarded. With the simulated data, comparisons of the Pb concentrations among the continents, sexes, and types of exposure were carried out. In addition, temporal trends of Pb concentrations in blood were also determined.

The statistical analyses were conducted using linear mixed-effects models (lmer, lme4 package, Bates *et al*. 2015). Thus, the variation within the different studies was considered a random effect, and the variation between studies was considered a fixed effect. The total Pb concentration for each continent, the ratio between the sexes, and among the types of exposure were compared through type III analysis of variance with Satterthwaite approximation for the calculation of degrees of freedom (lmerTest, Kuznetsova *et al*. 2017), followed by Tukey's multiple comparison test (emmeans, emmeans package, Lenth *et al*., 2018), and significant differences were reported using letter-based representations (cldList function, rcompanion package, Mangiafico, 2022).

The ratios of Pb concentration between sexes and among exposure types and control populations were obtained using empirical combinatorial analysis (expand.grid function; base package; Monte Carlo Method, Khitalishvili, 2016). The ratios calculated through all possible combinations are important in reducing the variance between different studies in the same period or different periods. Due to the high number of combinations produced with this technique, the analysis of variance of the ratios was performed using 100,000 ratio values randomly selected among all generated (sample, base package, R Core Team, 2020) for each level of the categorical variables.

For the analysis of the types of exposure, only studies that had control populations and one type of exposure that was evaluated by more than three studies were considered. The analyses of temporal trends (Pb concentration as a function of years and the period of Pb removal in gasoline) were performed through mixed-effects analysis of covariance using sampling year and continent variables followed by Tukey's multiple comparisons test to compare the slopes obtained among continents (emtrends, emmeans package, Lenth *et al*., 2018). When an effect was detected (anova function, lmerTest package, Kuznetsova et al., 2017), the regression equation associated with the

model's fixed effects (fixedef function, lme4 package, Bates et al., 2015) was reported together with the R^2 marginal and conditional values (r.squaredGLMM function, MuMIn package, Bartoń, 2022), which measure the variance explained by the fixed effects and the model as a whole, respectively.

Data were transformed to meet the assumptions of mixed linear models (normality, linearity, and homoscedasticity), when necessary, using a maximum likelihood function (optim.boxcox, boxcoxmix package, Almohaimeed & Einbeck, 2020). The models were validated using diagnostic graphs (Altman and Krzywinski, 2016). In all applicable cases, an *a priori* type I error of 5% ($\alpha = 0.05$) was assumed.

The map of the global distribution of Pb was constructed with the original dataset of each country using all the studies that presented some measure of centrality and sample size of populations randomly sampled and/or in which no type of exposure was reported. These data were used to calculate each country's weighted average Pb concentration.

4.3. Timeseries of the studies

An exponential increase in the number of publications referring to Pb contamination in human blood has been observed in the last 20 years (Figure 1). The data suggest that the topic is relatively poorly addressed, since the number of articles published per year is still quite restricted and until recently (between 2000 and 2014), the number of publications per year did not exceed 10 articles. The main interest in Pb research (64% of the articles) was the association of the element with some kind of exposure, mainly involving environmental exposure (24%), smoking (19%), occupational exposure (18%), and alcohol consumption (7%). This means that a few studies (36%) were dedicated to biomonitoring studies in random populations.

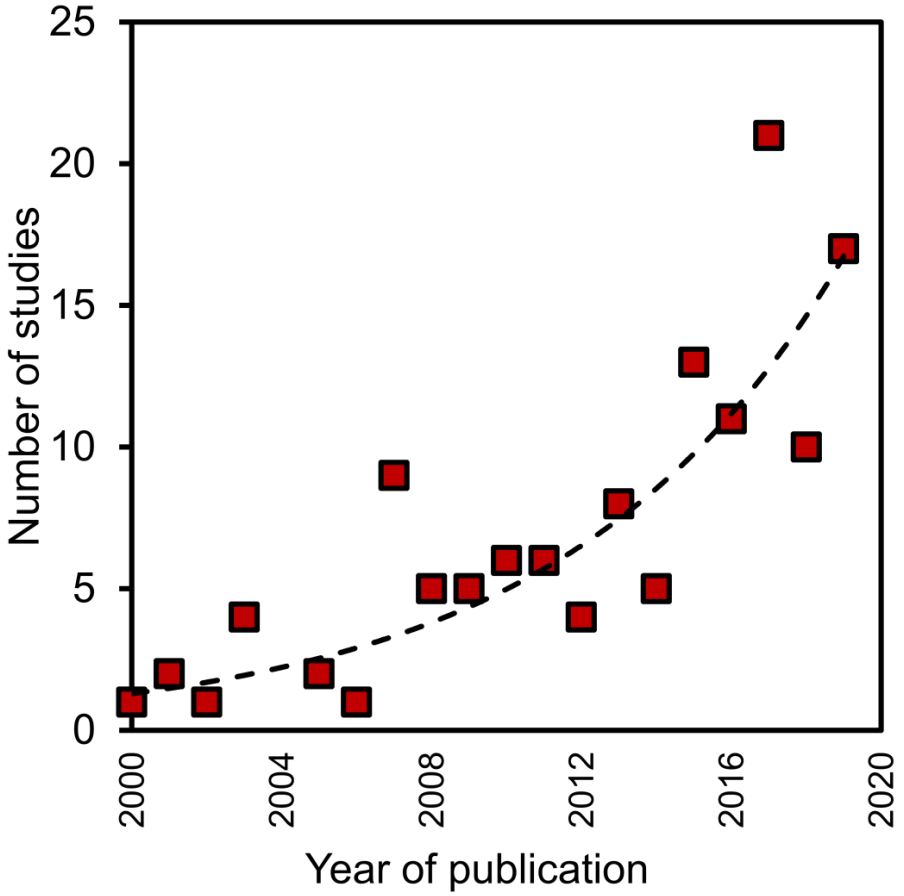

Figure 1. The number of publications that evaluated Pb concentration in human blood between 2000 and 2019. All studies that resulted from the systematic search were used to construct the graph (n = 132). The list of studies used to construct this graph can be found in Supplementary Table 1. Regression statistics: $Y = 2 \cdot 10^{-117} \cdot e^{0.1345 \cdot X}$, $R^2 = 0.73$, $p < 0.05$.

As the systematic search was performed using the term heavy metal, the selected studies did not contain data exclusively on Pb but also on 66 other elements that are toxic elements and macro- and micronutrients such as Cu, Se, Ca, Mg, P, Na, and S, in addition to rare earth elements such as Sc, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb and Lu (Figure S1). Among the elements analyzed in the different studies, Pb was the most studied, being present in 96% of the articles, followed by Cd with 71%, Hg with 45%, and Mn with 34%. The other elements were represented in less than 30% of the studies (Figure S1).

4.3. Global distribution of blood Pb concentrations

The lead concentrations evaluated in the populations of different countries showed great variation (2.07 to 298.10 μg · L^{-1}), with an overall weighted average of 34.61 μg · L^{-1} (Figure 2 and Table 1). The variation in the data can be explained, in part,

through a continental grouping. The highest concentrations were observed on the African continent in countries such as Ethiopia, Nigeria, Congo, Egypt, Botswana, Togo, South Africa, Morocco, and Senegal where values were at least twice as high as the global average (Figure 2 and Table 1). Elevated lead concentrations were also observed in Southeast and South Asian countries, in some South American countries such as Puerto Rico, Peru, Uruguay, and in North America and Mexico (Figure 2 and Table 1). The countries of the Northern Hemisphere, in general, had lower concentrations, as seen in the United States, Canada, most European countries, and northern Asia. European countries showed the smallest concentration variations (between 8.1 and 48.6 $\mu g \cdot L^{-1}$) (Figure 2 and Table 1). The data also reveal the poor representation of studies, mainly in Africa and South America, with only 12 and 13 countries represented, respectively (Figure 2 and Table 1).

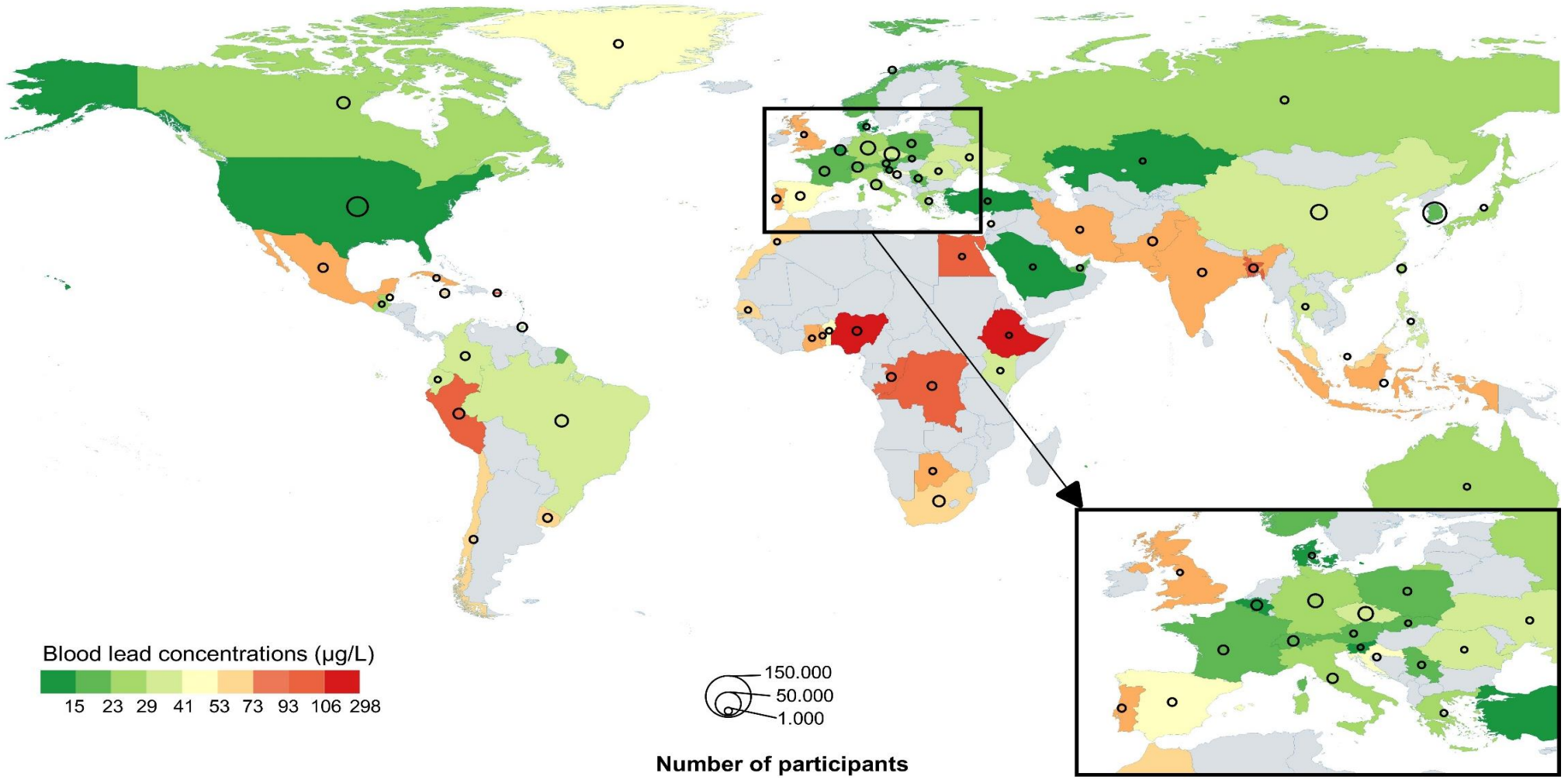

Figure 2. Map of the global distribution of Pb in human blood (µg·L^{-1}). The legend shows Pb concentrations as a weighted average considering all studies obtained for each country with the concentration and number of participants. For map construction, only studies that reported sampling of random populations or in which no risk factors for exposure or comorbidity existed were included for each country. Countries marked in gray do not have data. The list of studies used to construct the map can be found in Supplementary Table 1. Miller's projection system (ESPG 53003). Organization: Diego Lacerda.

Table 1. The weighted average of human blood Pb concentration in each country. The list of studies used to build this table can be found in Supplementary Table 1.

Continent/ Subcontinent	Country	Weighted average ($\mu g \cdot L^{-1}$)	Total participants	Total studies
North America	United States of America	11.70	21,085	9
	Canada	38.44	3,001	5
	Greenland	51.48	1,020	1
	Mexico	105.16	1,493	3
South America	Trinidad and Tobago	28.00	1,761	1
	Ecuador	31.70	69	1
	Colombia	32.84	1,126	4
	Brazil	34.78	5,215	12
	Belize	49.45	93	1
	Uruguay	61.95	1,166	5
	Jamaica	62.16	1,403	2
	Cuba	86.00	114	1
	Peru	87.60	3,042	4
	Puerto Rico	106.37	438	2
Africa	Kenya	37.00	65	1
	Benin	47.73	70	1
	Senegal	65.10	52	1
	Morocco	71.00	39	1
	South Africa	72.99	3,762	5
	Togo	82.27	70	1
	Botswana	88.00	213	1
	Egypt	97.50	45	1
	Democratic Republic of Congo	105.42	1,140	4
	Nigeria	277.53	1,159	5
	Ethiopia	298.10	36	1
Europe	Denmark	8.10	73	1
	Slovenia	13.40	42	1
	Belgium	14.79	2,878	2
	Suécia	18.78	2,717	5
	Sweden	19.40	57	1
	Austria	19.47	186	1
	Norway	19.90	360	2
	Serbia	21.17	305	1
	Poland	21.76	644	4
	France	22.80	1,992	1
	Greece	24.00	185	1
	Italy	27.94	2,416	6
	Germany	28.85	7,924	6
	Ukraine	31.65	212	1
	Czech Republic	31.67	8,466	3
	Romania	37.00	84	1
	Portugal	39.17	627	4
	Spain	45.89	956	4
	Croatia	48.66	440	3
Asia	Turkey	2.07	48	1
	Kazakhstan	10.37	25	1
	Saudi Arabia	11.22	20	1
	South Korea	19.45	32,052	10
	United Arab Emirates	23.08	74	1
	Taiwan	24.95	1,022	7
	Japan	25.34	104	2
	Russia	29.31	534	2
	Thailand	32.30	52	1
	Philippines	37.00	45	1
	China	41.07	11,029	11
	Lebanon	46.60	42	1
	Malaysia	65.40	47	1
	India	81.75	803	5
	Indonesia	86.00	397	1
	Iran	89.94	189	2
	Pakistan	91.15	1,322	5
	Bangladesh	95.10	981	2
Oceania	Australia	25.70	107	1
Global		34.61	127,134	162

4.4. Time trends in blood Pb concentration

The temporal trends of Pb concentrations in human blood globally and by continent are shown in Figure 3. It can be seen that there is an exponential decline in the trend in Pb concentrations on all continents over the years (Figure 3 A to E), which is also reflected in a global trend (Figure 3 F). Looking at each region, Asia and North America had the lowest slopes (more negative values), representing the greatest declines in lead concentrations over time (Figure 3 A and E), followed by Africa and South America (Figure 3 B and C). Europe's highest angular coefficient (less negative values) showed the smallest decline over the analyzed period (Figure 3 D). However, concentrations on the European continent were already relatively lower since the early 1990s. Despite the magnitude of the differences in angular coefficients among the continents, the Tukey test indicated a significant difference only between North America and South America (Figure 3).

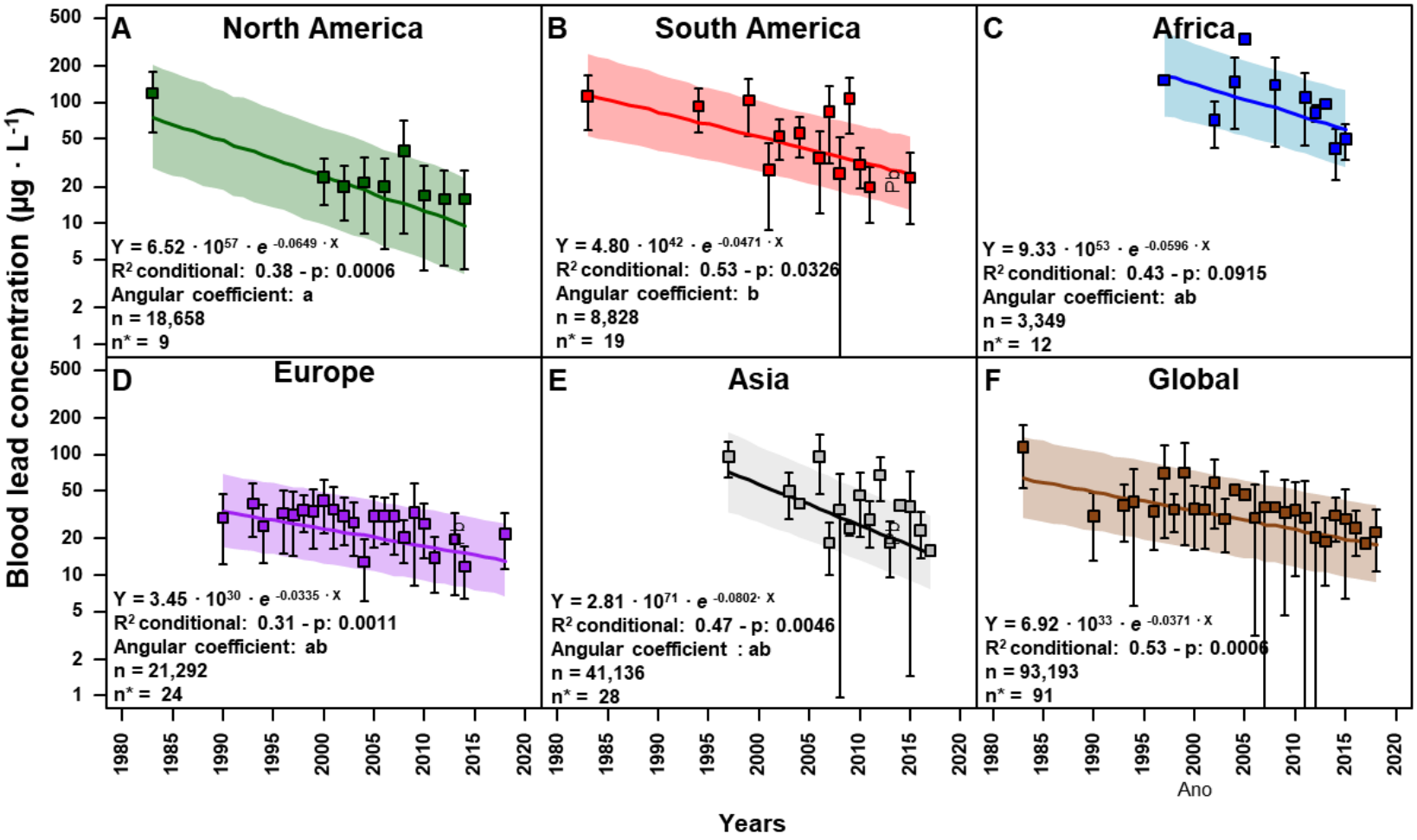

Figure 3. Regional (A to E) and global (F) temporal trends in human blood Pb concentrations (µg·L^{-1}). Symbols and bars represent the mean and standard deviation for each year, and the shaded area represents the 68% confidence interval of mixed-effect regression models. The different letters compare the angular coefficient between regions (A to E) according to Tukey's test ($p < 0.05$). The reported values represent: n = number of participants; n* = number of studies. For this analysis, only studies that reported sampling of random populations or in which no risk factors for exposure or comorbidity existed were included. The y-axis distances were log-transformed to optimize data visualization. The list of studies used for this analysis can be found in Supplementary Table 1.

There was a consistent pattern of decline in lead concentrations over time. After the phase-out, some studies associated the decrease in Pb concentration in human blood in different countries with the removal of tetraethyllead from gasoline (Meyer *et al*., 2003; Mathee *et al*., 2006; Singh and Singh, 2006; Nichani *et al*., 2006; Link *et al*., 2007; Wenneber *et al*., 2017; Han *et al*., 2018). Thus, it was expected that the reduction of the additive use in almost the entire world would be associated with a lower exposure that would decrease Pb blood concentration at a global level. The data show that the lead removal process was gradual across the world and relatively homogeneous across continents (Figure 4 and Supplementary Table 2). Removal began in Japan and was followed by some North and South American countries in the late 1980s and early 1990s. Subsequently, the process was followed by European countries in the mid-1990s and early 2000s. Finally, the countries of the Asian continent proceeded with the removal, and last, the countries of Africa did the same, and this process occurred until 2021 in Algeria (Figure 4 and Supplementary Table 2).

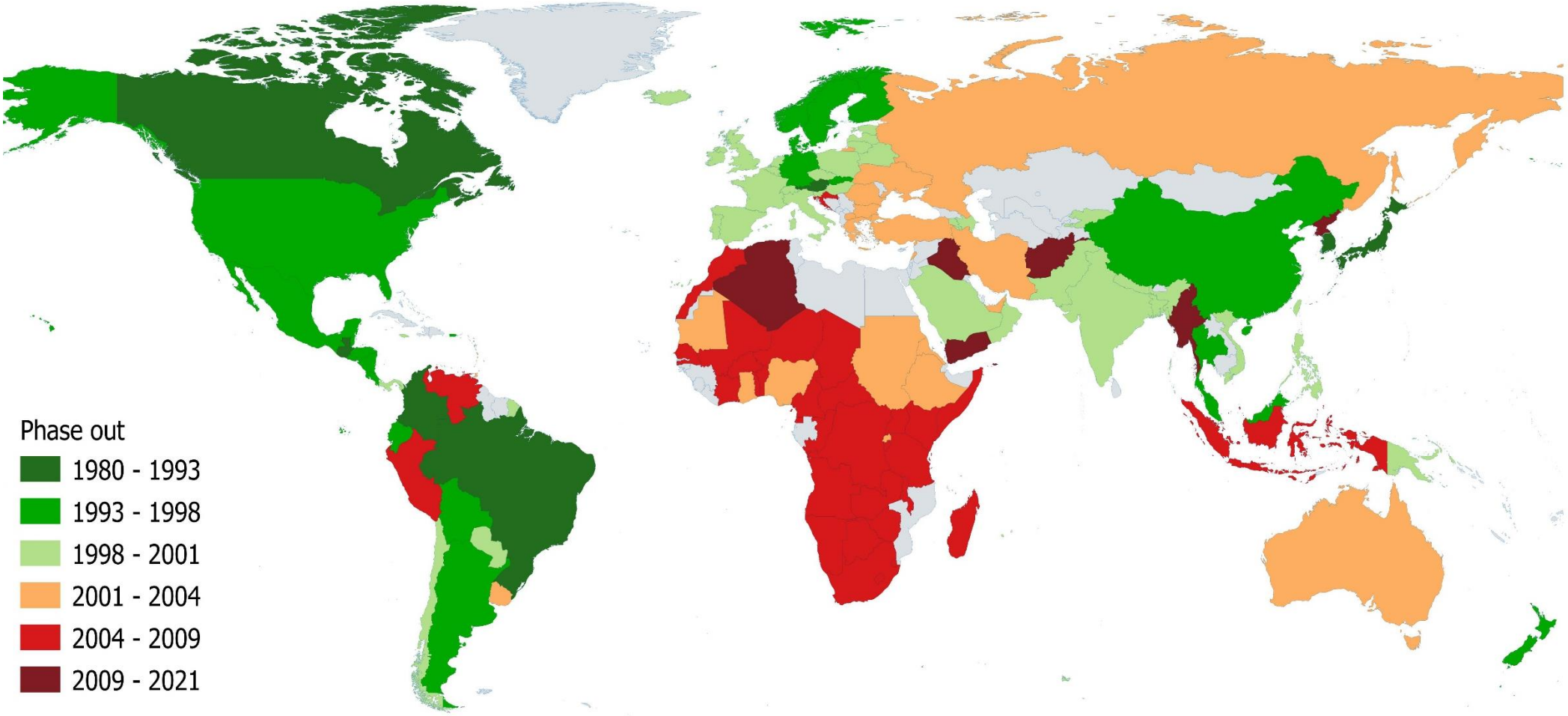

Figure 4. Map of the year of tetraethyllead removal from gasoline (phase-out). Data were not obtained for countries marked in gray. The list of references used for the construction of the map can be found in Supplementary Table S2. Miller projection system (ESPG 53003). Organization: Diego Lacerda.

Using each country's phase-out, a period of removal of Pb from gasoline (in years) was calculated for each study (Figure 5). These data were regressed with Pb blood concentrations, and a pattern very similar to the time trends shown in Figure 3 was observed, showing an exponential decrease in blood Pb concentrations directly associated with the period of removal of tetraethyllead from gasoline (Figure 5). Tukey's test did not show significant differences in angular coefficients among continents. Indeed, there is not much difference in magnitude among them. This shows that although countries differ in the period of Pb removal from gasoline, when this period is normalized by the year of removal, the effect observed across continents is the same, thus reflecting a global trend of exponential reduction in Pb concentrations in human blood as a function of the removal of the additive in gasoline (Figure 5).

The success of Pb removal from gasoline has not yet been quantified and reported in the scientific literature with this level of detail. This review provides a summary of regional and global time trends in human blood Pb concentrations. Furthermore, it demonstrated the homogeneous effect of removing the tetraethyllead additive from gasoline on the concentrations of this element in human populations worldwide. Notably, studies reporting exposure to Pb in other human matrices, such as hair, nails, and urine, were not included in this work. Therefore, the data presented here do not necessarily provide representative information on the levels of exposure of the population in each country or region but provide a great indication of the decrease in human exposure to the element.

The observed declines over time are consistent with time-series studies of blood Pb concentrations in some countries, such as South Africa, India, Germany, Sweden, and China (Meyer *et al*., 2003; Mathee *et al*., 2006; Singh e Singh, 2006; Nichani *et al*., 2006; Link *et al*., 2007; Wenneberg *et al*., 2017; Han *et al*., 2018), and in atmospheric lead concentrations (Gallon *et al*., 2005; Miralles *et al*., 2006; Migon *et al*., 2008; Walraven *et al*., 2014; Goia *et al*., 2017). The consistent declines according to the additive removal period, represented by the homogeneity of the angular coefficients of the different continents, confirm the association between the two variables and the effect caused worldwide. However, it is important to emphasize that high concentrations can still be observed in relatively recent studies, mainly in Africa, Asia, and South America (Babalola *et al*., 2007; Soto-Jimenez and Flegal, 2011;

Gebrie, Tessema, and Ambleu, 2014, Sani and Abdullahi, 2017; Akinwunmi *et al*., 2017).

Although the observed declining trends have relatively high coefficients of determination ($0.30 < R^2_{conditional} < 0.55$), both for annual trends and additive removal periods, these trends suffer from the scarcity of representative data in some regions. The lack of standardization in data reporting in most studies made it impossible to use them or led to the assumption of statistical errors that naturally propagated in the final estimation of the analyses. In addition, methodological differences in the quantification methods and analytical control of the results in the different studies represents another error component that could also interfere with our analyses. Finally, the phase-out was a process that varied from country to country (World Bank 1997; Meyer, Brown and Falk, 2008). The limitations described above must be kept in mind when interpreting our analyses. However, the results were quite consistent. Therefore, this study provides a good indication of decreasing lead exposure worldwide.

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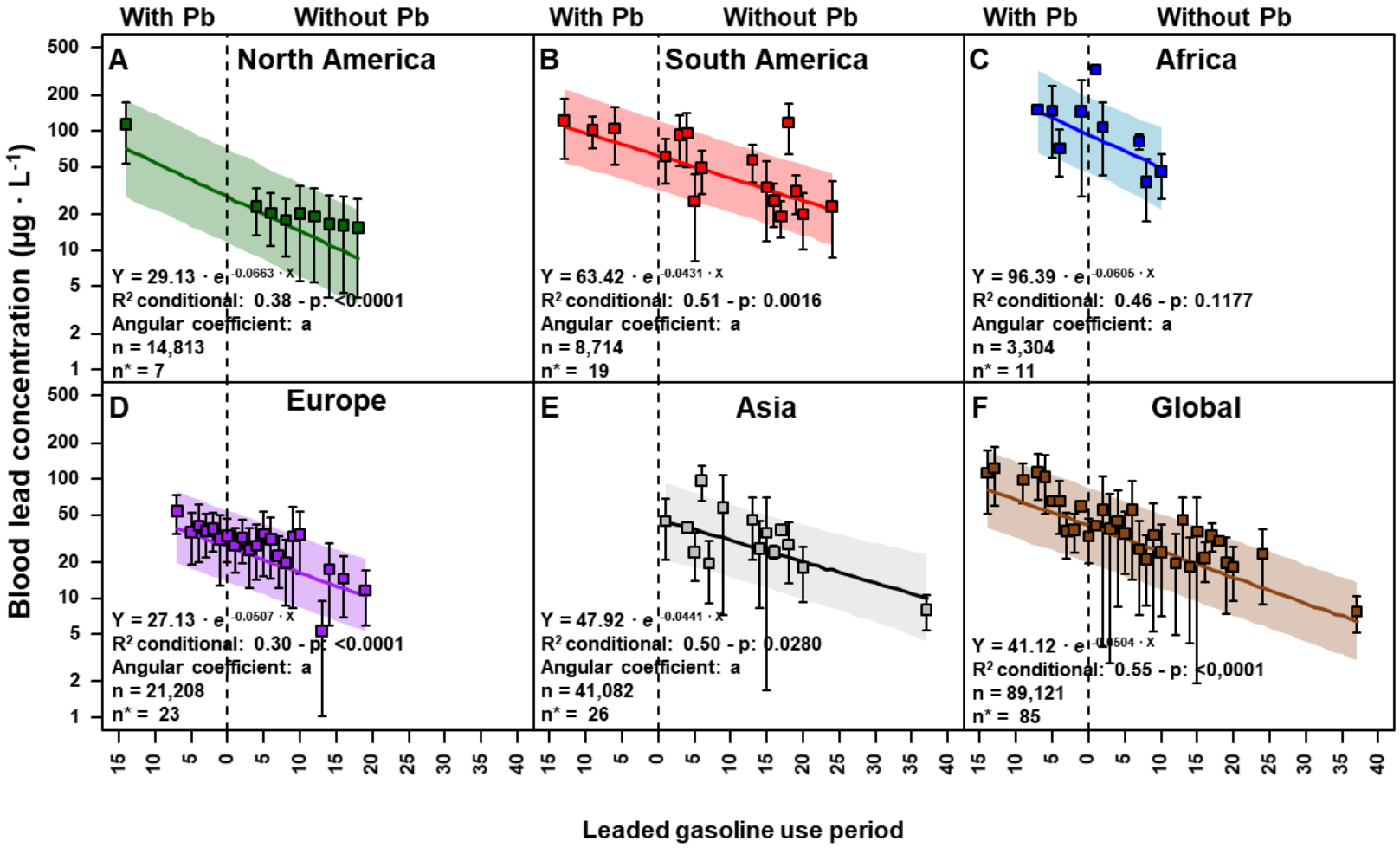

Figure 5. Regional (A to E) and global (F) associations of lead concentrations in human blood (µg · L^{-1}) with the period before (left of zero – with Pb) and after (right of zero – without Pb) the elimination of Pb in gasoline in each country. Symbols and bars represent the mean and standard deviation for each year, and the shaded area represents the 68% confidence interval of mixed-effect regression models. The different letters compare the angular coefficient among regions, and no significant difference was observed among them according to Tukey's test ($p < 0.05$). The reported values represent n = number of participants and n* = number of studies. For this analysis, only studies that reported sampling of random populations or in which no risk factors for exposure or comorbidity existed were included. The y-axis distances were log-transformed to optimize data visualization. The list of studies used for this analysis can be found in Supplementary Table 1.

4.5. Distribution of Pb concentrations between the regions

With the global trend of declining Pb concentrations over the years, the additive removal period influenced the overall medians for each continent. As mentioned, the African continent had the highest concentrations of Pb, followed by South America, Europe, Asia, and North America (Figure 6). Despite the large difference between the concentrations, the continents did not show significant differences according to the Tukey test ($p > 0.05$) since the random effect component of the model (internal variance of each study) is very high. However, when observing the magnitude of the differences, the African continent has the highest concentrations, followed by South America. The differences between Europe, Asia, and North America were not of great magnitude (Figure 5), and the following organization is possible: Africa > South America > Europe ≅ Asia ≅ North America. It is important to emphasize that this result should be analyzed with caution since, as observed, there is a strong temporal trend in the decline of Pb concentrations over the years, which may be reflected in higher or lower concentrations in each continent depending on when the study was carried out and the additive removal period in each country.

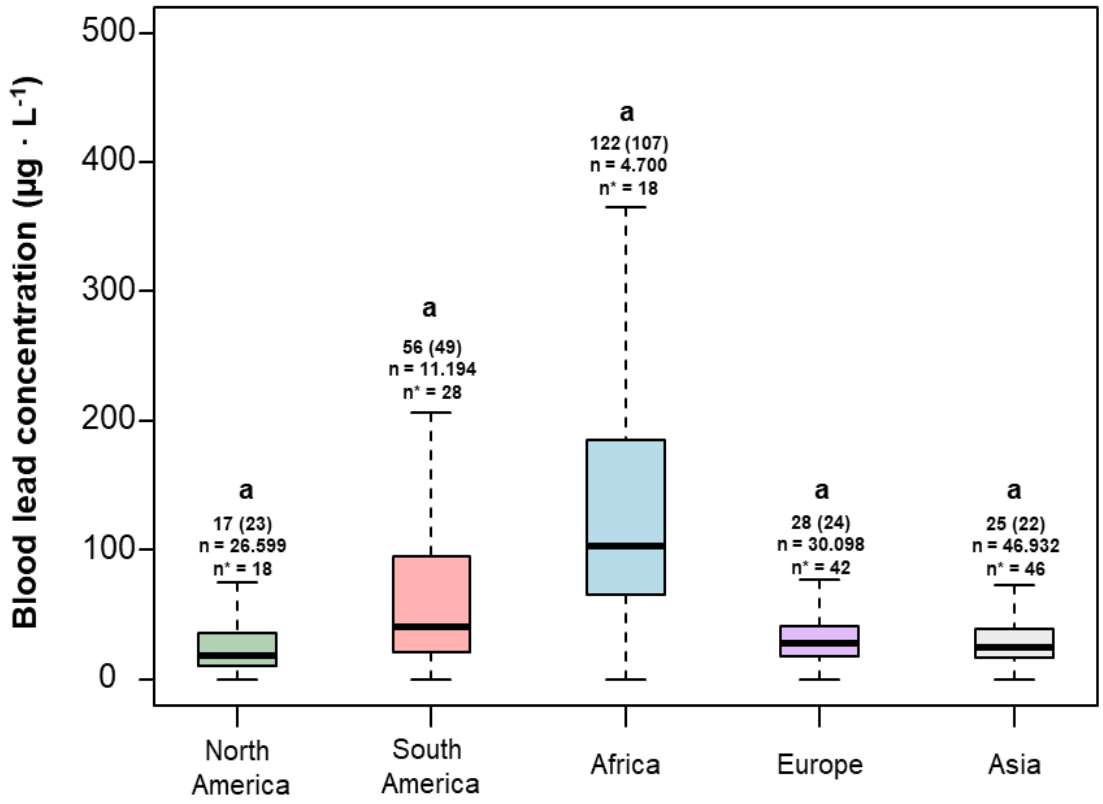

Figure 6. Distribution of human blood Pb concentrations (µg·L^{-1}) by continent. No significant differences were detected according to the Tukey test ($p > 0.05$). The reported values represent the median (interquartile range); n = number of participants; n* = number of studies. For this analysis, only studies that reported sampling of random populations or in which no risk factors for exposure or comorbidity existed were included. The list of studies used for this analysis can be found in Supplementary Table 1.

The high concentrations observed in African countries and in some Asian and South American countries directly reflect the year when lead was removed from gasoline (Figure 1 and Figure 4), leading to wide variation in concentrations across the world. In general, the higher the time that countries delayed the phase-out, the higher their median concentrations were. This variation in lead concentrations reported in different countries raises concern about the health problems caused by high exposure. The highest concentrations (µg · L^{-1}) were observed in countries such as Ethiopia (298), Nigeria (278), Puerto Rico (106), Congo (105), and Mexico (105), all with a weighted average greater than 100 µg · L $^{-1}$, indicating high and prolonged exposure in such countries. Blood concentrations between 100 and 300 µg·L^{-1} can cause intellectual deficits, changes in mood and neuromotor function in children and adults, as well as changes in metabolism and brain architecture in adults, in addition to

cardiovascular, respiratory, hepatic, renal, and gastrointestinal problems. (US-ASTDR, 2020). The US Centers for Disease Control and Prevention uses the value of 50 $\mu g \cdot L^{-1}$ as a health intervention value. The determination of safety levels for Pb is still a topic of discussion since different studies have shown toxic effects of the element at low and high concentrations (US-ASTDR, 2020). Given the downward trend over the years, it is possible to estimate that the mean concentration of Pb reached the limit established by the US-ASTDR first in Europe in 1980, followed by North America in 1989, South America and Asia in 2002 and, last, in Africa in 2018.

4.6. Influence of exposure sources and sex on blood lead concentration

Although the additive in gasoline has been the main problem involving Pb contamination and its removal has reduced diffuse exposure worldwide, some activities still contribute to exposure to this element (Figure 7 A). To assess the relationship between different types of exposure and blood Pb concentrations, data from exposed populations from different studies were classified into four categories according to exposure type: occupational exposure, environmental exposure, smoking, and alcohol consumption. In addition, the ratios of all possible combinations between the exposed groups and controls were determined to remove the time effect within each study. This analysis confirms the different types of exposure as sources of this element for the human organism (Figure 7 A).

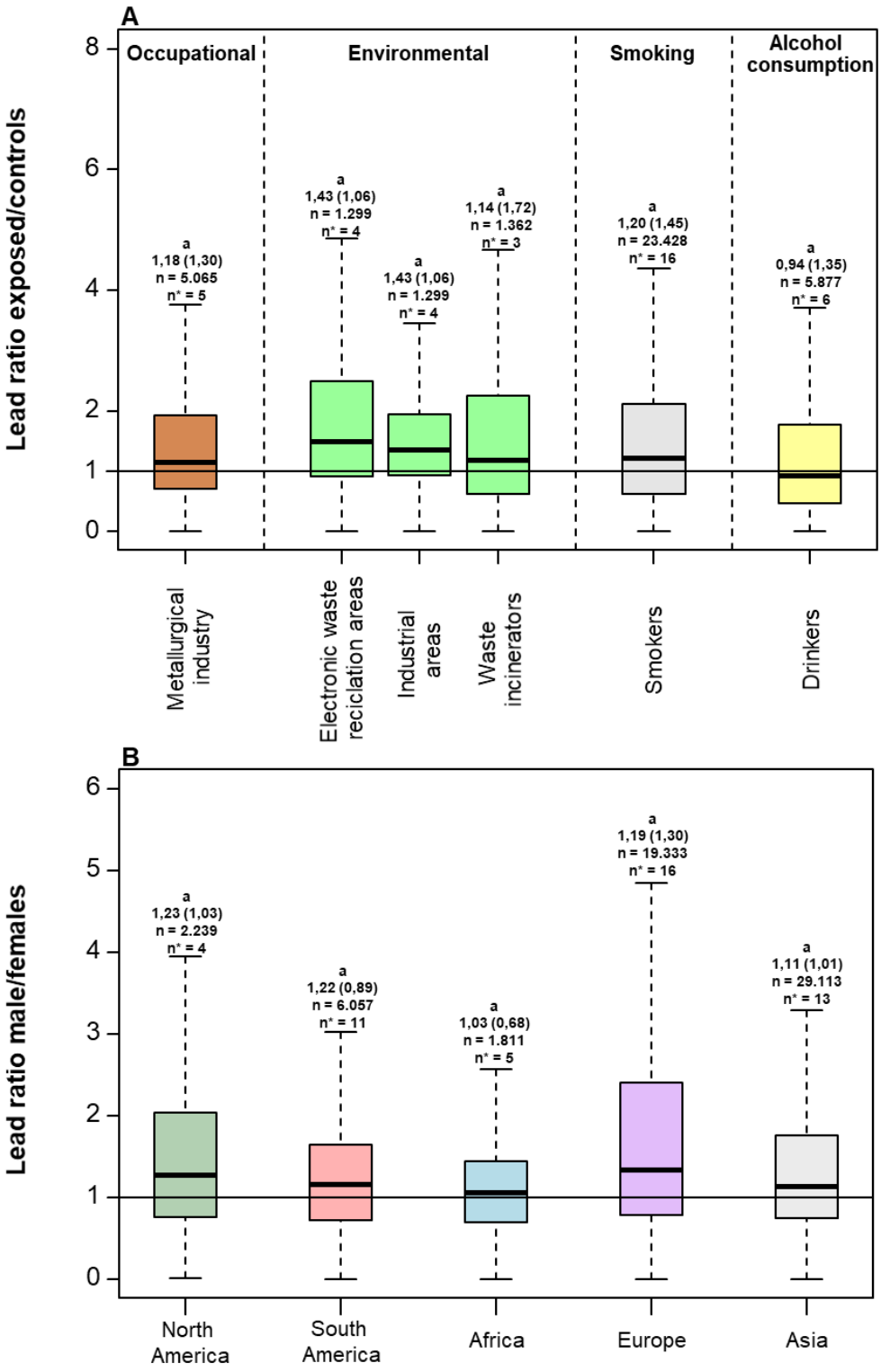

Figure 7. The ratio of human blood Pb concentrations between exposure types and control populations (A) and between sexes (male/female) by continent (B). The letters denote that there were no differences between types of exposure (Figure 7 A) and continents (Figure 7 B) according to Tukey's test ($p > 0.05$). The reported values represent the median (interquartile range); n = number of participants; n* = number of studies. For this analysis, only studies sampling populations with some occupational, environmental, or habitual exposure that had not reported any comorbidity were used. The list of studies used for this analysis can be found in Supplementary Table 1.

It is notable that workers in the metallurgical industry have median Pb concentrations 18% higher than their respective controls (Figure 7 A). Among the types of environmental exposure, which represent those individuals who live close to an activity that potentially contributes to Pb contamination, both individuals who live close to electronic waste recycling areas and industrial areas present median concentrations approximately 40% higher than the control group, and individuals who live near waste incineration areas have 14% higher concentrations (Figure 7 A). Smokers had median concentrations 20% higher than nonsmokers. Alcohol consumption was the only type of exposure that did not present an increase in concentrations (median = 0.94), showing that, in general, alcohol consumption did not contribute to the increase in the concentration of Pb in the blood (Figure 7 A). Some of the selected studies mentioned other forms of exposure not listed here. However, they were not evaluated in this work due to the lack of accurate information on the type of exposure, the lack of control groups, or the absence of more studies evaluating the same type of exposure.

The comparison between the sexes through the ratio of all possible combinations between male and female individuals within each study on different continents showed that men have higher lead concentrations. This was mainly in North America (23%), South America (22%), Europe (19%), and Asia (11%). On the African continent, men had concentrations only 3% higher than women (Figure 7 B). However, Tukey's multiple comparison test did not show significant differences in these ratios among the evaluated continents (Figure 7 B).

The removal of tetraethyllead and other actions, such as reducing the use of paints and other products containing Pb, did not prevent human exposure to Pb since many activities and life habits are still sources of this element. Pb is used in the production of batteries, glasses, enamels, plastics, ceramics, electronic products, and in the construction industry, among other activities. Approximately 12.1 million tons of Pb were consumed worldwide in only 2019 (ILZG, 2020). The data from the analyzed studies confirms the different sources of this element for the human organism. However, this assessment has limitations, such as the lack of detailed information and data reporting homogeneity for the types of activity/exposure. For example, many studies of individuals occupationally exposed to Pb in industries do not have accurate information about the activity performed by the worker or the level of exposure, making direct comparison impossible. Furthermore, some studies still have design errors

establishing individuals subject to the same atmospheric contamination as those occupationally exposed as a control population. Considering that atmospheric exposure is an important source of this element, choosing an adequate control group is fundamental for this type of evaluation. There was also a lack of studies assessing important categories of human Pb exposure (*e.g.,* electronic waste recyclers, battery factory workers, and miners).

The great variation in the Pb concentrations of exposed populations among the different studies was probably caused by differences in the sampling period and the level of exposure. Additionally, the differences in the sample size also influenced this analysis. These differences made the ratio distributions between the exposed and control groups quite asymmetrical, leading to significant differences between the mean and the median of the ratios. Alcohol consumption was the only route of exposure with a median of less than 1. Much of this result can be attributed to a single study in South Korea in which individuals who did not consume alcohol had higher concentrations than those who consumed alcohol. This study had a high sample size (2,320 participants), causing the median to be less than one. However, studies report that alcohol consumption increases lead exposure and induces changes in lead absorption, transport, and distribution, affecting blood concentration (Maranelli, Apostoli, and Ferrari, 1990; Schuhmacher, Domingo and Llobet, 1993; Weyermann and Brenner, 1997).

Although the median of the ratios between sexes was very close to one on the African continent, higher Pb concentrations in the blood of males are often reported in the literature, which has led to some discussion about the mechanisms involved in higher Pb concentrations observed in males compared to females. According to Lee and Kim (2014), differences in sex hormones is responsible for this. During the pubertal period, estrogen induces rapid bone formation and, consequently, the removal of lead from the blood into bones, explaining the lower lead concentrations in females. Another possible explanation could be attributed to the differences in erythrocyte concentrations between males and females. Over 99% of the lead present in the blood is found in red cells, and the higher count of such cells, usually observed in men, could explain the higher Pb concentrations in this group (Yip, Johnson, and Dallman, 1984; Bergdahl *et al*., 1997; Barbosa *et al*., 2006). We hypothesize that the differences in bone tissue also influence differential blood concentrations between the sexes. As

mentioned, after exposure, Pb accumulates in the skeleton, which accounts for approximately 90% of the body burden and acts as an endogenous source for the bloodstream, corresponding to 40-70% of the blood concentrations (Silbergeld *et al*., 1988; Smith, Osterloh, and Flegal, 1996). As men havet higher bone mass and density, this could partly explain the higher blood lead concentrations (Barry, 1975; Rabinowitz, 1991; Nieves *et al*., 2004; Lu *et al*., 2016). In addition, males also accumulate more lead in bones than women (Barry and Mossman, 1970; Barry, 1975).

4.7. Limitations of this review and recommendations for future studies

This study demonstrates and justifies the need to publish more accurate data on human blood Pb concentrations worldwide. Errors in the way data are reported may limit the analysis and interpretation of patterns. Even basic information such as sampling date and sample size is lacking in many studies. There is also a great need for accuracy in the statistics reported in the studies. For example, some reports present several measures of centrality but no dispersion measures.

In many cases, the measures of centrality presented are not adequate for the data distribution (*e.g.*, presenting mean and standard deviation for asymmetric data). In addition, many studies present the geometric mean and the geometric standard deviation as descriptive statistics, making it difficult to convert these data to the arithmetic mean and standard deviation. Developing countries, especially in Africa, Asia, and South America, have few studies that report blood Pb concentrations, and, in general, they are studies with small populations that may not adequately represent human exposure to Pb in these regions. We encourage researchers to share raw data in their published articles to enable accurate time-series evaluations.

Some studies report that blood Pb concentrations are higher in older individuals (Harlan, 1988; Lee; Chun and Song, 2005; Ettinger *et al*., 2020). However, in the present study we could not control this confounding factor due to the way in which the authors reported the data. In addition, most studies analyze wide age ranges. For example, Lourenço *et al*. (2013) evaluated the concentrations of metals in the blood of Portuguese people living near a deactivated uranium mine aged between 13 and 91, which, as in several other studies, does not allow for differentiating between adults and children. In addition, few articles reported Pb concentrations in adults and children evaluated in the same period. As time trends strongly influenced blood Pb

concentrations, the comparison between age groups could not be performed. These gaps and limitations in reporting or designing studies compromise the data evaluation and pattern observations and should be circumvented in future studies.

4.8. Conclusion

This review showed the importance of removing tetraethyllead in decreasing human exposure to Pb worldwide. The data showed a consistent trend of exponentially decreasing blood Pb concentrations after the additive removal period. In addition, the results also showed that, despite the removal of the additive providing lower levels of exposure, the general population remains exposed to the element through environmental and occupational sources and lifestyle habits such as smoking and alcohol consumption.

Through the interpretation of the data, it becomes clear how errors in experimental design and in data reporting influence the analysis and observation of patterns. Even though Pb is the element most studied by the articles analyzed, the lack of representation in several countries, especially in Africa and South America, justifies the need for greater monitoring of this element. In addition to the lack of representation in many countries, studies with more significant numbers of participants in random populations are needed to represent populations correctly.

Finally, the need for biomonitoring studies of chemical elements in human blood becomes clear through this review. Most of the reported elements (82%) were evaluated by less than 10% of the studies. Even elements of known toxicity, such as Hg, Mn, Cr, Se, As, Ni, Co, and Al, were reported in less than 50% of the studies. Given the lack of representative data on the concentrations of most elements in human populations, greater attention needs to be paid to human exposure levels and the possible adverse effects due to the lack of knowledge of their dynamics, natural concentrations, and risk factors.

4.9. References

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4.10. Supplementary material

Table S1. List of studies and inclusion criteria according to the analyzes performed. The studies are represented by codes described in List S1.

Analysis	Study code	Inclusion criteria in the analysis	Total studies	Total participants
Number of publications evaluating human blood lead concentrations between 2000 and 2019 and percentage of studies involving each element (Figure 1, Figure S1)	1, 2, 3, 4, 5, 6, 9, 10, 11, 13, 14, 15, 16, 17, 18, 19, 20, 22, 23, 24, 25, 26, 28, 29, 31, 32, 33, 34, 35, 38, 40, 41, 42, 43, 48, 49, 50, 51, 52, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 87, 88, 89, 90, 91,92, 93, 94, 95, 99, 100, 101, 103, 104, 105, 107, 108, 109, 110, 112, 114, 115, 116, 117, 118, 119, 121, 122, 123, 124, 125, 126, 127, 129, 130, 131, 132, 133, 135, 136, 137, 138, 139, 140, 142, 145, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162 and 163	Lead concentration and sample number	132	121,113
Global distribution of lead in the blood (Figure 2 and Table 1)	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162 and 163	Lead concentration and sample number; No type of exposure or comorbidity reported or values from control populations of the studies	162	124,134
Regional and global time trends in human blood lead concentrations (Figure 3)	6, 7, 8, 12, 15, 16, 17, 18, 19, 21, 23, 24, 27, 28, 29, 30, 31, 32, 33, 34, 36, 39, 41, 44, 45, 46, 47, 48, 49, 61, 62, 68, 69, 72, 75, 77, 78, 79, 80, 81, 82, 83, 84, 87, 89, 90, 91, 92, 94, 95, 97, 101, 102, 103, 104, 105, 107, 108, 109, 110, 111, 116, 118, 119, 121, 125, 128, 129, 131, 133, 136, 138, 140, 141, 142, 143, 144, 148, 150, 151, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162 and 163	Lead concentration, sample number and dispersion measure; Date of collection of blood samples or time intervals equal to or less than three years; No type of exposure or comorbidity reported or values from control populations of the studies.	91	93,193

Analysis	Study code	Inclusion criteria in the analysis	Total studies	Total participants
Regional and global time trends in human blood lead concentrations from gasoline removal (Figure 5)	6, 7, 8, 12, 15, 16, 17, 18, 19, 21, 23, 24, 27, 28, 29, 30, 31, 32, 33, 34, 36, 39, 44, 45, 46, 47, 48, 49, 61, 62, 68, 69, 72, 75, 77, 78, 79, 80, 81, 82, 83, 87, 89, 90, 91, 92, 94, 95, 97, 101, 102, 103, 104, 105, 108, 109, 110, 111, 118, 119, 121, 125, 128, 129, 133, 136, 138, 140, 141, 142, 143, 144, 148, 150, 151, 153, 154, 155, 156, 158, 159, 160, 161, 162 and 163	Lead concentration, sample number, and dispersion measure; Date of collection of blood samples or time intervals equal to or less than three years; No type of exposure or comorbidity reported or values from control populations of the studies. gasoline removal (Figure 5)	85	89,121
Mixed effect analysis of variance of the distribution of lead concentrations in human blood by continent (Figure 6)	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 52, 53, 55, 56, 57, 59, 60, 61, 62, 63, 64, 65, 67, 68, 69, 70, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 87, 88, 89, 90, 91, 92, 93, 94, 95, 97, 98, 99, 100, 101, 102, 103, 104, 105, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 121, 122, 123, 124, 125, 126, 127, 128, 129, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162 and 163	Lead concentration, sample number, and dispersion measure; No type of exposure or comorbidity reported or values from control populations of the studies.	151	119,523
Mixed effect analysis of variance of the ratio of lead concentrations in human blood between exposed individuals and control populations (Figure 7A)	1, 3, 4, 7, 9, 12, 17, 19, 22, 23, 26, 28, 31, 47, 48, 52, 53, 56, 57, 76, 80, 87, 88, 90, 92, 94, 102, 109, 114, 115, 121, 123, 124, 127, 133, 136, 144, 155, 161, 162 and 163	Lead concentration, sample number and dispersion measures; Only studies that present lead levels of exposed populations and control populations.	37	37,299

Analysis	Study code	Inclusion criteria in the analysis	Total studies	Total participants
Mixed effect analysis of variance of the ratio of lead concentrations in human blood by continent between sexes (Figure 7B)	2, 6, 7, 8, 9, 14, 16, 17, 19, 22, 23, 24, 29, 30, 31, 32, 35, 36, 39, 42, 46, 48, 54, 74, 78, 87, 88, 91, 92, 105, 108, 109, 112, 113, 121, 122, 123, 127, 133, 136, 141, 143, 144, 145, 150, 154, 155, 162 and 163	Lead concentration, sample number and dispersion measures; Both sexes evaluated in the same study; No type of exposure or comorbidity reported or values from control populations from exposure studies.	49	58,553

List S1. List of studies resulting from the systematic search. The numbers represent the code of each study according to supplementary table 1.

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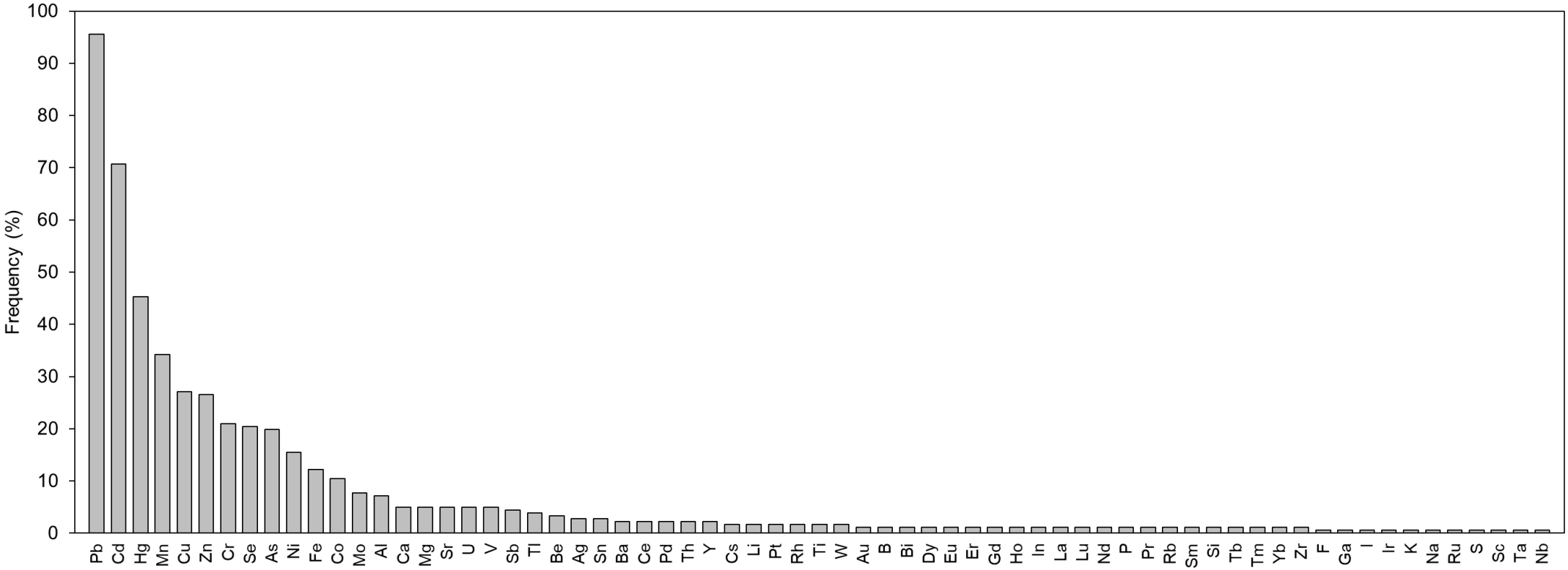

Figure S1. Percentage of studies with each element.

Table S2. List of countries ranked by the phase-out of leaded gasoline for each continent or subcontinent.

Continent	Country	Year of elimination of Pb in gasoline	Reference
North America	Canada	1993	UNITED NATIONS ORGANIZATION. **Phasing lead out of gasoline: An examination of policy approaches in different countries**. UNEP/Earthprint, 1999. Available in:<https://www.un.org/esa/gite/iandm/unep-lead.pdf>. Accessed on August 10, 2020.
	United States of America	1996	UNITED NATIONS ORGANIZATION. **Phasing lead out of gasoline: An examination of policy approaches in different countries**. UNEP/Earthprint, 1999. Available in:<https://www.un.org/esa/gite/iandm/unep-lead.pdf>. Accessed on August 10, 2020.
	Mexico	1997	CARAVANOS, Jack *et al*. Blood lead levels in Mexico and pediatric burden of disease implications. **Annals of Global Health**, v. 80, n. 4, p. 269-277, 2014.
South America	Bermuda	1990	WORLD BANK. **Vehicular air pollution: experiences from seven Latin American urban centers**. 1997. Available in: <http://www.rhd.gov.bd/Documents/ExternalPublications/WorldBank/TransSectPub/contents/documents/B11.pdf>. Accessed on August 10, 2020.
	Antigua and Barbuda	1991	WORLD BANK. **Vehicular air pollution: experiences from seven Latin American urban centers**. 1997. Available in: <http://www.rhd.gov.bd/Documents/ExternalPublications/WorldBank/TransSectPub/contents/documents/B11.pdf>. Accessed on August 10, 2020.
	Brazil	1991	GOLDEMBERG, José. The Brazilian biofuels industry. **Biotechnology for Biofuels**, v. 1, n. 1, p. 6, 2008.
	Colombia	1991	WORLD BANK. **Vehicular air pollution: experiences from seven Latin American urban centers**. 1997. Available in: <http://www.rhd.gov.bd/Documents/ExternalPublications/WorldBank/TransSectPub/contents/documents/B11.pdf>. Accessed on August 10, 2020.
	Guatemala	1991	WORLD BANK. **Vehicular air pollution: experiences from seven Latin American urban centers**. 1997. Available in: <http://www.rhd.gov.bd/Documents/ExternalPublications/WorldBank/TransSectPub/contents/documents/B11.pdf>. Accessed on August 10, 2020.
	Argentine	1996	WORLD BANK. **Vehicular air pollution: experiences from seven Latin American urban centers**. 1997. Available in: <http://www.rhd.gov.bd/Documents/ExternalPublications/WorldBank/TransSectPub/contents/documents/B11.pdf>. Accessed on August 10, 2020.
	Belize	1996	CHARALAMBOUS, Andreas *et al*. Screening for lead exposure in children in Belize. **Revista Panamericana de Salud Pública**, v. 25, p. 47-50, 2009.
	Bolivia	1996	WORLD BANK. **Vehicular air pollution: experiences from seven Latin American urban centers**. 1997. Available in: <http://www.rhd.gov.bd/Documents/ExternalPublications/WorldBank/TransSectPub/contents/documents/B11.pdf>. Accessed on August 10, 2020.
	Costa Rica	1996	WORLD BANK. **Vehicular air pollution: experiences from seven Latin American urban centers**. 1997. Available in: <http://www.rhd.gov.bd/Documents/ExternalPublications/WorldBank/TransSectPub/contents/documents/B11.pdf>. Accessed on August 10, 2020.

Continent	Country	Year of elimination of Pb in gasoline	Reference
	Honduras	1996	WORLD BANK. **Vehicular air pollution: experiences from seven Latin American urban centers**. 1997. Available in: <http://www.rhd.gov.bd/Documents/ExternalPublications/WorldBank/TransSectPub/contents/documents/B11.pdf>. Accessed on August 10, 2020.
	Nicaragua	1996	WORLD BANK. **Vehicular air pollution: experiences from seven Latin American urban centers**. 1997. Available in: <http://www.rhd.gov.bd/Documents/ExternalPublications/WorldBank/TransSectPub/contents/documents/B11.pdf>. Accessed on August 10, 2020.
	Puerto Rico	1996	DIGNAM, Timothy *et al*. Prevalence of elevated blood lead levels and risk factors among residents less than 6 years of age, Puerto Rico-2010. **Journal of Public Health Management and Practice**, v. 22, n. 1, p. E22, 2016.
	Ecuador	1997	WORLD BANK. **Vehicular air pollution: experiences from seven Latin American urban centers**. 1997. Available in: <http://www.rhd.gov.bd/Documents/ExternalPublications/WorldBank/TransSectPub/contents/documents/B11.pdf>. Accessed on August 10, 2020.
	El Salvador	1997	WORLD BANK. **Vehicular air pollution: experiences from seven Latin American urban centers**. 1997. Available in: <http://www.rhd.gov.bd/Documents/ExternalPublications/WorldBank/TransSectPub/contents/documents/B11.pdf>. Accessed on August 10, 2020.
	Barbados	2000	WORLD BANK. **Vehicular air pollution: experiences from seven Latin American urban centers**. 1997. Available in: <http://www.rhd.gov.bd/Documents/ExternalPublications/WorldBank/TransSectPub/contents/documents/B11.pdf>. Accessed on August 10, 2020.
	Paraguay	2000	WORLD BANK. **Vehicular air pollution: experiences from seven Latin American urban centers**. 1997. Available in: <http://www.rhd.gov.bd/Documents/ExternalPublications/WorldBank/TransSectPub/contents/documents/B11.pdf>. Accessed on August 10, 2020.
	Chile	2001	SAX, Sonja N. *et al*. Trends in the elemental composition of fine particulate matter in Santiago, Chile, from 1998 to 2003. **Journal of the Air and Waste Management Association**, v. 57, n. 7, p. 845-855, 2007.
	Jamaica	2001	WORLD BANK. **Elimination of lead in gasoline in Latin America and the Caribbean**. 1997. Available in: <https://esmap.org/sites/default/files/esmap-files/RP_LACleadelimination1997.pdf>. Accessed on August 10, 2020.
	Panama	2001	WORLD BANK. **Elimination of lead in gasoline in Latin America and the Caribbean**. 1997. Available in: <https://esmap.org/sites/default/files/esmap-files/RP_LACleadelimination1997.pdf>. Accessed on August 10, 2020.
	Saint Lucia	2002	WORLD BANK. **Vehicular air pollution: experiences from seven Latin American urban centers**. 1997. Available in: <http://www.rhd.gov.bd/Documents/ExternalPublications/WorldBank/TransSectPub/contents/documents/B11.pdf>. Accessed on August 10, 2020.
	Uruguay	2003	MAÑAY, Nelly *et al*. Lead contamination in Uruguay: the “La Teja” neighborhood case. In: **Reviews of Environmental Contamination and Toxicology**. Springer, New York, NY, 2008. p. 93-115.
	Trinidad and Tobago	2004	RAJKUMAR, Wayne Simon *et al*. Blood lead levels in primary school children in Trinidad and Tobago. **Science of the Total Environment**, v. 361, n. 1-3, p. 81-87, 2006.

Continent	Country	Year of elimination of Pb in gasoline	Reference
	Peru	2005	WORLD BANK. **An opportunity for a different Peru: prosperous, equitable, and governable**. 2006. Available in: <https://openknowledge.worldbank.org/bitstream/handle/10986/6633/382860ENGLISH0101OFFICIAL0USE0ONLY1.pdf?sequence=1andisAllowed=y>. Accessed on August 10, 2020.
	Venezuela	2005	OUR WORLD IN DATA. **How the world eliminated lead from gasoline**. Available in: <https://ourworldindata.org/leaded-gasoline-phase-out> accessed on April 12, 2022.
Africa	South Sudan	1999	OUR WORLD IN DATA. **How the world eliminated lead from gasoline**. Available in: <https://ourworldindata.org/leaded-gasoline-phase-out> accessed on April 12, 2022.
	Sudan	1999	OUR WORLD IN DATA. **How the world eliminated lead from gasoline**. Available in: <https://ourworldindata.org/leaded-gasoline-phase-out> accessed on April 12, 2022.
	Ethiopia	2003	WORLD BANK. **The WORLD BANK clean air initiative in Sub-Saharan African cities**. 2003 Available in: <http://documents1.worldbank.org/curated/en/353171468761104841/pdf/284530PAPER0SSA0Clean0air0no1014.pdf>. Accessed on August 10, 2020.
	Eritrea	2004	UNITED NATIONS ORGANIZATION. **Lessons learned from the Partnership for Clean Fuels and Vehicles**. 2020. Available in:<https://wedocs.unep.org/bitstream/handle/20.500.11822/25154/GAELP_ADDIS_6_Lessons_UNEP_PCFV.pdf?sequence=1andisAllowed=y>. Accessed on: August 10, 2020.
	Ghana	2004	UNITED NATIONS ORGANIZATION. **Lessons learned from the Partnership for Clean Fuels and Vehicles**. 2020. Available in:<https://wedocs.unep.org/bitstream/handle/20.500.11822/25154/GAELP_ADDIS_6_Lessons_UNEP_PCFV.pdf?sequence=1andisAllowed=y>. Accessed on: August 10, 2020.
	Mauritania	2004	UNITED NATIONS ORGANIZATION. **Lessons learned from the Partnership for Clean Fuels and Vehicles**. 2020. Available in:<https://wedocs.unep.org/bitstream/handle/20.500.11822/25154/GAELP_ADDIS_6_Lessons_UNEP_PCFV.pdf?sequence=1andisAllowed=y>. Accessed on: August 10, 2020.
	Nigeria	2004	OJO, J. O.; OKETAYO, O. O.; HORVAT, M. **Marked lowering in Pb blood levels follows lead phase-out from gasoline in Nigeria**. 2010. Available in: <https://inis.iaea.org/collection/NCLCollectionStore/_Public/41/131/41131315.pdf>. Accessed on August 10, 2020.
	Rwanda	2004	UNITED NATIONS ORGANIZATION. **Lessons learned from the Partnership for Clean Fuels and Vehicles**. 2020. Available in:<https://wedocs.unep.org/bitstream/handle/20.500.11822/25154/GAELP_ADDIS_6_Lessons_UNEP_PCFV.pdf?sequence=1andisAllowed=y>. Accessed on: August 10, 2020.
	Southern Sudan	2004	UNITED NATIONS ORGANIZATION. **Lessons learned from the Partnership for Clean Fuels and Vehicles**. 2020. Available in:<https://wedocs.unep.org/bitstream/handle/20.500.11822/25154/GAELP_ADDIS_6_Lessons_UNEP_PCFV.pdf?sequence=1andisAllowed=y>. Accessed on: August 10, 2020.
	Sudan	2004	UNITED NATIONS ORGANIZATION. **Lessons learned from the Partnership for Clean Fuels and Vehicles**. 2020. Available in:<https://wedocs.unep.org/bitstream/handle/20.500.11822/25154/GAELP_ADDIS_6_Lessons_UNEP_PCFV.pdf?sequence=1andisAllowed=y>. Accessed on: August 10, 2020.

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	Angola	2005	OUR WORLD IN DATA. **How the world eliminated lead from gasoline**. Available in: <https://ourworldindata.org/leaded-gasoline-phase-out> accessed on April 12, 2022.
	Benin	2005	BODEAU-LIVINEC, Florence *et al*. Elevated blood lead levels in infants and mothers in Benin and potential sources of exposure. **International Journal of Environmental Research and Public Health**, v. 13, n. 3, p. 316, 2016.
	Botswana	2005	WISTON, Modise. Status of air pollution in Botswana and significance to air quality and human health. **Journal of Health and Pollution**, v. 7, n. 15, p. 8-17, 2017.
	Burkina Faso	2005	UNITED NATIONS ORGANIZATION. **Lessons learned from the Partnership for Clean Fuels and Vehicles**. 2020. Available in:<https://wedocs.unep.org/bitstream/handle/20.500.11822/25154/GAELP_ADDIS_6_Lessons_UNEP_PCFV.pdf?sequence=1andisAllowed=y>. Accessed on: August 10, 2020.
	Burundi	2005	OUR WORLD IN DATA. **How the world eliminated lead from gasoline**. Available in: <https://ourworldindata.org/leaded-gasoline-phase-out> accessed on April 12, 2022.
	Cameroon	2005	UNITED NATIONS ORGANIZATION. **Lessons learned from the Partnership for Clean Fuels and Vehicles**. 2020. Available in:<https://wedocs.unep.org/bitstream/handle/20.500.11822/25154/GAELP_ADDIS_6_Lessons_UNEP_PCFV.pdf?sequence=1andisAllowed=y>. Accessed on: August 10, 2020.
	Central African Republic	2005	UNITED NATIONS ORGANIZATION. **Lessons learned from the Partnership for Clean Fuels and Vehicles**. 2020. Available in:<https://wedocs.unep.org/bitstream/handle/20.500.11822/25154/GAELP_ADDIS_6_Lessons_UNEP_PCFV.pdf?sequence=1andisAllowed=y>. Accessed on: August 10, 2020.
	Chad	2005	UNITED NATIONS ORGANIZATION. **Lessons learned from the Partnership for Clean Fuels and Vehicles**. 2020. Available in:<https://wedocs.unep.org/bitstream/handle/20.500.11822/25154/GAELP_ADDIS_6_Lessons_UNEP_PCFV.pdf?sequence=1andisAllowed=y>. Accessed on: August 10, 2020.
	Democratic Republic of the Congo	2005	UNITED NATIONS ORGANIZATION. **Lessons learned from the Partnership for Clean Fuels and Vehicles**. 2020. Available in:<https://wedocs.unep.org/bitstream/handle/20.500.11822/25154/GAELP_ADDIS_6_Lessons_UNEP_PCFV.pdf?sequence=1andisAllowed=y>. Accessed on: August 10, 2020.
	Equatorial Guinea	2005	OUR WORLD IN DATA. **How the world eliminated lead from gasoline**. Available in: <https://ourworldindata.org/leaded-gasoline-phase-out> accessed on April 12, 2022.
	Eswatini	2005	OUR WORLD IN DATA. **How the world eliminated lead from gasoline**. Available in: <https://ourworldindata.org/leaded-gasoline-phase-out> accessed on April 12, 2022.
	Gabon	2005	OUR WORLD IN DATA. **How the world eliminated lead from gasoline**. Available in: <https://ourworldindata.org/leaded-gasoline-phase-out> accessed on April 12, 2022.
	Ivory Coast	2005	UNITED NATIONS ORGANIZATION. **Lessons learned from the Partnership for Clean Fuels and Vehicles**. 2020. Available in:<https://wedocs.unep.org/bitstream/handle/20.500.11822/25154/GAELP_ADDIS_6_Lessons_UNEP_PCFV.pdf?sequence=1andisAllowed=y>. Accessed on: August 10, 2020.

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	Lesotho	2005	OUR WORLD IN DATA. **How the world eliminated lead from gasoline**. Available in: <https://ourworldindata.org/leaded-gasoline-phase-out> accessed on April 12, 2022.
	Madagascar	2005	OUR WORLD IN DATA. **How the world eliminated lead from gasoline**. Available in: <https://ourworldindata.org/leaded-gasoline-phase-out> accessed on April 12, 2022.
	Malawi	2005	OUR WORLD IN DATA. **How the world eliminated lead from gasoline**. Available in: <https://ourworldindata.org/leaded-gasoline-phase-out> accessed on April 12, 2022.
	Mali	2005	OUR WORLD IN DATA. **How the world eliminated lead from gasoline**. Available in: <https://ourworldindata.org/leaded-gasoline-phase-out> accessed on April 12, 2022.
	Namibia	2005	OUR WORLD IN DATA. **How the world eliminated lead from gasoline**. Available in: <https://ourworldindata.org/leaded-gasoline-phase-out> accessed on April 12, 2022.
	Niger	2005	UNITED NATIONS ORGANIZATION. **Lessons learned from the Partnership for Clean Fuels and Vehicles**. 2020. Available in:<https://wedocs.unep.org/bitstream/handle/20.500.11822/25154/GAELP_ADDIS_6_Lessons_UNEP_PCFV.pdf?sequence=1andisAllowed=y>. Accessed on: August 10, 2020.
	Senegal	2005	UNITED NATIONS ORGANIZATION. **Lessons learned from the Partnership for Clean Fuels and Vehicles**. 2020. Available in:<https://wedocs.unep.org/bitstream/handle/20.500.11822/25154/GAELP_ADDIS_6_Lessons_UNEP_PCFV.pdf?sequence=1andisAllowed=y>. Accessed on: August 10, 2020.
	Somalia	2005	OUR WORLD IN DATA. **How the world eliminated lead from gasoline**. Available in: <https://ourworldindata.org/leaded-gasoline-phase-out> accessed on April 12, 2022.
	Tanzania	2005	UNITED NATIONS ORGANIZATION. **Lessons learned from the Partnership for Clean Fuels and Vehicles**. 2020. Available in:<https://wedocs.unep.org/bitstream/handle/20.500.11822/25154/GAELP_ADDIS_6_Lessons_UNEP_PCFV.pdf?sequence=1andisAllowed=y>. Accessed on: August 10, 2020.
	Togo	2005	UNITED NATIONS ORGANIZATION. **Lessons learned from the Partnership for Clean Fuels and Vehicles**. 2020. Available in:<https://wedocs.unep.org/bitstream/handle/20.500.11822/25154/GAELP_ADDIS_6_Lessons_UNEP_PCFV.pdf?sequence=1andisAllowed=y>. Accessed on: August 10, 2020.
	Uganda	2005	OUR WORLD IN DATA. **How the world eliminated lead from gasoline**. Available in: <https://ourworldindata.org/leaded-gasoline-phase-out> accessed on April 12, 2022.
	Zambia	2005	OUR WORLD IN DATA. **How the world eliminated lead from gasoline**. Available in: <https://ourworldindata.org/leaded-gasoline-phase-out> accessed on April 12, 2022.
	Zimbabwe	2005	OUR WORLD IN DATA. **How the world eliminated lead from gasoline**. Available in: <https://ourworldindata.org/leaded-gasoline-phase-out> accessed on April 12, 2022.
	Kenya	2006	NGO, Nicole S. *et al*. Occupational exposure to roadway emissions and inside informal settlements in sub-Saharan Africa: A pilot study in Nairobi, Kenya. **Atmospheric Environment**, v. 111, p. 179-184, 2015.

Continent	Country	Year of elimination of Pb in gasoline	Reference
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	South Africa	2006	SOUTH AFRICA. **Regulations regardind petroleum products specifications and standards Nº . R. 627**. Department of Minerals and Energy. 2006. Available in: <http://www.energy.gov.za/files/policies/regulations_petroleumproducts_standards_2006.pdf>. Accessed on: August 10, 2020.
	Congo	2009	TUAKUILA, Joel *et al*. Blood lead levels in children after phase-out of leaded gasoline in Kinshasa, the capital of Democratic Republic of Congo (DRC). **Archives of Public Health**, v. 71, n. 1, p. 5, 2013.
	Algeria	2021	OUR WORLD IN DATA. **How the world eliminated lead from gasoline**. Available in: <https://ourworldindata.org/leaded-gasoline-phase-out> accessed on April 12, 2022.
Europe	Austria	1993	UNITED NATIONS ORGANIZATION. **Phasing lead out of gasoline: An examination of policy approaches in different countries**. UNEP/Earthprint, 1999. Available in:<https://www.un.org/esa/gite/iandm/unep-lead.pdf>. Accessed on August 10, 2020.
	Finland	1994	UNITED NATIONS ORGANIZATION. **Phasing lead out of gasoline: An examination of policy approaches in different countries**. UNEP/Earthprint, 1999. Available in:<https://www.un.org/esa/gite/iandm/unep-lead.pdf>. Accessed on August 10, 2020.
	Slovakia	1994	UNITED NATIONS ORGANIZATION. **Phasing lead out of gasoline: An examination of policy approaches in different countries**. UNEP/Earthprint, 1999. Available in:<https://www.un.org/esa/gite/iandm/unep-lead.pdf>. Accessed on August 10, 2020.
	Denmark	1995	UNITED NATIONS ORGANIZATION. **Phasing lead out of gasoline: An examination of policy approaches in different countries**. UNEP/Earthprint, 1999. Available in:<https://www.un.org/esa/gite/iandm/unep-lead.pdf>. Accessed on August 10, 2020.
	Sweden	1995	UNITED NATIONS ORGANIZATION. **Phasing lead out of gasoline: An examination of policy approaches in different countries**. UNEP/Earthprint, 1999. Available in:<https://www.un.org/esa/gite/iandm/unep-lead.pdf>. Accessed on August 10, 2020.
	Germany	1996	UNITED NATIONS ORGANIZATION. **Phasing lead out of gasoline: An examination of policy approaches in different countries**. UNEP/Earthprint, 1999. Available in:<https://www.un.org/esa/gite/iandm/unep-lead.pdf>. Accessed on August 10, 2020.
	Norway	1997	ORGANIZATION FOR ECONOMIC COOPERATION AND DEVELOPMENT. **OECD Environmental Performance Reviews: Norway**. OECD, 2011. Available in: <https://www.oecd-ilibrary.org/docserver/9789264098473-en.pdf?expires=1603993061andid=idandaccname=ocid54025470andchecksum=E14BC87C82FB327886FA7FC452FB3EFC>. Accessed on August 10, 2020.
	Italy	1999	ORGANIZATION FOR ECONOMIC COOPERATION AND DEVELOPMENT. **OECD Environmental Performance Reviews: Italy**. OECD, 2013. Available in: <https://www.oecd-ilibrary.org/docserver/9789264186378-en.pdf?expires=1603992958andid=idandaccname=ocid54025470andchecksum=3F0A9A210C88BB3FA22F38E5D9688F27>. Accessed on August 10, 2020.

Continent	Country	Year of elimination of Pb in gasoline	Reference
	Portugal	1999	ORGANIZATION FOR ECONOMIC COOPERATION AND DEVELOPMENT. **OECD Environmental Performance Reviews: Portugal**. OECD, 2011. Available in:<https://www.oecd-ilibrary.org/docserver/9789264097896-en.pdf?expires=1603993370andid=idandaccname=ocid54025470andchecksum=49839E8E6450B3806299B9EBB514DCB7>. Accessed on August 10, 2020.
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5. CONSIDERAÇÕES FINAIS

O desenvolvimento desta tese contribui com dados importantes sobre a aplicação das razões isotópicas na avaliação de preferências alimentares e também com o conhecimento sobre outros fatores que possivelmente influenciam a composição isotópica de carbono e nitrogênio. A utilização desta ferramenta pode auxiliar em avaliações mais precisas dos padrões alimentares, especialmente a composição isotópica de carbono foram as que melhor se relacionaram com a frequência de consumo de diferentes fontes de proteína, além de se associarem também com outras variáveis sócio ambientais como a altitude e a etnia e com marcadores do sangue.

Este trabalho também traz dados inéditos sobre a exposição dos agricultores residentes na da microrregião do Caparaó Capixaba a elementos com reconhecida toxicidade. As elevadas concentrações observadas na população referência mostram que há uma exposição elevada e concomitante e aos elementos avaliados, ressaltando a necessidade de estudos de monitoramento de substâncias tóxicas não só para esta população, mas também para outras regiões do Brasil, visto que muitos países já realizam este tipo de monitoramento.

A partir da avaliação da associação das concentrações dos elementos avaliados com a composição isotópica de carbono e nitrogênio, com fatores sócio ambientais como a idade, o composição corporal, a altitude, gênero e o consumo de álcool e tabaco o estudo contribui com dados que auxiliam na compreensão da dinâmica envolvendo a exposição humana a estes elementos bem como fatores de risco associados ao aumento dessa exposição.

As associações observadas entre os elementos e os marcadores do sangue são de grande relevância, pois despertam preocupação sobre possíveis efeitos à saúde dos indivíduos causados pela exposição a estes elementos. Como discutido, não é possível atribuir casualidade baseando-se em avaliações como esta, contudo, estes dados são importantes para a literatura, no sentido que, corroboram estudos prévios realizados em outras populações e, podem ser utilizados para embasar futuras investigações sobre a dinâmica destes elementos.

Por fim, os resultados obtidos no terceiro capítulo mostraram a importância do fim da utilização do chumbo tetraetila como aditivo da gasolina na diminuição da exposição humana, e certamente de outros organismos, a este elemento. A diminuição exponencial das concentrações sanguíneas ao longo dos anos em todos os continentes mostra a diminuição da contaminação atmosférica, que reduziu os níveis deste elemento nas populações em todo o mundo. Estes dados ressaltam a importância dos estudos de biomonitoramento, que permitem a compilação de dados e a avaliação dos padrões de exposição a longo prazo, podendo auxiliar na tomada de decisão por parte dos governos, como foi o caso do chumbo tetraetila, e de outros elementos para os quais existem regulamentações sobre níveis permitidos em produtos e alimentos. Os dados mostraram ainda, que há muito o que ser estudado, pouco se sabe sobre os níveis de exposição para a maioria dos elementos, e muito menos sobre a sua dinâmica e possível toxicidade.

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