TRACE ELEMENTS IN HUMAN SAMPLES AND FATTY ACID COMPOSITION OF HUMAN MILK IN SLOVENIAN POPULATION

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Doctoral Dissertation
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Doctoral Dissertation

ELEMENTI V SLEDOVIH V HUMANIH VZORCIH IN MAŠČOBNE KISLINE V MATERINEM MLEKU PRI SLOVENSKI POPULACIJI

Doktorska disertacija

Supervisor: Prof. Dr. Milena Horvat

Ljubljana, Slovenia, September 2020

Ne želite si biti to, kar niste, ampak to, kar ste.
Prizadevajte si, da bi vedno bolj napredovali v dobrem na poti,
po kateri hodite.
Potrpežljivo hodite z majhnimi koraki,
dokler ne boste imeli nog za tek ali, še bolje, kril za letenje.

Sv. Frančišek Saleški

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Abstract

Children's development and their health outcome is one of major public health concerns. Quality of the maternal diet and lifestyle before the pregnancy, during pregnancy, and the breastfeeding period have an important influence on the child's development later in life. Exposure to potentially toxic chemical elements and nutritional status of essential elements and fatty acids (FAs) during pregnancy and the breastfeeding period are known for potential adverse development and health effects in children.

In this thesis, the exposure assessment to potentially toxic chemical elements and the nutritional status of essential elements and FAs were investigated in the primiparous mothers in different geographical areas of Slovenia. The study builds on the rich data obtained in the first national human biomonitoring study (HBM, 2007-2014), which included 535 first-time mothers.

The first objective was to investigate the relationship between the composition of FAs, their stable carbon isotopes ($\delta^{13}C_{FA}$) and trace elements composition in human milk and mother's dietary habits and living area. The underlying purpose of the first objective was to improve the understanding of the exposure to selected elements through determination of indicatory FAs and their compound-specific stable isotope signatures. For this reason, the associations between the maternal diet and FAs composition in 74 samples of mature human milk from two contrast Slovenian areas were investigated. The results of the data from questionnaires revealed statistically significant differences in the dietary intake of game, freshwater fish, and fresh and frozen seafood between the study areas. In the coastal area, despite the higher intake of fresh seafood, the levels of saturated and monounsaturated FAs were higher and the levels of polyunsaturated fatty acids (PUFAs), omega-3 (ω-3), and omega-6 (ω-6) FAs were lower compared to those in the inland Pomurje region. The ω-6/ω-3 ratio did not differ significantly between the study areas. This finding was not expected and indicates a discrepancy between the measured and self-reported data. The latter is a consequence of insufficient data information on dietary supplements, which can be an important source of essential elements and FAs. This is the first study to investigate the possibility to use stable carbon isotopes of FAs in human milk as a biomarker of seafood consumption.

Additional data evaluation using data mining approaches confirmed that using the classifier for fresh seafood intake based on the seven variables, including elemental composition, levels of FAs, and their stable isotopes in milk, enables distinguishing between lower and higher fresh seafood consumption with 84% accuracy. Clustering could be used to divide the data sets into two with 90% accuracy. A clustering approach based on the percentage of the certain FAs and $\delta^{13}C_{FA}$ is an accurate indicator to distinguish between higher and lower fresh seafood intake. This confirmed our hypothesis that stable carbon isotope analysis in combination with elemental analysis is an important tool to distinguish between diets, particularly for fresh seafood. These findings suggest that in human-related studies related to the risks and benefits of seafood consumption, the analyses of FAs, their stable carbon isotopes, and elemental compositions can significantly improve precision of the data on seafood consumption.

The second objective of the study was to examine the relation between the child's birth weight and maternal blood levels of toxic and potentially toxic elements and socio-demographic characteristics and maternal dietary habits. The results of the multiple regression results of 494 Slovenian mother-child pairs from 12 different areas revealed that statistically significant ($p < 0.5$) and positive association between manganese (Mn) in maternal blood and birth weight. This could be explained by the essentiality of Mn in fetal development as an important cofactor in enzyme reactions in bone formation and in metabolic regulation for amino acid, lipid, protein and carbohydrate levels. Besides blood Mn levels, maternal age, pre-pregnancy body mass index, estimated gestational age at birth and gender of the child were the main predictors of the child's birth weight.

The overall conclusion of this work was that the routine HBM studies intended for spatial and time trends exposure to potentially toxic chemicals of population can benefit greatly from additional biomarker analysis, including compound-specific carbon isotope signatures, which are useful for source apportionment of nutritional and toxic compounds. Moreover, data mining approaches may further improve data interpretation beyond classical statistical treatment of data in HBM studies.

Povzetek

Otrokov razvoj in njegovo zdravstveno stanje sta eni glavnih skrbi javnega zdravja. Kakovost materine prehrane in življenjskega sloga pred in med nosečnostjo ter v obdobju dojenja pomembno vplivata na otrokov razvoj. Izpostavljenost potencialno strupenim kemijskim elementom in prehranski status esencialnih elementov ter maščobnih kislin (MK) med nosečnostjo in dojenjem sta prepoznana kot potencialno odgovorna za škodljive učinke na razvoj in zdravje pri otrocih.

V tej doktorski nalogi smo pri prvesnicah iz različnih območij Slovenije preučevali oceno izpostavljenosti potencialno toksičnim elementom in prehranskemu statusu esencialnih elementov ter MK. Doktorska naloga temelji na bogatih podatkih, pridobljenih v prvi nacionalni študiji humanega biomonitoringa (HBM, 2007–2014), ki je vključevala 535 prvesnic.

Prvi cilj je bil raziskati povezave med sestavo MK, razmerjem stabilnih izotopov ogljika ($\delta^{13}C_{FA}$) v MK in sestavo elementov v sledovih v materinem mleku in materinimi prehranskimi navadami ter območjem bivanja. Osnovni namen prvega cilja je bil izboljšati razumevanje izpostavljenosti izbranim elementom z določitvijo indikativnih MK in njihovih specifičnih izotopskih vrednosti. Zaradi tega smo raziskali povezavo med materino prehrano in kompozicijo MK v 74 vzorcih zrelega mleka iz dveh kontrastnih slovenskih območij: obalnega območja in pomurske regije. Rezultati podatkov iz vprašalnikov so pokazali statistično pomembne razlike med prehranskim vnosom divjačine, sladkovodnih rib ter sveže in zamrznjene morske hrane med izbranima območjema. V obalnem območju so bile kljub večjemu vnosu sveže morske hrane ravni nasičenih in enkrat nenasičenih MK-jev višje, ravni večkrat nenasičenih MK (ang. polyunsaturated fatty acids; PUFA), omega-3 (ω-3) in omega-6 (ω-6) MK pa nižje v primerjavi z ravnmi v notranjosti Slovenije (pomurska regija). Razmerje ω-6/ω-3 med študijskima območjema ni pokazalo statistično značilnih razlik. Ugotovitev ni bila pričakovana in kaže na neskladje med izmerjenimi in samo-poročanimi podatki. Slednje je posledica pomanjkljivih podatkov o prehranskih dopolnilih, ki so lahko pomemben vir esencialnih elementov ter MK. To je prva študija, ki je vključevala uporabo razmerja stabilnih izotopov ogljika v MK v materinem mleku kot biomarkerja vnosa morske hrane.

Dodatno vrednotenje podatkov z uporabo pristopov za rudarjenje le-teh je potrdilo, da uporaba »klasifikatorja« za vnos sveže morske hrane, ki temelji na sedmih spremenljivkah, vključno z elementarno sestavo, sestavo MK in njihovega razmerja stabilnih izotopov ogljika v mleku, omogoča razlikovanje med nižjim in višjim vnosom sveže morske hrane s 84 % natančnostjo. »Podatkovni grozd« je mogoče uporabiti za delitev nabora podatkov na dva dela z 90 % natančnostjo. »Grozdni pristop«, ki temelji na ravni nekaterih MK in $\delta^{13}C_{FA}$ vrednosti, je natančen pokazatelj za razlikovanje med višjim in nižjim vnosom sveže morske hrane. To je potrdilo našo hipotezo, da je analiza razmerja stabilnih izotopov ogljika ($\delta^{13}C_{FA}$ vrednosti) v kombinaciji z elementarno analizo pomembno orodje za razlikovanje med uživanjem hrane, zlasti če ta temelji na sveži morski hrani. Te ugotovitve kažejo, da lahko v populacijskih študijah, ki so povezane s tveganji in koristmi uživanja morske hrane

pri ljudeh, analiza MK in $\delta^{13}C_{FA}$ vrednosti ter elementna sestava materinega mleka znatno izboljšajo natančnost podatkov o uživanju sveže morske hrane.

Drugi cilj študije je bil raziskati povezavo med porodno težo otroka in koncentracijo elementov v materini krvi, socialno-demografskimi značilnostmi in materino prehrano. Rezultati multiple regresije 494 slovenskih parov mati–otrok iz 12 različnih območij so razkrili statistično pomembno ($p < 0{,}5$) in pozitivno povezavo med koncentracijami mangana (Mn) v materini krvi in porodno težo. To bi lahko razložili z esencialnostjo Mn v plodovem razvoju kot pomembnega kofaktorja pri encimskih reakcijah tvorbe kosti in pri presnovnem uravnavanju ravni aminokislin, lipidov, beljakovin in ogljikovih hidratov. Poleg vsebnosti Mn v krvi, so bili osnovni prediktorji otrokove telesne teže: materina starost, indeks telesne mase pred nosečnostjo, ocenjena gestacijska starost ob porodu in spol otroka.

Splošni zaključek tega dela je, da lahko rutinske študije HBM, ki so namenjene ocenjevanju izpostavljenosti ljudi potencialno škodljivim kemikalijam ter ugotavljanju prostorskih in časovnih trendov, veliko pridobijo z dodatno analizo biomarkerjev, vključno s specifičnim izotopskim odtisom, ki se uporablja za določitev vira prehranskih in toksičnih komponent. Poleg tega lahko »pristopi rudarjenja s podatki« še izboljšajo razlago podatkov, ki presega klasično statistično obdelavo v študijah HBM.

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Abbreviations

%	... Percentage
‰	... Per mil
AA	... Arachidonic acid
ALA	... Alpha–linolenic acids
ANOVA	... Analysis of variance
As	... Arsenic
C=C	... Carbon double bond
Cd	... Cadmium
CDC	... Centers for Disease Control and Prevention, Canada
CLA	... Conjugated linoleic acid
Co	... Cobalt
CO2	... Carbon dioxide
Cr	... Chromium
Cu	... Copper
CVAFS	... Cold vapor atomic fluorescence spectrometry
δ	... Delta notation
$\delta^{13}C_{FA}$	... Stable carbon isotope $^{13}C/^{12}C$ ratio of FAs
DHA	... Docosahexaenoic acid
DMA	... Direct Mercury Analyzer
EA-IRMS	... Element Analyzer Isotope Ratio Mass Spectrometry
EDTA	... Disodium ethylene diamine tetra acetic acid
EPA	... Eicosapentaenoic acid
FA	... Fatty acid
FAME	... Fatty acid methyl ester
Fe	... Iron
FID	... Flame ionization detection
GC	... Gas chromatography
GC-C-IRMS	... Gas chromatography-combustion-isotope ratio mass spectrometry
H_2SO_4	... Sulfuric acid
HBM	... Human biomonitoring
$HClO_4$	... Hydrochloric acid
Hg	... Mercury
HNO_3	... Nitric acid
I	... Iodine
IAEA	... International Atomic Energy Agency
ICP-MS	... Inductively-coupled plasma mass spectrometry

IUPAC	. . . International Union of Pure and Applied Chemistry
KP	. . . Area of Koper, Coastal Slovenia
LA	. . . Linoleic acid
LC FAs	. . . Long-chain fatty acids
LOD	. . . Limit of detection
MBFAs	. . . Methyl-branched fatty acids
MeHg	. . . Methylmercury
mL	. . . Milliliter
Mn	. . . Manganese
Mo	. . . Molybdenum
MS	. . . Area of Pomurje, Inland Slovenia
MUFA	. . . Monounsaturated fatty acid
ng	. . . Nano gram
NIMD	. . . National Institute for Minamata Disease
NMEC	. . . The Republic of Slovenia National Medical Ethics Committee
p	. . . Statistical significance
PAM	. . . Partitioning around medoids
Pb	. . . Lead
PCB	. . . Polychlorinated biphenyls
PHIME	. . . Public Health Impact of Long-term Mixed Element Exposure in Susceptible Population Strata
PUFA	. . . Polyunsaturated fatty acid
Se	. . . Selenium
SFA	. . . Saturated fatty acid
ω	. . . Omega
WHO	. . . World Health Organization
Zn	. . . Zinc

Chapter 1

Introduction

Children's development and their health outcome is one of major public health concerns. Quality of the maternal diet and lifestyle before the pregnancy, during pregnancy, and the breastfeeding period have an important influence on the child's development later in life. Exposure to potentially toxic chemical elements and nutritional status of essential elements and fatty acids (FAs) during pregnancy and the breastfeeding period are known for potential adverse development and health effects in children.

In this thesis, the exposure assessment to potentially toxic chemical elements and the nutritional status of essential elements and FAs were investigated in the primiparous mothers in different geographical areas of Slovenia. The study was built on the rich data obtained in the first national human biomonitoring study (HBM, 2007–2014), which included 535 first-time mothers.

1.1 Human Biomonitoring

HBM is increasingly used as a tool in environmental health and medicine to understand the presence of chemicals in the human body and their effects on human health. It is a method for assessing human exposure to natural or synthetic chemicals/compounds or their effects and susceptibility. The HBM includes collecting samples from human volunteers (urine, blood, hair, saliva, nails, breast milk or other human tissue) and measuring of those indicators of chemical uptake and effect – biomarkers (CDC, 2009; DEMOCOPHES, 2013; Ladeira & Viegas, 2016). The methodology of any biomonitoring study is the core of it and it depends on the pourpouse of each HBM. The four stages include: 1) study design – planning; 2) study conduct – implementation; 3) data analysis; and 4) communication, interpretation and implementation of results. The schematic diagram of various considerations in the design of HBM study is presented in Figure 1.1 and described in Committee on Human Biomonitoring for Environmental Toxicants (2006). **Preparing and designing** any biomonitoring study includes several key components, such as consideration of the study hypothesis, the properties of biomarkers which will be used, the selection of the population to be involved in the study, ethical and communication concern. The next stage is the **implementation of the HBM study**, which involves: a) enrolment and consent of individuals of the target population; b) sample collection and processing; c) laboratory analysis; and d) statistical analyses and communication of results. After having all the data from questionnaires, laboratory analysis and hypothesis of the HBM study, the **data analysis** can start. First, basic statistics should be done. Statistical analysis comprises weighting the samples on the basis of population, parameters describing distribution, creating a good dataset and constructing complicated statistic models for the study (Becker et al., 2008; Committee on Human Biomonitoring for Environmental

Toxicants, 2006). After running the data through statistical analysis, it is time for interpretation of the results, to confirm the hypothesis or to deny it. The fourth stage are **communications and implementations of results** to individuals, health organizations/clinical centres, general public and policy-makers and agency (Committee on Human Biomonitoring for Environmental Toxicants, 2006).

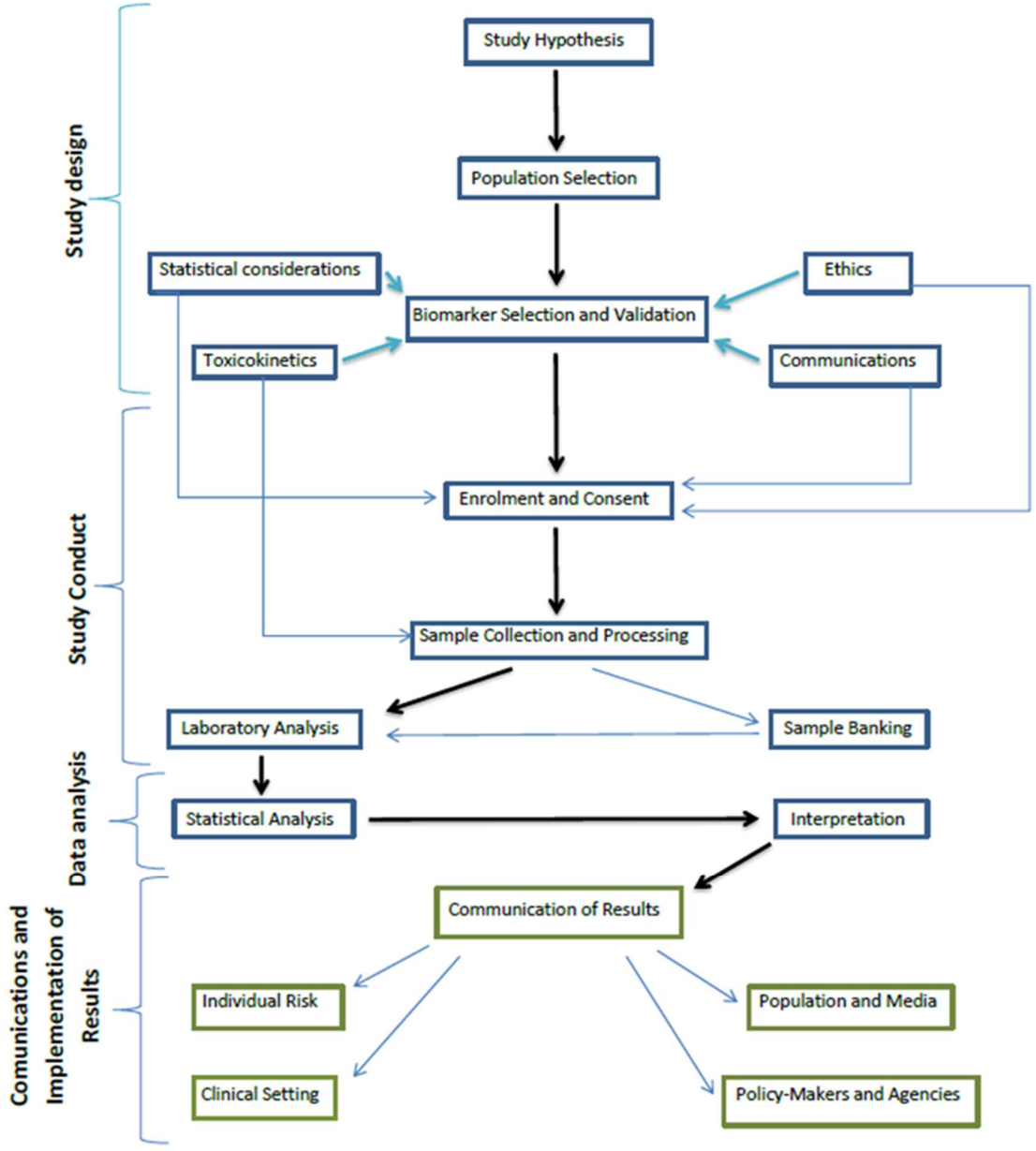


Figure 1.1: Stages of a biomonitoring study (Committee on Human Biomonitoring for Environmental Toxicants, 2006).

There are different legislative drivers/bases (public concern, hazardous properties, regulatory demand, predicted exposure levels, recommendations of the agencies or organizations such as the World Health Organization, European Environmental Agency, etc.) on which the HBM studies were implemented on, such as the EU Environment and Health Strategy; Action Plan (2004-2010); WHO, the Fifth Ministerial Conference on

Environment and Health, Parma 2010 and so on. In Slovenia, for example, HBM is commanded in the Slovenian ACT on CHEMICALS (Official Gazette of the Republic of Slovenia, No. 16/2008) (more will be presented in Section 1.5).

Well-established examples of HBM in the world are: in Canada (Canadian Health, 2010; Health Canada, 2013), USA (CDC, 2009, 2013), Germany (Becker et al., 2008; Schulz, Wilhelm, Heudorf, & Kolossa-Gehring, 2011), Flanders (Belgium) (Schoeters, Colles, et al., 2012; Schoeters, Den Hond, et al., 2012), Czech Republic (Cerná, Krsková, Cejchanová, & Spěváčková, 2012; Cerná et al., 2007), Poland (Polish Mother and Child Cohort; REPRO_PL) (Nofer Institute of Occupational Medicine Department of Environmental Epidemiology, n.d.) and in harmonized EU-projects and programs: COPHES and DEMOCOPHES (DEMOCOPHES, 2013; "Harmonized human biomonitoring on a European scale: experiences in seventeen countries," 2015), and current HBM4EU ("HBM4EU," n.d.). HBM4EU is a joint project of 30 countries, the European Environment Agency and the European Commission, co-funded under Horizon 2020 with aims of coordinating and advancing HBM in EU. It is generating evidence of the actual exposure of citizens to chemicals and the possible health effects in order to support policy making ("HBM4EU," n.d.).

Such programs enabled a greater number of samples in the human body and tissue, data-sets and the opportunity to increase the knowledge to use for exposer and risk assessment in environmental and occupational health. HBM can be useful for different purposes, depends on the aim: for obtaining trends in population, it can be a part of epidemiological studies, as an integral part or it can focus on a narrower part.

The stages of HBM mentioned above are dependent on the **objectives of HBM**, which can be short-term (to obtain data on exposure of a selected population) or long-term (determine the potential effect of various chemicals on health; monitor changes due to exposure over a long period and spatially; provide measures in the future To minimize any such exposure; review the effectiveness of measures to reduce risks to health and environment; science-based risk evaluation; time trends) ("Crome-Life," n.d.). There are different types of HBM surveys: cross-sectional surveys (assess at one moment of time) or longitudinal surveys (repeated measurements on sample individuals over time).

In general, in HBM design, especially in the context of exposure assessment, the **questionnaires** which are key-important, represent the highest uncertainty. In the preparation of HBM, questionnaires are really important and depend on the purpose of the HBM. But even with quality-prepared questionnaires, the answers of participants are sometimes uncertain (Galbete et al., 2018). This can be obtained especially when looking for associations between pollutant sources and the uncertainty of questionnaires. An example of such a study was a research project within the EU 6th Framework Program PHIME (Public health impact of long-term, low-level mixed element exposure in susceptible population strata), where one of the goals was to epidemiologically assess the impact of selected potentially toxic elements exposure through foods and characterized benefits of exposure to essential elements and other dietary components (such as FAs) and to describe some aspects of risk/benefit relationships. For a good evaluation of dietary habits, especially seafood consumption, for example of women in the childbearing period, it is important to know how big the **sample size** should be, what to ask in questionnaires, how to ask to find possible associations between the right cause and the right consequence of this cause. For risk assessment of seafood, health benefits have to be balanced with the potential health risk. There are articles, which discussed benefits and risks of maternal seafood consumption on children's development. On the one hand, seafood is an important source of nutrients beneficial for human health including omega-3 (ω-3) FAs, iodine (I), selenium (Se), proteins, fats, fat-soluble vitamins (vitamin D). On the other hand, seafood could also be a source of contaminants such as methylmercury (MeHg), arsenic (As),

polychlorinated biphenyls (PCBs), dioxins pharmaceuticals, microplastics, endocrine disruptors, which can be present at even higher concentrations. For example, MeHg that is a neurotoxin that can cause long-term development delays in children and has been linked to impaired cardiovascular health in adults, is the dominant mercury (Hg) species in fish and bioaccumulates in the aquatic food web. Consumption of contaminated fish is a primary vector of human exposure to MeHg that was demonstrated in numerus studies (Jacobs, Sioen, Marques, & Verbeke, 2018; Łuczyńska, Paszczyk, Nowosad, & Łuczyński, 2017; A. Miklavčič et al., 2011; Ana Miklavčič et al., 2013, 2011; Ruxton, Reed, Simpson, & Millington, 2004; Tamburlini & Barbone, 2007; Valent, Horvat, Mazej, Stibilj, & Barbone, 2011; Valent et al., 2013).

The key part of the design is to prepare good questionnaires, which will be able to detect the precise and real data from participants. But it is not necessary that even on a well-designed questionnaire the participants would know the precise answer, imprecise could be the observation of dietary habits. There are different food questionaries' in use, from the frequency questionnaires (example: how often do you eat a specific item) to a precise food diary (participants write precise information for each meal). Food diary is hard to be feasible for many participants, but it could be appropriate for some sub-groups or studies with less participants. How to prepare, with the already known facts and experiences from other HBM studies, the questionnaires with which we will be able to get reliable answers? Or is there some other way to get the reliable answers? One way for the improvements, which is crucial for interpretation, can also be the determination of chemicals. For instance, for seafood consumption associations with elements Hg, As and Se are known (Ana Miklavčič et al., 2013). Further on Valent et al. (2013) and Strain et al. (2008, 2015) presented their findings in the field of prenatal MeHg exposure and polyunsaturated fatty acids (PUFAs) from seafood consumption with child developments for northern Italy and Seychelles, respectively (J. J. Strain et al., 2008; J. Strain et al., 2015; Valent et al., 2013). In Italy (N = 606) mean weekly fish intake during pregnancy was less than 2 servings and the levels of Hg in biological samples were low and only moderately correlated with fish intake, also maternal ω-3 PUFA correlations with fish intake were poor (Valent et al., 2013). In 2008, mother-child pairs from Seychelles (N = 229) reported an intake of 9 fish meals per week. Their data highlight the potential importance to child development of the prenatal availability of omega-3 long chain PUFA (ω-3 LC PUFA) from fish and the overall diet and indicate possible attenuation of such effects by ω-6 LC PUFA and MeHg. Their data indicate that confounding of adverse effects of MeHg by nutrients, or conversely, confounding of beneficial effects of LC PUFA by MeHg, should be considered when evaluating data from observation studies (J. J. Strain et al., 2008). Later research of mothers from Seychelles (N = 1265) reported an average of 8.5 fish meals per week. Despite that, they found no overall adverse association between prenatal MeHg exposure and neurodevelopment outcomes through 20 months of age. Conforming with their past findings in multiple cohorts from their population, they supported the importance of maternal PUFA status, and they suggested that the development consequences of exposure to MeHg from fish and maternal PUFA status are more complex than previously thought (J. Strain et al., 2015). The findings of study on pregnant Seychellois women indicated that a diet rich in fish, particularly oily fish, is associated with higher maternal concentrations of ω-3 LC PUFA at 28 weeks' gestation. The diet pattern with a high amount of fish and fruit was positively associated with docosahexaenoic acid (DHA) and total ω-3 FA status during pregnancy. Considering the importance of ω-3 PUFA for the neurodevelopment of the child through life, dietary recommendations for fish consumption during pregnancy should consider the importance of fish consumption and its association with the increased PUFA status (Conway et al., 2018).

The PHIME study above was the basis to set the objectives of this dissertation. Our motivation was to improve the methodology – find a better marker of seafood consumption for future studies. Therefore, we took milk samples from two completely different areas of Slovenia: inland (area of Pomurje) and coastal (area of Koper) and examined if there were any opportunities to get better information on dietary habits through determinations of FAs and their stable carbon isotope ratios in human milk.

1.2 Biomarkers

Biomarkers were defined by the World Health Organization (WHO) in 1993 (World Health Organization (WHO), 1993) in connection with risk assessment, where the term biomarker is used in a broad sense to include almost any measurement reflecting an interaction between a biological system and an environmental agent, which may be chemical, physical or biological. Analysis of biological tissues for chemicals, metabolites of chemicals, enzymes, molecular, genetic, and immunologic and other biochemical substances, or physiologic observations has been used to document the interaction of chemicals within biological systems. Measurements of these biomarkers are recognized as providing data linking exposure to a chemical with an internal dose and outcome and as relevant to the process of risk assessment (INTARESE (Integrated Assessment of Health Risk of Environmental Stressors in Europe), 2007; World Health Organization (WHO), 1993). In 2012, Knudsen et al. introduced definitions of three identified biomarker classes: "biomarker of exposure; biomarker of effect, and biomarker of susceptibility" (Kundsen, Hundebøll, & Merlo, 2012). The simplified flow chart of these classes of biomarkers is shown in Figure 1.2 (Committee on Human Biomonitoring for Environmental Toxicants, 2006). Owen et al. (2008) have identified several required elements, which include identification of the assessment endpoint at the outset, rational selection of biomarker(s) measurement endpoint, biomarker validation (see Table 1.1) and qualification (Owen, Galloway, Hagger, Jones, & Depledge, 2008; World Health Organization (WHO), 2001).

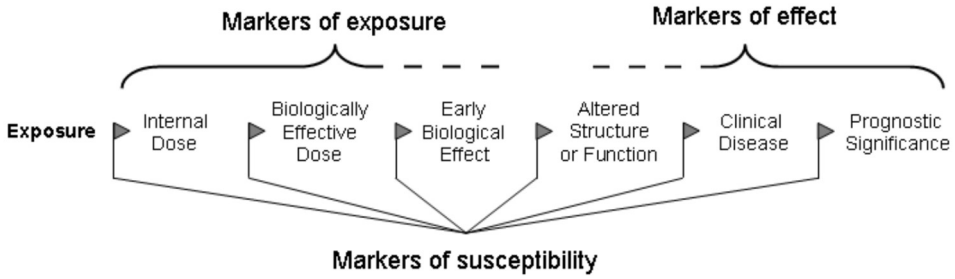


Figure 1.2: Simplified flow chart of classes of biomarkers (Committee on Human Biomonitoring for Environmental Toxicants, 2006).

WHO stated that biomarkers should be viewed as another set of tools available for researchers and risk assessors and not as a replacement for traditional approaches. There is also a need for a long-term commitment to the assessment of the validity of biomarkers for risk assessment, environmental health research and public health practice (World Health Organization (WHO), 2001). Examples of overview of biomarkers in HBM include a review about biomarkers of exposure in environment-wide association studies as opportunities to decode the exposure using human biomonitoring data written by Steckling et al. (2018) (Steckling et al., 2018) and Ladeira et al. (2016) wrote an overview on biomarkers and their application in occupational and environmental health (Ladeira &

Viegas, 2016). Biomarkers may be applied whether exposure has come from dietary, environmental or occupational sources (Ladeira & Viegas, 2016). Biomarkers could be used to elucidate cause-effect and dose-effect relationships in health risk assessment, in clinical diagnosis or/and for monitoring purposes (World Health Organization (WHO), 1993). To choose a suitable biomarker, it is also important to take into account the matrix in which the substance is measured. For instance, for elemental concentrations in urine, it is possible to use "normalization of urine biomarkers" and the results express normalized for specific gravity, levels of creatinine in urine or leave results as they are in ng/mL or ng/g, in the study Stajnko et al. (2017) the difference in-between these options was discussed (Stajnko et al., 2017).

Table 1.1: Characteristics of biomarkers.

Biomarker type	**Characteristic of validity**
Exposure*	Consistently linked with exposure at relevant levels of exposure with confounding and background exposures assessed[a]
Effect*	Consistently linked with increased risk with confounding and effect modifying factors assessed
Susceptibility*	Can distinguish subgroups at risk given specific exposure
Nutritional status	*Can provide nutritional status of individuals*

[a] Biomarkers of exposure may also be validated by establishing a constant link to an adverse health effect or to the concentration of the chemical in the target organ.

[*] (World Health Organization (WHO), 2001).

Characteristics of different types of biomarkers are presented in Table 1.1.

A **biomarker of exposure** is an exogenous substance/chemical or its metabolite, or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment in an organism (Kundsen et al., 2012). Specific chemicals and associated biomarkers of exposure include for example: blood lead (Pb), blood Hg, urinary 1-hydroxypyrene for polycyclic aromatic hydrocarbons, urinary phthalate metabolites, urinary cotinine for environmental tobacco smoke, and urinary pesticide metabolites (Fan, 2014). It may be used to assess and confirm the exposure of individuals or populations to a particular substance, providing a connection between external exposures and internal dosimetry. In evaluating exposure, distinction is made between the external dose, which is defined as the amount of a chemical agent in environmental contact with the organism, as determined by personal or area monitoring, and the internal dose, which is the total amount of a chemical agent absorbed by the organism over a period of time. Biomarkers of exposure reflect the distribution of the chemical (or its metabolite) throughout the organism. In principle, this distribution can be tracked through various biological levels (e.g., tissue, cell, etc.) to the ultimate target (World Health Organization (WHO), 1993).

A **biomarker of effect** is a measurable biochemical, physiological, behavioural or other alteration in an organism that, depending upon the magnitude, can be recognized as associated with an established or possible health impairment or disease (Kundsen et al., 2012). Some biomarkers, which Fan (2014) mentioned are: organophosphates and carbamates and blood cholinesterase inhibition, triazines and pituitary luteinizing hormone surge attenuation, nitrate and methemoglobinemia, Se and selenosis, tetrachlorodibenzo-p-dioxin and response markers, polychlorinated biphenyls and blood thyroxine decrease, benzene and blood cell counts; cancer – related biomarkers (DNA and protein adducts, genetic changes) (Fan, 2014). They can be used to document either preclinical alterations or adverse health effects elicited by external exposure and absorption of a chemical. This way, the linkage of biomarkers between exposure and effect contributes to the definition of dose-response relationships (World Health Organization (WHO), 1993).

A **biomarker of susceptibility** is an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance (Kundsen et al., 2012). Some examples of biomarkers of susceptibility: metabolic enzymes, genes, genetic polymorphism and studies using "omics" methodology (Fan, 2014). It can help to elucidate the degree of the response to exposure elicited in individuals (World Health Organization (WHO), 1993).

Biomarkers of nutritional status

Biomarkers of nutritional status can be markers of exposure or effect or susceptibility to understand the complex relationships between nutrition and health (Picó, Serra, Rodríguez, Keijer, & Palou, 2019). For instance, **biomarkers of exposure** include different types of biomarkers used to evaluate dietary intake of nutrients, non-nutritive food components or dietary patterns. **Biomarkers of effects** are biomarkers that are related to a target function or a biological response. These biomarkers do not only reflect intake but also nutrient metabolism and, possibly, effects on physiological or disease processes. It should be noted that various factors may influence biomarker responses, that a biomarker may not reflect, the effect of a single nutrient but the interactions of various nutrients. These include internal responses in the body, exposure to other chemicals, individual variability (including genetic), gender, age sensitivity, nutrition, activity patterns, and pharmaceutical use, among others. **Biomarkers of health and disease and physiological status** are biomarkers, which indicate an end-point, relate to a state of health and/or disease risk. These markers reflect the different intermediate disease phenotypes or the severity of the disease. Understanding all the various possible modifying factors is important for risk assessments since they can affect any step in the continuum of events (Fan, 2014; Picó et al., 2019). Biomarkers provide a more proximal measure of nutrient status than dietary intake. A **nutritional biomarker** should have a characteristic that can be objectively measured in different biological samples, can be used as an indicator of nutritional status with respect to the intake or metabolism of dietary constituents, can be a biochemical, functional or clinical index of status of an essential nutrient or other dietary constituent (Dragsted et al., 2018; Picó et al., 2019; Potischman & Freudenheim, 2003).

One challenge in nutritional studies is the valid and reliable assessment of food intake, as well as its effects on the body. Usually in nutritional as well as HBM studies, food intake measurement is based on self-reported dietary intake questionnaires, which have inherent limitations. Limitations can be improved by the use of biomarkers, capable of objectively assess food consumption without the bias of self-reported dietary assessment. Another aim in such studies is to determine the biological effects of foods and their impact on health.

System analysis of dynamic responses could help to identify biomarkers indicative of intake and effects on the body at the same time, possibly in relation to individuals' health/disease states. That kind of biomarkers may be used to quantify intake and validate intake questionnaires, analyse physiological or pathological responses to certain food components or diets, identify persons with specific dietary deficiency, provide information on inter-individual variations to achieve optimal health for particular phenotypes, currently referred to as "precision nutrition." This way, holistic approaches using global analysis methods, capable of gathering high amounts of data, appear to be very useful to identify new biomarkers and to enhance understanding of the role of food in health and disease (Picó et al., 2019).

As mentioned above, measurement of markers describing the organism's biological nutritional status and its sensitivity could be introduced in HBM studies. Is it enough to measure biomarkers in HBM studies that tell us just how exposed we are to any of the potentially harmful substances? We could introduce biomarkers to tell us more about the nutritional status. What would improve the interpretation of the results? The suggestion would be that, with minimal effort (both by investigators and researchers), the uncertainties obtained from the questionnaires could be greatly reduced. In this work, we suggest improving the fingerprint of nutritional status, which also defines susceptibility. A status that describes the biological nutrition of an organism and its sensitivity to substances that adversely affect health. HBM is facing challenges in terms of interpretation due to the large sample size required especially for low exposure scenarios to overcome the uncertainties in statistical analyses. Recruiting participants in HBM is challenging and can be ethically controversial for certain goals. Why would we take biological material from so many people, if we can take it from fewer? Therefore, it is important to improve the analytical approach itself as well as the approach in analyzing the obtained data, such as use of modern data mining techniques. In our work we included FAs to the list of classical exposure biomarkers in HBM in order to assess the nutritional status of vulnerable individuals, in our case lactating women, which may define their susceptibility to harmful contaminants.

As suggested above, biomarkers of good nutrition may be, for example, FAs in human milk or the essential trace elements in breast milk, blood, urine or hair, which will be presented in the next two sections. Human milk was selected as a matrix since it is, on the one hand, a good reflection of the mother's body burden and nutritional status and it represents, on the other hand, the dietary exposure of breast-fed infants. It can, furthermore, allow conclusions on mother-child transfer of nutrients as well as potentially toxic chemicals. Birth weight can be taken as an indicator of children's health in response to the maternal nutritional status during pregnancy as obtained via blood levels.

1.3 Fatty Acids and Their Stable Isotope Ratios of Carbon

FAs are one of numerous chemical compounds among monoglycerides, diglycerides, triglycerides, phosphatides, cerebrosides, sterols, terpenes and fatty alcohols from which fats or lipids consist of. In nature, they can exist as a pure substance or as parts of more complex molecules. FAs consist of a hydrocarbon chain skeleton of different lengths with a carboxyl group at one end. Depending on the number of double bonds (C=C), FAs can be saturated (no double bond, SFA), monounsaturated (one double bond, MUFA), or polyunsaturated (two or more double bonds, PUFA). FAs have an essential role for energetic, metabolic, and structural activities (Brondz, 2002; Lobb & Chow, 2008). The position of C=C and the total change length determine the PUFAs nomenclature. The term ω-3 indicates that the first C=C is located between the third and fourth carbon from

the methyl (CH_3) end of the molecule (Ruxton et al., 2004). Two main groups of PUFAs are LC ω-3 and ω-6 FAs. Some FAs, which are essential for humans and necessary for human health cannot be synthesized by human body. PUFAs with double bonds at position 6 or 3 are one of them. Those should get through diet (Connor, 2000).

Lipid content and FA profile are considered to have an important role in the human diet (Birch, Birch, Hoffman, & Uauy, 1992; Z. Y. Chen et al., 1997; Connor, 2000; Innis, 2007, 2014; R. Jensen, 1999; Keim et al., 2012; Koletzko, Mrotzek, & Bremer, 1988; Ruxton et al., 2004; Williams, 2000). Consumption of SFAs is associated with a higher incidence of cardiovascular diseases, whereas unsaturated FAs are beneficial for human health. For example, ω-3 PUFAs play an essential role in the development of neurological functions in the early years of life, and conjugated linoleic acids (CLAs) have anti-diabetic, anti-atherogenic, and anti-carcinogenic health effects (Lock & Bauman, 2004; Nunes & Torres, 2010). The ω-6 and ω-3 PUFA contents can influence the optimal growth and development of the child (Birch et al., 1992; Z. Y. Chen et al., 1997; Innis, 2007, 2014; R. Jensen, 1999; Keim et al., 2012; Koletzko et al., 1988; Ruxton et al., 2004). FAs were also related to chronic diseases such as cardiovascular disease, diabetes, cancer, arthritis, obesity and asthma (Ruiz-Rodriguez, Reglero, & Ibañez, 2010). Since FAs composition of food products, especially for babies, for example human milk, has been really interesting in research because of its implication for human health, Williams (2000) suggested that the quantity and quality of fat in diet should be considered (Williams, 2000).

FAs in human milk originate from the mobilization of maternal body stores of FAs, diet, or endogenous synthesis from precursor FAs by the liver or breast tissue (Francois, Connor, Wander, & Connor, 1998; R. Jensen, 1999; R. G. Jensen, 1996; Sauerwald, Demmelmair, & Koletzko, 2001). FAs in human milk originate from three sources: (1) mobilisation from maternal endogenous stores of FAs owing to deficient energy intake, (2) endogenous synthesis of FAs from precursor FAs when excess energy is fed, and (3) diet composition (Francois et al., 1998; R. Jensen, 1999; R. G. Jensen, 1996; Sauerwald et al., 2001). Francois et al. (1998) found that a single meal of specific fats could significantly affect the FA profile of human milk for 1–3 days, with maximum levels of specific FAs occurring within a 24-h period (Francois et al., 1998).

Content of fatty acids in food

SFAs are known to be mainly present in dairy products (e.g., milk, butter, cream, and cheese) and in animals, and their products (e.g., biscuits, crackers, cakes, pastries, sauces, fast food, and hamburgers)(Chow, 2007; Francois et al., 1998; Nishimura et al., 2014; Tvrzicka, Kremmyda, Stankova, & Zak, 2011).

MUFAs have been used as a substitute for SFAs in nutrition. Their main sources in human diet are butter, swine and bovine meat, mackerels, nuts (almonds, peanuts, Brazil nuts, cashew nuts, hazelnuts, and pistachio nuts), seeds, olives, olive oil, and rapeseed oil (Chow, 2007; Francois et al., 1998; Nishimura et al., 2014; Tvrzicka et al., 2011).

In the human diet, PUFAs are more beneficial. They are present in higher amounts in vegetable oils. Foods rich in ω-3 PUFAs include fish, salmon, trout, herring, mackerel, tuna fish, sardines, wolf-fish, flounder, seafood, fish oils, soybean oil, and rapeseed oil. The main dietary sources of ω-6 PUFAs are vegetable oils and margarines, mayonnaise, meat, sunflower, corn oil, soybean oil, grapeseed, and sesame seeds (Chow, 2007; Francois et al., 1998; Nishimura et al., 2014; Tvrzicka et al., 2011). The parent compound of ω-6 family is linoleic acids (LA) and of ω-3 family alpha–linolenic acids (ALA). They can be converted to their long-chain PUFAs, LA to arachidonic acid (AA) and ALA to eicosapentaenoic acid (EPA) and DHA (Connor, 2000). Due to health aspects, a number of authorities and scientific associations have issued policy recommendations that ultimately aim to reduce

intake of SFA and trans-fatty acids, which are published in "Policy recommendations on fat intake" (The Joint Research Centre (JRC), 2020).

Stable carbon isotope ratios of fatty acids

Stable carbon isotope ratios in FAs can give additional information about the origin and sources of FAs present in human milk. The stable isotope ratios of light elements are a promising set of candidate biomarkers because they vary naturally and reproducibly among foods, and those variations are captured in molecules such as FAs and tissues with high fidelity.

Stable carbon isotope ratio measurements are expressed in delta notation (δ) relative to the Vienna-Pee Dee Belemnite (V-PDB) standard in per mil (‰) and defined as $\delta^{13}C = [(R_s - R_{ref})/R_{ref}] \times 1000$, where R is the $^{13}C/^{12}C$ ratio in sample 's' and reference material 'ref', respectively (Brand, Coplen, Vogl, Rosner, & Prohaska, 2014). Because tissue from living organisms typically has a lower abundance of ^{13}C, $\delta^{13}C$ values from biological studies are rather unfortunately almost always negative.

$\delta^{13}C$ could also be used for tracking dietary habits, since the FAs are connected also to dietary habits. This is because variations in the isotopic composition of carbon ($\delta^{13}C$) occur owing to differences in the fixation pathways of CO_2, which are correlated with environmental and physiological conditions (Boschker & Middelburg, 2002). All plants in naturally synthesized organic compounds assimilate carbon isotope ^{12}C in preference to ^{13}C, resulting in different $\delta^{13}C$ owing to different enzymatic processes and sizes of metabolic carbon pools (Hayes, 1993). Thus, $\delta^{13}C$ differences were observed between different types of plants: C3 plants (tree, shrubs, temperate grasses) has $\delta^{13}C$ values from −35 to −22‰, $\delta^{13}C$ values in C4 plants (sugar cane, subtropical grasses, maize) range from −17 to −9‰, while crassulacean acid metabolism (CAM) plants has $\delta^{13}C$ values between −27 and −12‰ (Meier-Augenstein, 1999; Smith & Epstein, 1971; Tykot, 2006). Further, differences in $\delta^{13}C$ between terrestrial C3 plants and plants in marine environment can be also observed with higher $\delta^{13}C$ values observed in marine plants (Richards & Hedges, 1999). On average, people in Slovenia consume 1–2 servings of fish per week, which is likely a small source of carbon compared to the major C3 and C4 sources such as wheat and corn. However, if people consume a very large proportion of calories from fish and other marine foods, their $\delta^{13}C$ values will be influenced by those sources. Animal products are another important source of elevated $\delta^{13}C$ in the diet; therefore, biomarkers that can differentiate carbon contributed by sugars and carbon contributed by protein (e.g., meat, eggs) are of particular interest and relevant. This point may be obvious, but is worth stating: all of the carbon, in our bodies derives from the food we eat; there are no other sources and there is no way for the body to synthesize them *de novo*.

1.4 Trace Elements

According to the International Union of Pure and Applied Chemistry (IUPAC) Golden book (2014), the trace elements are elements which have an average concentration of less than about 100 parts-per-million atoms (ppma; 10^{-6}) or less than 100 μg/g (IUPAC (International Union of Pure and Applied Chemistry), 2014). Most of the investigated trace elements are metals (e.g. copper (Cu), zinc (Zn), lead (Pb), cadmium (Cd), mercury (Hg)), exceptions are metalloids (selenium (Se), arsenic (As)). Metals are natural chemical elements, which have metallic properties (e.g. metallic shine, high electrical and thermal conductivity), metalloids have both metallic and non-metallic properties (Lazarini & Brenčič, 2004; Maret, 2016). Trace elements are found throughout the environment in

ambient air, drinking water, soil or/and dust. They originate from natural sources and processes and could be increased due to the elevated human activity (industry). Elements are transported into the human body by inhalation, ingestion and/or dermal absorption. Trace elements have a different role in the organism, some are essential, others are potentially toxic or toxic (Nordberg, Fowler, & Nordberg, 2015).

An element is considered essential to a living organism when the element is an integral part of an organic structure performing a vital function in that organism and a decrease in concentration below a certain limit results consistently in a reduction in a physiologically important function. But even essential elements, when present in excess, have the potential to turn harmful (Aggett, Nordberg, & Nordberg, 2015; World Health Organization (WHO), 1996). Elements, which were classified as essential for human health by WHO (1996) are: chromium (Cr), cobalt (Co), Cu, iron (Fe), molybdenum (Mo), Se and Zn (World Health Organization (WHO), 1996), later in the last decade manganese (Mn) was added to this list (Aggett et al., 2015). They may serve as cofactors or as constituents of complex molecules and although the requirements are smaller but their status of them necessary to follow has to be strictly regulated to ensure sufficient amount for the proper functioning of the organism. Essential element deficiencies are reported from about 40% of the world's population, mostly from developing countries. This is probably a result of a poor diet and can lead to endocrine, cardiovascular, skeletal, gastrointestinal, kidney, genetic, and ophthalmologic disorders (Nriagu & Szefer, 2007). Furthermore, similarities between metabolism of essential and toxic elements should be taken into account, if there is not enough essential element, it could come to a higher absorption of a potentially toxic one. An example is a deficiency in Zn and Fe could lead to higher absorption of Cd and Pb (Goyer, 1995). Another important role of essential trace elements is in detoxification processes. For example: beneficial effects of selenium in populations exposed to toxic elements (As, Cd, Hg) are known, various complexes can be formed (Rahman et al., 2019).

Other, non-essential elements are xenobiotics, i.e., which have no useful role in human physiology and most other living organisms and, even worse, as in the case of Pb and Hg, may be toxic even at trace levels of exposure (Howard Hu, 2002). Toxic elements may accumulate undetected in the body over time, and can affect the membrane or structural proteins, enzymes, membrane lipids, or DNA and may also displace an essential element from its binding site and thereby disrupting protein function or it may cause neurotoxicity by different mechanisms: inorganic element transformation to organic species, sulfhydryl group binding, and/or and free radical generation which can lead to lipid peroxidation, cellular membrane degeneration, and ultimately cell death (Metametrix, 2011). Toxicity of elements and on the other hand also bioavailability are determined by the chemical form of the element. Oxidation state and solubility are especially important factors for bioavailability (Christensen, 1995).

In the past more studies had focus on occupationally exposed subject. In recent years it has been demonstrated in epidemiological studies that exposure of general population to low levels of toxic elements could make an important contribution to the global burden of disease and additionally a deficient intake of essential elements through food also give rise to the global burden of disease (Christensen, 1995; Ivanenko et al., 2013; Kabir et al., 2012; Nordberg, Fowler, & Nordberg, 2014; Nordberg, Fowler, Nordberg, & Friberg, 2007; Schroijen et al., 2008; Vrijens et al., 2013; Wilhelm et al., 2007).

In this dissertation, some most relevant toxic and potentially toxic elements, listed among the 7 top chemicals on the Priority list of Hazardous Substances(ATSDR, 2019), were addressed: As, Pb, Hg and Cd; along with the exposure to non-essential trace elements, also the status of essential trace elements (Se, Zn, Cu and also Mn) was evaluated as it may play in toxicity of non-essential elements as described above.

Selected population groups are one of the groups that are the most susceptible to increased exposure to those elements i.e. infants and children because of higher absorption rates and diminished ability to detoxify many exogenous compounds, relative to that of adults (Grandjean & Landrigan, 2006). A developing fetus is particularly susceptible due to a partial lack of a blood-brain barrier (Andersen, Nielsen, & Grandjean, 2000) and rapid and tightly controlled growth of brain during the critical period of nine months. Increased level of contaminants in maternal blood during pregnancy could have the potential influence to the developing fetus. It is known that several of these elements move from mother to fetus through the umbilical cord and to the child through the mother's breast milk (Röllin, Rudge, Thomassen, Mathee, & Odland, 2009; Rudge et al., 2009). Of concern are especially long-term, subtle effects that might influence reproductive health and/or pregnancy outcomes (new-born weight, length, etc.), reduce defense against diseases, affect children's mental development, or increase the risk of cancer (Stillerman, Mattison, Giudice, & Woodruff, 2008). In the study performed by Polanska et al. (2018), findings showed that fetal exposure to even very low Pb levels might affect the early cognitive domain, with boys being more susceptible than girls (Polanska et al., 2018).

1.5 First Slovenian HBM in the Frame of Presented Work

In Slovenia, there are different legislative drivers/bases on which the HBM studies were implemented on: EU Environment and Health Strategy; Action Plan (2004–2010); WHO, the Fifth Ministerial Conference on Environment and Health, Parma 2010; and in Slovenia Act for Chemicals (Official Gazette of the Republic of Slovenia, No. 110/2003), which in the Chapter IX pointed out the protection of human health and the environment, and in ARTICLE 49 the protection of people or the environment, prohibitions and restrictions, and in ARTICLE 51.a biomonitoring of chemicals, which protect human health and the environment, the monitoring of the presence of chemicals and their breakdown products in people and organisms shall be conducted in professionally justified intervals of time. Thus, consequentially, Slovenia has a National Human Biomonitoring program. This first National HBM survey consisted of the pilot phase (2007–2010) and the second phase (2011–2014). This HBM survey included samples of urine, hair, blood and milk form 50 women and 50 men from the same area (20–40 years old) in 12 different areas of Slovenia (Snoj Tratnik et al., 2019). The first national HBM survey in Slovenia examined Cd, Pb, Hg, As, Mn, Se, Cu and Zn in a childbearing population (18–49 years) selected from lactating primiparous women and men (N = 1084).

The main aims of the survey were to estimate trace elements' levels and geographical differences in order to identify sources of possible exposures, estimate babies' exposure via maternal milk and set the national reference values for selected elements in the selected population. The study population was selected evenly from 12 study areas across Slovenia, including rural, urban and presumably metal-contaminated areas, and biological matrices were used to assess internal exposure (Figure 1.3). Within 6–8 weeks after delivery, venous blood, spot urine, scalp hair and breast milk samples were collected to determine the selected elements.

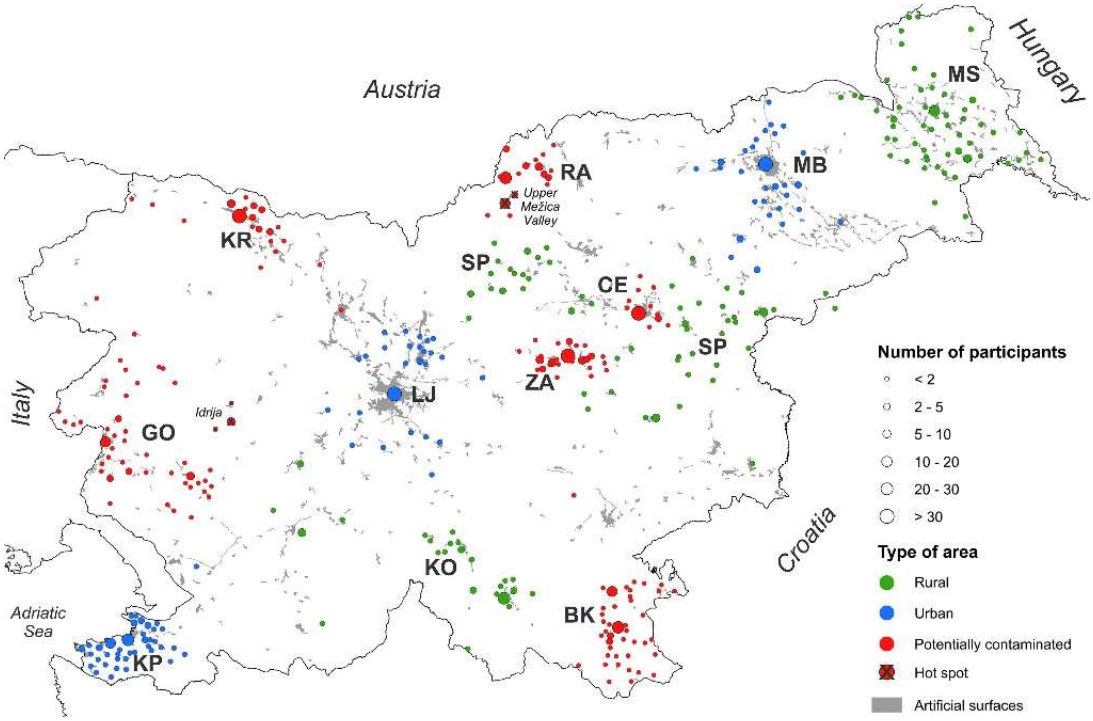


Figure 1.3: Geographical distribution of participants in the Slovenian HBM survey. Circles depict the total number of people recruited in an individual settlement. Study areas: BK = Bela Krajina, CE = Celje, GO = Idrija and Posočje, KO = Kočevje and Cerknica, KP = Koper, KR = Jesenice, LJ = Ljubljana, MB = Maribor, MS = Pomurje, RA = Mežica Valley, SP = Savinjsko-Posavska, ZA = Zasavje (Snoj Tratnik et al., 2019).

Mothers completed a questionnaire to report their age, prior areas of residence, familial background, social factors, education, weight before and during pregnancy, height and weight post-partum, parity, detailed reproductive, medical and occupational history, lifestyle, relevant paternal/pregnancy-related information, information about the new-born child, and dietary information (frequencies of food consumption and source of each type of them – vegetable, fruits, nuts, milk and dietary products, eggs, poultry, game, other meat, freshwater fish, and fresh, frost and tinned-fish or other seafood, coffee or tea and alcohol drinks). Regarding seafood, participants had to mark how often they consumed (1) seafood without any conservation processing, that is, fresh seafood; (2) seafood which was frozen before consumption (for transport, storage, etc.), that is, frozen seafood; and (3) seafood which was canned for storage, that is, canned seafood, because such food processing can cause some changes in the FA profile. For example, Barrow et al. (2008) wrote about differences between freezing items and long-term storage in a freezer. In this study, seafood refers to all types of fish and shellfish, that is, any type of sea life regarded as food by humans.

The first national HBM in Slovenia was approved by the Republic of Slovenia National Medical Ethics Committee (NMEC) with numbers of accordance 42/12/07 and 53/07/09. All participants provided informed consent and their data were pseudo-anonymized. Further, participants had the right to withdraw at any time during the study period.

The data analysis included descriptive statistics and multiple linear regression using elemental concentrations in biological matrices, questionnaire data and environmental datasets. The sources of potential exposure, tested in relation to the concentrations in the biological matrices, were selected from available data, starting from the general knowledge

that exposure routes in non-occupationally exposed humans may be direct, through environmental compartments (air, soil and water) or food and lifestyle habits. Extensive sample size database accompanied with life-style and environmental data improved the prediction of exposure patterns, set the reference values for the childbearing population living in Slovenia, and provided a strong basis for evaluating spatial and temporal trends in exposure. Results are presented in IJS-DP-11651 (Mazej et al., 2014) and Snoj Tratnik et al. 2019, (Snoj Tratnik et al., 2019).

The population groups for the purpose of this thesis were assessed as part of a first national HBM study in Slovenia, biological matrices (milk, blood, urine and hair) and data-set were used to assess internal exposure. Snoj Tratnik et al. (2019) have described the methodology in detail. These samples and data represents an excellent basis for further study and include: a) The study population for the main part of the dissertation included two groups of lactating women, one group was from the coastal area around Koper (KP, N = 36) and another was from the inland area of Pomurje (MS, N = 38); and b) 535 pregnant women from 12 regions across Slovenia. Data set included information from questionnaires and analytical results of selected elements in milk, blood, urine and hair sample.

The main goal of the first part of the thesis was to obtain and evaluate the composition of fatty acids (FAs) and stable isotope ratios $^{13}C/^{12}C$ ($\delta^{13}C_{FA}$) of individual FAs in human milk and define their sources in connections with mother's dietary habits and living place. In the frame of finding possible biomarker(s) as an indicator of lactating women's dietary habits, (especially for seafood *vs* terrestrial food) the FAs and $\delta^{13}C_{FA}$ were determined in milk samples in a sub group of the first Slovenian HBM. Further, the association between the selected elements and FAs obtained in the body through seafood consumption, and the concentration of selected elements in human milk samples was also investigated. To improve the methods for accessing exposure to selected elements through determination of indicatory FA and their $\delta^{13}C_{FA}$ values in human milk samples, specific data mining analyses of the dataset were applied (Jagodic, Potočnik, et al., 2020). In Chapter 1.6, the influence of the maternal diet on the nutritional value of milk samples is presented. The second part of the dissertation was related to the investigation of the associations between the child's birth weight and maternal blood levels of selected elements and maternal lifestyle (Jagodic et al., 2017) (Chapter 1.7).

In addition, the dissertation combined the use of the composition of FAs and their $\delta^{13}C_{FA}$ values in human milk samples. To our knowledge such an approach was used for the first time in HBM studies.

The following sub-chapters include the methods for determination of FA composition and corresponding isotope composition of carbon in individual FAs, elemental composition and statistical evaluation of the data. The last two sub-chapters are dedicated to the presentation of results already published in papers that are part of the dissertation.

Composition and stable carbon isotope ratios of fatty acids in maternal milk

The identification and quantification of FAs in milk were performed using an in-situ trans-esterification method in which human milk triacylglycerides were extracted. The total lipid content from milk samples was extracted using a mixture of dichloromethane sodium hydroxide in methanol (Park & Goins, 1994). Fatty acids methyl esters (FAMEs) were characterized using an Agilent 6890N (Network GC System) gas chromatograph equipped with an Omegawax 320 capillary column and flame ionization detection (FID). The individual FAs were identified and quantified by comparing their retention times with

those of a standard FAME Mix (Supelco 37 component FAME Mix) and expressed as the percent of total identified FAs, a more detailed description can be found in Jagodic et al. (2020) (Jagodic, Potočnik, et al., 2020; Jagodic, Snoj Tratnik, et al., 2020).

$\delta^{13}C_{FA}$ was determined by a gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) using an Agilent 6890N GC coupled to an IsoPrime GV IRMS via a combustion interface. Separation was performed using a DB-1MS capillary column with He as a carrier (Gams Petrišič & Ogrinc, 2013). C19:0 FAME (methyl nanodecanoate) with $\delta^{13}C$ values of -30 ± 0.1‰ (prior determined on Elemental analyzer IRMS (EA-IRMS) was used as a laboratory reference material. For data normalization, a single-point normalization method was used. The precision of measurements expressed as the standard deviation of the laboratory reference material was 0.3 to 0.5‰. A more detailed description of the methodology can be found in Jagodic et al. (2020) (Jagodic, Potočnik, et al., 2020).

Levels of selected elements in maternal blood and milk

In the frame of the first national HBM in Slovenia, chemical analyses of selected trace elements in matrixes were done. The results of elemental composition in milk and blood samples of primiparas were used in the presented dissertation, and are briefly presented below.

Levels of selected toxic and potentially toxic elements were analyzed using an Inductively-coupled plasma mass spectrometry (ICP-MS) for As, Cd, Cu, Mn, Pb, Se and Zn for blood and/or milk samples; a Direct Mercury Analyzer (DMA 80) for Hg in blood, and cold vapor atomic-absorption spectrometry (CVAAS) for Hg in milk.

For determination of As, Cd, Cu, Mn, Pb, Se and Zn in maternal whole blood, aliquots of a whole blood sample were diluted ten times with an alkaline solution containing Triton X-100 and disodium ethylene diamine tetra acetic acid (EDTA) (adapted from (Barany et al., 1997)). For multi-elemental analysis of milk samples, two sample preparation methods were followed. Briefly, for Cu, Zn, and Se, an aliquot of milk was transferred into quartz tubes. The samples were microwave-digested with nitric acid and hydrogen peroxide in a closed vessel (Microwave System Ethos 1). For As, Cd, and Pb, an aliquot of milk was diluted five times with an alkaline solution containing ammonia Triton X-100 and EDTA similar as for whole blood samples (adapted from (Barany et al., 1997)).

The concentration of total Hg in whole blood was determined using DMA-80. For Hg in milk samples, an aliquot of a milk sample was taken in a glass volumetric flask, to which 2 mL of a mixture of HNO_3 -$HClO_4$ (1:1, v/v) and H_2SO_4 were added. For digestion, the flasks were heated at 220°C for 20 min. After cooling, samples were diluted with Milli-Q water. The total Hg concentration in milk was determined using CVAFS and Tekran 2600 (Akagi, 1997; Snoj Tratnik et al., 2019).

To ensure quality, blank samples, control samples, and reference materials were run together with the samples on a daily basis. To achieve the quality control, the labs also collaborated on yearly bases in different inter-laboratory comparisons (e.g. HBM4EU ("HBM4EU," n.d.), G-EQUAS – the German external quality assessment scheme (Institute and Out-Patient Clinic for Occupational and Social & Erlangen-Nuremberg and Environmental Medicine of the University, 2020), National Institute for Minamata Disease (NIMD) (NIMD, 2020) and so on). One of them was also the collaboration in an inter-comparison of selected elements in the IAEA-153 powder milk (Hoffman et al., 2018).

A more detailed description of the methodology can be found in literature (Jagodic, Potočnik, et al., 2020; Jagodic, Snoj Tratnik, et al., 2020; Ana Miklavčič et al., 2013; Potocnik, Necemer, Mazej, Jacimovic, & Ogrinc, 2016; Snoj Tratnik et al., 2019).

Statistical analyses

The analytical data were further prepared for statistical evaluation. The concentrations of elements and the percentage of FAs below the limit of detection (LOD) were arbitrarily assigned a value of half the LOD. For parametric statistical tests, non-normally distributed data were log10-transformed. Based on the food intake categories from the questionnaire, the daily intake (meals per day) was estimated, as described by Valent et al. (2013). Individual FAs were evaluated based on the peak area and expressed as the percent of total identified FAs. For the composition and $\delta^{13}C_{FA}$ values of FAs, the dataset excluded variables with less than 10 determinant cases in total amount. In case for $\delta^{13}C_{FA}$ the following FAs C18:3ω6, C18:1ω9t, C22:2, C23:0, C24:1ω9, and C24:0 were excluded in statistical evaluation. The percentage of combined FAs (PUFA, MUFA, SFA, ω-6 and ω-3 FAs, etc.) were included in statistical analyses and were calculated as described in the papers: Jagodic, Potočnik, et al., 2020; and Jagodic, Snoj Tratnik, et al., 2020.

Statistical analyses were performed using the statistical programs SPSS and STATA 12/SE. Graphical plots were created using OriginPro2017. Statistical significance was defined as $p < 0.05$ and marginal significance, as $0.05 < p < 0.1$. Descriptive statistics, univariate analyses, and multiple linear regression were used to examine possible associations. Univariate analyses included the Spearman test for categorical data and the Pearson test for continuous data, analysis of variance (ANOVA), chi-square tests (Pearson and Fischer's), and Mann-Whitney U-test (Field, 2009; Košmelj, 2007). Data mining approaches for supervised (i.e. classification) and unsupervised (i.e. clustering) learning were applied to identify fresh seafood intake (< 1 per month *vs* at least 1 per month) (Adankon & Cheriet, 2009; Eftimov et al., 2017; Granitto, Furlanello, Biasioli, & Gasperi, 2006; Kaufman & Rousseeuw, 1990; Steel & Torrie, 1960). Clustering was performed to understand how a sample might comprise distinct subgroups given a set of variables. The (dis)similarity between the participants was calculated using the Gower distance from the R package 'cluster' (Maechler, 2019). After calculating the distances between the participants, the 'partitioning around medoids' (PAM) algorithm was used to perform clustering (Kaufman & Rousseeuw, 1990).

Specific information of data treatment is available in each publication (Jagodic, Potočnik, et al., 2020; Jagodic et al., 2017; Jagodic, Snoj Tratnik, et al., 2020).

1.6 Influence of Dietary Habits on Composition and Stable Carbon Isotope Ratios of Fatty Acids in Maternal Milk of Slovenian Primiparous Women

Human milk is considered an essential and irreplaceable part of a healthy diet and is known as the best nutrition source for new-borns owing to its high protein, vitamin, mineral, and FA contents (Bratanič, 2018; Chu, 2013; Gura, 2014; Krawinkel, 2011; WHO, 2011). According to literature, the mother's diet and living environment can affect the levels of elements and FA compositions in human milk (R. Jensen, 1999; Kelishadi et al., 2012; Nishimura, Castro, Jordao, & Sartorelli, 2013; Ruan et al., 1995; Rudge et al., 2009). FA composition of human milk has attracted much interest from the viewpoint of a child's nutrition owing to the potential health impact of certain FAs on the child (Röllin et al., 2009; Rudge et al., 2009). Consumption of SFAs is associated with a higher incidence of cardiovascular diseases, whereas unsaturated FAs are beneficial for human health. For example, ω-3 PUFAs play an essential role in the development of neurological functions in the early years of life, and CLAs have anti-diabetic, anti-atherogenic, and anti-carcinogenic

health effects (Lock & Bauman, 2004; Nunes & Torres, 2010). The ω-6 and ω-3 PUFA contents can influence the optimal growth and development of the child (Birch et al., 1992; Z. Y. Chen et al., 1997; Innis, 2007, 2014; R. Jensen, 1999; Keim et al., 2012; Koletzko et al., 1988; Ruxton et al., 2004). Therefore, it is important to have a sufficient amount of them in human milk.

Apart from the beneficial components, several potentially toxic substances can also move from the mother to the child through human milk, and their levels are commonly determined in human milk as biomarkers of infant exposure (Rudge et al., 2009). For instance, seafood is an important source of PUFAs and also of potentially toxic elements. Therefore, seafood intake should be evaluated in the context of potential risks and benefits in health-related HBM, especially in infants and children as they are the most susceptible groups (Andersen et al., 2000; Bravi et al., 2016; Grandjean & Landrigan, 2006; Mozaffarian D, 2006; J. Strain et al., 2015; Tatsuta, Nakai, Sakamoto, Murata, & Satoh, 2018; Valent et al., 2013; Zeilmaker et al., 2013). One of the critical steps in determining the risks and benefits from seafood intake via human milk is to associate external and internal exposures.

Because the FA composition is known to be correlated with diet, the analysis of FAs and their $\delta^{13}C_{FA}$ values could also be used for tracking dietary habits. Like it was mentioned above, this is because variations in the isotopic composition of ^{13}C occur owing to differences in the fixation pathways of CO_2, which are correlated with environmental and physiological conditions (Boschker & Middelburg, 2002; Hayes, 1993; Meier-Augenstein, 1999; Smith & Epstein, 1971; Tykot, 2006). In general, higher $\delta^{13}C$ values are related to a marine diet. Only a few studies have measured $\delta^{13}C_{FA}$ values in milk, and these were mainly related to determining the geographical origin of milk (Ehtesham, Hayman, McComb, Van Hale, & Frew, 2013) or the dietary habits of animals (Richter et al., 2012). Richter et al. (2012) tested the potential use of $\delta^{13}C_{FA}$ values as tracers for the transformation of FA from diet to milk with a focus on the metabolic origin of c9,t11-18:2 (Richter et al., 2012). To the best of our knowledge, Vetter et al. (2007) performed the only study determining $\delta^{13}C$ values in human milk, where $\delta^{13}C$ values of individual methyl-branched fatty acids (MBFAs) were compared to $\delta^{13}C$ values of straight-chain FAs in food (Vetter, Gaul, Thurnhofer, & Mayer, 2007). Their results illustrate that MBFAs have distinctive $\delta^{13}C$ values and must originate from other sources and/or from very different substrates. Owing to the potential of this technique, we investigated whether it could be used as a tool for obtaining maternal dietary information (particularly for seafood intake) through the analysis of maternal milk samples (Jagodic, Potočnik, et al., 2020).

As it has already been stressed out above in Chapter 1.2, data obtained from questionnaires are frequently imprecise. Oken et al. (2014) compared three methods for assessing fish intake through questionnaires via correlation with analytical results of mercury in blood and hair and plasma DHA, where, for example, plasma DHA concentration correlated with fish intake assessed with a one-question screener but not with the other two. They concluded that a longer questionnaire does not provide an advantage over shorter ones in evaluating the intake of fish, DHA, and mercury compared with biomarkers (Oken et al., 2014). The alternative independent approach is to estimate seafood consumption frequency through the FA and stable carbon isotope composition of individual FAs in human milk (Jagodic, Potočnik, et al., 2020). For example, the DHA concentration in human milk is known to be directly correlated with fish consumption habits (Cohen, Bellinger, Connor, & Shaywitz, 2005; Leino, Karjalainen, & Tuomisto, 2013).

The FA composition of mature human milk was obtained from two population groups living in geographically distinct areas in Slovenia: Koper (KP), a coastal area, and Pomurje (MS), an inland area. Accordingly, possible difference in dietary habits, especially in

seafood intake, and in the FA composition of milk samples were explored. The association between dietary habits and FA composition in maternal milk samples was investigated (Jagodic, Potočnik, et al., 2020; Jagodic, Snoj Tratnik, et al., 2020). The FA composition of the colostrum in three different areas in Slovenia—Koper, Ljubljana, and Celje (N. Fidler, Salobir, & Stibilj, 2000; Nataša Fidler & Salobir, 2000)—and in mature milk in Ljubljana (Benedik, 2015) have been examined before, thereby allowing a comparison between the existing and new data for mature milk from the Koper and Pomurje areas (Jagodic, Snoj Tratnik, et al., 2020).

Specifically, in the dissertation, the aim was to test whether the levels of selected elements and indicatory FAs and their $\delta^{13}C_{FA}$ in human milk could represent a more reliable marker of dietary habits and sources of food consumed as assessed from the questionnaire data. We focused on seafood as a source of potentially toxic elements and nutritional FAs. Potentially toxic and essential elements and FA compositions and their $\delta^{13}C_{FA}$ were measured in human milk (Jagodic, Potočnik, et al., 2020). Some of the most relevant potentially toxic and essential elements (ATSDR, 2019) were selected for the study: Pb, Hg, Cd, As, Zn, Cu, and Se, based on the selection criteria described by Snoj Tratnik et al. (Snoj Tratnik et al., 2019). To test this hypothesis, the analytical results of % FAs, $\delta^{13}C_{FA}$, and elemental composition in human milk were statistically evaluated to discriminate between (1) mothers living in different geographical areas (coastal (KP) and inland (MS)) with significantly different seafood consumption patterns; (2) intake of terrestrial-based food and seafood, and (3) different types of seafood (fresh/canned/frozen). In addition to classical statistical approaches, the present study applied up-to-date data mining approaches which enabled better interpretation of a large-scale database and consideration of more variables simultaneously, unlike in classical statistical approaches (Jagodic, Potočnik, et al., 2020).

1.7 Maternal Trace Elements Status and Child's Birth Weight in the Slovenian Study Population

Birth weight is the main factor influencing infant mortality rates, health of children and a healthy later life. Birth weight is associated with the gender of the child, the anthropometry of the mother (e.g., poor pre-pregnancy weight, weight gain during pregnancy, shorter gestational age), parity, multiple birth, complicated pregnancy (e.g., maternal anaemia, pregnancy diabetes, poor antenatal care, problems with placenta, maternal infections), restricted foetal growth, poor nutritional intake of women, and deprived socio-economic conditions (Anjum, Javed, Afzal, & Sheikh, 2011; Muthayya, 2009; Stillerman et al., 2008; UNICEF & WHO, 2004; World Health Organization (WHO), 1992). Effects are also associated with physically demanding work during pregnancy, environmental tobacco smoke, intensity and duration of cigarette smoking during pregnancy, taking illicit drugs and consuming alcohol (Anjum et al., 2011; Horta, Victora, Menezes, Halpern, & Barros, 1997; Muthayya, 2009; Stillerman et al., 2008; UNICEF & WHO, 2004; World Health Organization (WHO), 1992). In addition, maternal health in relation to pregnancy, the mother's genetic background, age, location of residence and education also influence birth weight (Anjum et al., 2011; UNICEF & WHO, 2004).

Various associations between trace elements and birth weight have been observed. Studies have shown significant direct correlations between birth weight and maternal levels of Zn and Cu in serum (Abass et al., 2014). As for Mn, it has been suggested that Mn levels in maternal whole blood influence birth weight (Vigeh et al., 2008; Zota et al., 2009), while the absence of a correlation between birth weight and Mn, Cu, Zn and Se in maternal blood has also been reported (Al-Saleh, Nandakumaran, Al-Shammari, Al-Falah, & Al-

Harouny, 2004; Bermúdez, García-Vicent, López, Torró, & Lurbe, 2015; Mora et al., 2015). Levels of Mn in maternal blood have been found to increase throughout the pregnancy (ATSDR, 2012; Hansen et al., 2011), while lower or higher Mn concentrations in maternal blood have been associated with a low birth weight (U-shaped relationship) (L. Chen et al., 2014; Eum et al., 2014). Toxic elements (Cd, Pb, As and Hg) may have a negative effect on birth weight at relatively low levels of exposure. Xu et al. (2011) suggested that the presence of As in maternal blood has a negative influence on birth weight and on gestational age in boys (Xu et al., 2011). Studies investigating the effects of Cd exposure during pregnancy have been inconclusive: some studies (IkehTawari, Anetor, & M. A., 2013; Johnston, Valentiner, Maxson, Miranda, & Fry, 2014; Sun et al., 2014) report negative associations, some positive (Bloom et al., 2015), and others no association at all (Dwivedi, Jain, & Jain, 2013; Odland, Nieboer, Romanova, Thomassen, & Lund, 1999). Exposure to Pb was found to be associated with birth weight either negatively (Gundacker et al., 2010; Odland et al., 1999), or insignificantly (Bloom et al., 2015; Dwivedi et al., 2013; Sun et al., 2014). Non-significant correlations have been observed for Hg in maternal blood (Bloom et al., 2015; Dwivedi et al., 2013; Tsuzuki, Morimoto, Hosokawa, & Matsushita, 2013).

Attention was devoted to exposure to essential and toxic substances during pregnancy and the breastfeeding period, since these are recognized as being potentially responsible for adverse development and health in children. The main objective of the Jagodic et al. (2017) study was to examine the relation between birth weight and maternal blood levels of toxic and potentially toxic elements determined within the first Slovenian HBM program and taking into account the socio-demographic characteristics and diet of the mothers (Jagodic et al., 2017). In this study, we look at a mother's nutrition as reflected in her blood levels – the concentrations of the elements in light of her baby's adequate nutrition during pregnancy. We looked at both the elemental composition and the other information available to us from the questionnaires. The study was published from the dataset available at that time. In the future, we would suggest looking at the positive effects of seafood and other essential nutritional components as these can also reduce the effects of potentially toxic components. The article is a good example of a beginner's research study. In any case, a similar study should consider better planning for such observations in the future (more optimal time for maternal blood collection, determination of FAs in maternal blood, etc.).

Chapter 2

Aims and Hypothesis

The first objective of the dissertation was to investigate the relationship between the composition of fatty acids (FA), their stable carbon isotope ratios ($\delta^{13}C_{FA}$) and trace element composition in human milk and mother's dietary habits and living area. The underlying purpose of the first objective was to improve the understanding of exposure to selected elements through determination of indicatory fatty acids and their compound-specific stable isotope signatures. We estimated the association between selected elements obtained in the body through seafood consumption and their concentration in human milk samples. We intended to improve the methods for assessing exposure to selected elements through determination of indicatory FA and their $\delta^{13}C_{FA}$ values in human milk samples as an indicator of dietary habits, especially for seafood.

The second objective of the dissertation was to investigate the associations between the levels of selected elements in maternal blood samples, maternal dietary and other life-style habits and the child's birth weight. Attention was devoted to exposure to essential and toxic elements during pregnancy and the breastfeeding period, because they are recognized as being potentially responsible for adverse development and health effects in children.

Hypotheses:

a) Fatty acid composition of maternal milk and their $\delta^{13}C_{FA}$ could be used as an indicator of dietary habits, especially for seafood *vs* territorial food consumption.

b) The methods for assessing the source of exposure to metals and metalloids could be improved through determination of indicatory fatty acids and their $\delta^{13}C_{FA}$ in human milk samples as an indicator of dietary habits, especially for seafood.

c) Maternal dietary and other lifestyle habits are associated with the status of trace elements in her body and can therefore influence child's health as indicated through birthweight.

Chapter 3

Scientific Publications

Publications included in this dissertation are listed in such a manner that their consecutive appearances follow the general dissertation content. Candidate's contributions to individual scientific article are described before each article. In the dissertation, publications appear in the following order:

Jagodic, M., Snoj Tratnik, J., Potočnik, D., Mazej, D., Ogrinc, N., Horvat, M. Dietary habits of Slovenian inland and coastal primiparous women and fatty acid composition of human milk samples. Food and Chemical Toxicology, 2020, Vol. 141, 111299, doi: 10.1016/j.fct.2020.111299,

Jagodic, M., Potočnik, D., Snoj Tratnik, J., Mazej, D., Pavlin, M., Trdin, A., Eftimov, T., Kononenko, L., Ogrinc, N., Horvat, M. Selected elements and fatty acid composition in human milk as indicators of seafood dietary habits. Environmental research: multidisciplinary journal of environmental sciences, ecology, and public health, 2020, Vol. 180, 108820, doi: 10.1016/j.envres.2019.108820,

Jagodic, M., Snoj Tratnik., J., Mazej, D., Stajnko, A., Pavlin, M., Krsnik, M., Kobal, A. B., Kononenko, L., Odland J. Ø., Horvat, M. Birth weight in relation to maternal blood levels of selected elements in Slovenian mothers: a cross-sectional study. Journal of health science, 2017, Vol. 5, 95-106, doi: 10.17265/2328-7136/2017.02.005.

3.1 Dietary Habits of Slovenian Inland and Coastal Primiparas on Fatty Acid Composition of Their Milk samples

Jagodic, M., Snoj Tratnik, J., Potočnik, D., Mazej, D., Ogrinc, N., Horvat, M. Dietary habits of Slovenian inland and coastal primiparous women and fatty acid composition of human milk samples. Food and Chemical Toxicology, 2020, Vol. 141, 111299. https://doi.org/10.1016/j.fct.2020.111299

This study aimed to obtain FA patterns in mature milk arising from different dietary habits in two geographically different areas in Slovenia: Koper (KP), a coastal area, and Pomurje (MS), an inland area. Maternal diet influences the quality of human milk. The quantity and composition of FAs in milk can influence a child's growth and development. Therefore, associations between the maternal diet and the FA composition in 74 mature human milk samples were investigated.

The results revealed statistically significant differences in the dietary intake of game, freshwater fish, and fresh and frozen seafood between the study areas. Among the mean percentages of 35 individual FAs, 19 were higher in KP and 16 were higher in MS. In KP, despite the higher intake of fresh seafood, the levels of saturated and monounsaturated FAs were higher and the levels of PUFAs, ω-3, and ω-6 were lower compared to those in MS. The ω-6/ω-3 ratio did not differ significantly between the study areas. This finding was not expected and indicates a discrepancy between the measured and self-reported data – the latter lack reliable data on dietary supplements. Therefore, determination of FA profile is important as a beneficial factor in environmental health studies.

The candidate's contribution to this paper consisted of literature overview, extraction of data from published papers and performing analytical and statistical analyses of this case study. The candidate prepared the tables, and drafted the manuscript in collaboration with other co-authors.

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Dietary habits of Slovenian inland and coastal primiparous women and fatty acid composition of their human milk samples

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ABSTRACT

The quantity and composition of fatty acids (FAs) in milk can influence an infant's growth and development through maternal diet. Therefore, associations between maternal diet and FA composition in 74 mature human milk samples were investigated. This study aimed to obtain FA patterns in mature milk arising from different dietary habits in two geographically different areas in Slovenia: Koper (KP), a coastal area, and Pomurje (MS), an inland area. The results revealed statistically significant differences in the dietary intake of game, freshwater fish, and fresh and frozen seafood between the study areas. Among the mean percentages of 35 individual FAs in milk, 19 were higher in KP and 16 were higher in MS. In KP, despite the higher intake of fresh seafood, the levels of saturated and monounsaturated FAs were higher and the levels of PUFAs, ω-3, and ω-6 were lower compared to those in MS. The ω-6:ω-3 ratio did not differ significantly between the study areas. This finding was not expected and indicates a discrepancy between the measured and self-reported data - the latter lack reliable data on dietary supplements. Therefore, determination of FA profile is important as a promising biomarker of dietary intake in environmental health studies.

1. Introduction

The maternal diet and living environment are known to affect the quality of an infant's first food, namely, maternal milk via breastfeeding (Jensen, 1999; Kelishadi et al., 2012; Nishimura et al., 2013; Ruan et al., 1995). Human breast milk is an important part of the diet of infants. It contains many of nutrients that are essential for the health of new-borns (Chu, 2013; Krawinkel, 2011; WHO, 2011). The fatty acid (FA) composition of human milk has attracted much interest from the viewpoint of a child's nutrition owing to the potential health impact of certain FAs on the infant (Röllin et al., 2009; Rudge et al., 2009). Consumption of unsaturated FAs (UFAs) is considered beneficial for human health; therefore, it is important to have a sufficient amount of them in human milk. The nutraceutical components of human milk are omega-3 polyunsaturated FAs (ω-3 PUFA), which play an essential role in development in the early years of life and can improve neurological functions, and conjugated linoleic acids (CLA), which has anti-diabetic, anti-atherogenic, and anti-carcinogenic effects (Andersen et al., 2000; Birch et al., 1992; Chen et al., 1997; Grandjean and Landrigan, 2006; Innis, 2007; Jensen, 1999; Koletzko et al., 1988; Lock and Bauman, 2004; Nunes and Torres, 2010; Ruxton et al., 2004).

FAs in human milk originate from the mobilization of maternal body stores of FAs, diet, or endogenous synthesis from precursor FAs by the liver or breast tissue (Francois et al., 1998; Jensen, 1999, 1996; Sauerwald et al., 2001). Francois et al. (1998) reported that a single meal of a specific fat could significantly affect the human milk FA profile for 1–3 days (Francois et al., 1998). What, then, are the sources of FAs in human diet? Saturated FAs (SFAs) are known to mainly be present in dairy products (e.g., milk, butter, cream, and cheese) and in food containing animal fat products such as biscuits, crackers, cakes, pastries, sauces, fast food, and hamburgers. Monounsaturated FAs (MUFAs) have been used as a substitute for SFAs in nutrition, since the diet rich in MUFAs are preferred to those high in SFAs. Their main sources in human diet are butter, swine and bovine meat, mackerel, nuts (almonds, pea-, Brazil, cashew, hazel-, and pistachio nuts), seeds, olives, olive oil, and rapeseed oil. In the human diet, PUFAs are beneficial for human health, since some of them are even essential nutrients and cannot be synthesized in humans. They are present in higher amounts in vegetable oils, such as soybean and rapeseed oil. Foods rich in ω-3 PUFAs include also fatty fish (salmon, trout, herring, mackerel,

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tuna fish, sardines, wolf-fish, and flounder), seafood (molluscs, shellfish, crustaceans, echinoderms) and fish oils. The main dietary sources of ω-6 PUFAs are vegetable oils and margarines, mayonnaise, meat, sunflower, corn oil, soybean oil, grapeseed, and sesame seeds (Chow, 2007; Francois et al., 1998; Nishimura et al., 2014; Tvrzicka et al., 2011).

The World Health Organisation (WHO) recommends exclusive breastfeeding for infants for the first 6 months followed by nutritionally adequate and safe complementary feeding with continued breastfeeding up to 2 years of age or beyond (WHO, 2011). This overall strategy is supported also by Slovenian national policies, laws, and programs that promote and support breastfeeding and protect the rights of working mothers breastfeeding both in healthcare institutions and in society (Bratanič, 2018). In Slovenia, information on individual durations of breastfeeding is fragmented, and there is no central system to monitor the duration of exclusive breastfeeding. Therefore, it is important to evaluate the dietary habits from pregnant and lactating woman. In 2007 and 2008, the National institute for public health and their partners performed a survey on dietary habits of Slovenian inhabitants (N = 1193), reported by the main groups of food (Gabrijelčič Blenkuš et al., 2009). Most of the participants consumed milk at least once per day (58.5%), yogurt (30.0%) and cheese (36.4%) 2–3 times per week and the rest of dairy products 1–3 times per month. Fish, seafood and eggs were mainly consumed 1–3 times per month, while different types of meat - poultry, beef and pork - were consumed once per week, respectively. However, respondents with tertiary or higher education consumed sea fish frequently (64.1%). It is also important to note, that inhabitants of eastern Slovenia consume freshwater fish more often than the inhabitants of the western part, which preferred marine over freshwater fish. The largest proportion of respondents used vegetable (64.5%) and olive oil (25.1%) for preparation of their meals. The largest proportion of respondents used butter, greaves, mayonnaise and other vegetable grease only 1–3 times per month (Gabrijelčič Blenkuš et al., 2009). Owing to the importance of the quality of human milk and its dependence on the maternal diet, the present study evaluated FA composition of mature human milk and its association with dietary habits, especially seafood intake. For this purpose, two study areas with presumed differential dietary habits were selected, one in the coast (Koper, KP) and the other inland (Pomurje, MS). We hypothesised that this would reflect on the composition of FAs determined in milk samples of the study areas residents and give a background information for potential use of FAs in milk as indicators of specific dietary habits in environmental health studies. The latter was evaluated together with stable isotopes of FAs in a separate paper (Jagodic et al., 2020). The FA composition of the colostrum in three different areas in Slovenia—Koper, Ljubljana, and Celje (Fidler et al., 2000; Fidler and Salobir, 2000)—and in mature milk in Ljubljana (Benedik, 2015) has been examined before, thereby allowing a comparison between existing and new data for mature milk from the Koper and Pomurje areas.

2. Materials and methods

2.1. Description of study population

The study population included primiparous women (19–39 years old) who gave birth to a live born child in two different areas in Slovenia: Koper (KP), a coastal area, and Pomurje (MS), an inland area (Fig. 1). The study groups were assessed as part of a First National Human Biomonitoring study in Slovenia (HBM; 2007–2015), which has been described in detail elsewhere (Jagodic et al., 2020; Snoj Tratnik et al., 2019).

The primiparous women from KP and MS were recruited between 2011 and 2014. Biological samples (maternal blood, urine, milk, and hair) were collected between 5 and 11 weeks after delivery. Mothers collected maternal milk from multiple feeds in maximum 6 days. Through questionnaires, information about their age, residence history,

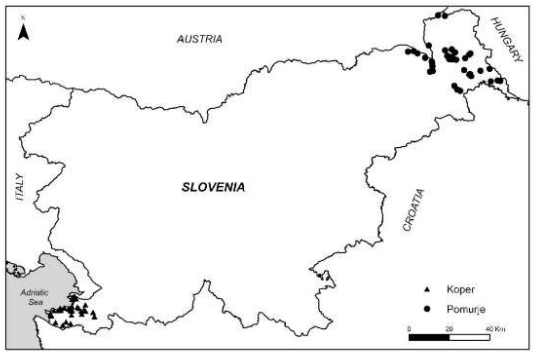


Fig. 1. Sampling areas: Koper (KP), a coastal area, and Pomurje (MS), an inland area (Jagodic et al., 2020).

family background, social factors, education, weight before pregnancy, height and weight post-partum, parity, detailed reproductive history, medical and occupational history, lifestyle, relevant paternal/pregnancy related information, information about the new-born child, and dietary information for last year (frequencies of food consumption and their sources—vegetables, fruits, nuts, milk and dairy products, eggs, poultry, game, other meat, freshwater fish, and fresh, frozen, and tinned-fish or other seafood, coffee or tea and alcoholic drinks, different dietary oils and dietary supplements) were obtained. For seafood, more detailed specifications were used: (1) seafood without any preservation processes = fresh seafood, (2) seafood that was frozen before consumption (for transport, storage, or other purposes) = frozen seafood, and (3) seafood that was canned for storage purposes = canned seafood. In this study, the term "seafood" refers to all types of sea fish and shellfish, that is, any form of sea life regarded as food by humans. Participants reported also if they were taking dietary supplements, but specific information on the supplement was not collected.

A total of 74 women in KP (36) and MS (38) signed informed consent, provided biological samples, and filled in the questionnaires as described by Jagodic et al. (2020). Recruitment and sampling in the framework of the First National HBM in Slovenia are described in detail by Snoj Tratnik et al. (2019).

2.2. Ethical considerations

First National HBM in Slovenia was approved by the Republic of Slovenia National Medical Ethics Committee (NMEC) with numbers of accordance 42/12/07 and 53/07/09. The study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants signed informed consent and all samples and accompanying data were pseudo-anonymised. Participants had a right to withdraw from the study at all stages of the study period.

2.3. Sample preparation and chemical analysis

FAs in human milk were identified and quantified by an in-situ transesterification method. Briefly, human milk triacylglycerides were extracted using the in-situ transesterification method (Park and Goins, 1994). Fatty acid methyl ester (FAME) characterization was performed using an Agilent 6890N (Network GC System) gas chromatograph with a flame ionization detector (GC-FID) equipped with a capillary column (Omegawax 320, 30 m × 0.32 mm × 0.25 μm, Supelco).

Individual FAs were identified and quantified by comparing their retention times with those of the standard (Supelco 37 component FAME Mix), which was analysed after every 10 samples and expressed

as the weight percent of the sum of all identified FAs. In each set of samples, a blank sample was also analysed to check the quality of the data. The precision of this method, based on replicates of real samples, was up to 5%. Further, the limit of detection (LOD) was 0.004%. A more detailed description of the methodology can be found in Jagodic et al., (2020).

2.4. Statistical analysis

Non-normally distributed data were log10-tranformed. The percentage of FAs below the LOD were arbitrarily assigned the value of ½ LOD. Based on the food intake categories in the questionnaire, daily intake (meals per day) was estimated (never – 0, less than once per month – 0.02, 1–3 times per month – 0.07, once per week – 0.14, 2–4 times per week – 0.43, 5–6 times per week – 0.79, once per day – 1, more than once per day – 2.5) as described by Valent et al. (2013). Information on the type of seafood (fresh, frozen, or canned) was also included in the questionnaire. The sum of all seafood meals was calculated.

The percentage of FAs were calculated after determining individual FA as described by Jagodic et al. (2020). FAs were grouped as follows: SFA = C4:0 + C6:0 + C8:0 + C1:0:0 + iC11:0 + C11:0 + C12:0 + iC13:0 + C13:0 + iC14:0 + C14:0 + iC15:0 + aC15:0 + C15:0 + C16:0 + iC17:0 + aC17:0 + C17:0 + iC18:0 + C18:0 + C2:0:0 + C21:0 + C22:0 + C23:0 + C24:0; MUFA = C:14:1ω5 + C:15:1 + C:16:1ω9 + C:16:1ω7 + C:17:1 + C:18:1ω9 + C:18:1ω7 + C20:1ω9 + C22:1ω9; PUFA = C18:2ω6c + C18:2ω6t + C18:3ω6 + C18:3ω3 + CLAc9t11C18:2 + C20:2ω6- + C20:3ω6 + C20:3ω3 + C20:4ω6 + C20:5ω3 + C22:2ω6 + C22:4ω6 + C22:5ω6 + C22:5ω3; ω-3 FA = C18:3ω3 + C20:3ω3 + C20:5ω3 + C22:5ω3; ω-6 FA = C18:2ω6c + C18:2ω6t + C18:3ω6 + C20:2ω6 + C20:3ω6 + C20:4ω6 + C22:2ω6 + C22:4ω6 + C22:5ω6; ω-6 LCP = C22:5ω6 + C22:4ω6 + C22:2ω6 + C20:4ω6 + C20:3ω6 + C20:2ω6; ω-3 LCP = C22:5ω3 + C20:5ω3 + C20:3ω3. LPC: ω-6 LCP + ω-3 LCP; C18:2ω6 = C18:2ω6(t + c).

Statistical analyses was performed using Microsoft Excel and SPSS and STATA statistical programs. Statistical significance was defined as $p < 0.05$ and marginal significance, as $0.05 < p < 0.1$. Descriptive statistic, univariate analyses, and simple and multiple linear regression were used to examine possible associations between the FA composition and lifestyle, especially dietary habits. Univariate analyses included: Spearman's test for categorical data, Pearson's test for continuous data, analysis of variance (ANOVA), chi-square tests (Pearson's and Fischer's), and Mann-Whitney *U* test.

3. Results and discussion

3.1. General characteristics of study population

The general characteristics of study groups were obtained through questionnaires. The average maternal age was 29.2 (23.0–36.0) years for KP and 28.8 (20.0–38.0) years for MS. The respective average pre-pregnancy body mass indexes were 23.3 (17.9–35.2) kg/m^2 and 23.3 (17.7–35.1) kg/m^2. In KP, 55%, 33%, and 12% of the participating women had completed university, secondary school, and primary school, respectively. In MS, the corresponding numbers were 46%, 49%, and 5%, respectively. The average infant birth weights were 3.39 (2.3–4.4) and 3.26 (2.3–3.4) kg in KP and MS, respectively. The average infant birth lengths were 53.6 (47.0–59.0) and 50.7 (48.0–54.0) cm in KP and MS, respectively. The average infant weights on the sampling day were 4.29 (3.10–6.30) and 4.51 (2.79–5.50) kg in KP and MS, respectively. The average infant ages on the day of sampling were 1.76 (1.43–2.47) and 1.70 (1.30–2.43) months in KP and MS, respectively. Among the described characteristics, only birth length differed statistically between the areas, being higher in KP.

3.2. Dietary habits of study population

Tables S1 and S2 present the results of the food-frequency questionnaire. Statistically significant differences between the areas were observed in the daily intake of game (higher in KP), freshwater fish (lower in KP), fresh seafood (higher in KP), and frozen seafood (lower in KP) (Table S1) and in the frequency of "morning tea intake" (higher in KP), olive oil use (more in KP), and sunflower oil use (more in MS) (Table S2). The results of comparisons between KP and MS for the intake of freshwater fish, fresh seafood, and frozen seafood and the use of olive oil and sunflower oil confirmed the expected dietary differences because of geographical and agriculture factors. MS has higher local production of sunflowers than KP, and KP has higher local production of olive oil and fresh seafood than MS. The questionnaires showed no statistically significant differences between the two areas for total seafood intake (Table S1). Statistical difference was observed for fresh seafood intake, which was mainly caused by the fact that people in KP eat more fresh seafood because of its higher availability in this coastal area. Therefore, the question arises whether analyses of FAs in human milk provide better information about maternal dietary habits.

3.3. FA composition of mature milk of study population

The percentage of FAs in maternal milk in KP and MS is shown in Fig. 2 and in Table S3, which is the same as that reported by Jagodic et al. (2020). A total of 49 FAs were detected. We were not able to distinguish chromatographic peak for FAs: C22:6ω3 and C24:1ω9, which were not separated on the column used. Further in the paper, we used record "C22:6ω3/C24:1ω9" for their common peak. The mean percentages of individual FAs differed statistically significantly between the study areas for 35 FAs. Specifically, in 19 cases, the percentages were higher in KP than in MS, including for C20:5ω3 and C22:6ω3/C24:1ω9 which are typically found in fish and are required for optimal function of the visual process and brain and nervous systems (Boyer et al., 2005; Harris et al., 1984; Innis, 2007; Jensen, 1999). In the other 16 cases, the percentages were higher in MS than in KP, including for C18:2ω6, C18:3ω3, and C20:4ω6 which are important, even essential, PUFAs for the growth of humans and infants (Boyer et al., 2005; Innis, 2007; Jensen and Lapillonne, 2009; Jensen, 1999; Ruan et al., 1995; Sauerwald et al., 2001). C22:6ω3 and C20:4ω6 are known to be important PUFAs in the neuron-rich grey matter of the brain, and C22:6ω3 status is connected to the immune system (Lauritzen and Carlson, 2011). UFAs are considered beneficial to human health, as noted above. The nutraceutical components were ω − 3 PUFAs, which play essential roles in development in the early years of life and can improve neurological functions, and CLA, which has anti-diabetic, anti-atherogenic, and anti-carcinogenic effects (Lock and Bauman, 2004; Nunes and Torres, 2010).

The mean percentages of FAs in different groups differed statistically significantly between the study areas: only MUFAs had higher levels in KP than in MS, while PUFAs, ω-3 FAs, ω-6 FAs, iC13:0/C13:0, ω-6 + ω-3 FA, ω-6 LCP, ω-3 LCP, LCPs, and ω-6/ω-3 LCPs all had higher levels in MS than in KP.

Table S3 shows a comparison of the mean percentage of FAs in mature milk from KP and MS determined in the present study with reported data for some other parts of Slovenia, specifically, colostrum data from Celje, Ljubljana, and Koper (Fidler et al., 2000) and mature milk from Ljubljana (Benedik, 2015).

In the latter study, the percentage of FAs in mature milk was first evaluated according to the lactation status (colostrum *vs* mature milk). Changes in the content of certain FAs are known to depend on the time of lactation (colostrum *vs* mature milk) (Chen et al., 1997; Guesnet et al., 1993; Wu et al., 2010). Colostrum showed a higher FA percentage compared to mature milk for 82% of cases in Ljubljana (colostrum) (Fidler et al., 2000) *vs* Ljubljana (mature) (Benedik, 2015) and for 83% of cases in Koper (Fidler et al., 2000) *vs* KP (Table S3). Results from KP

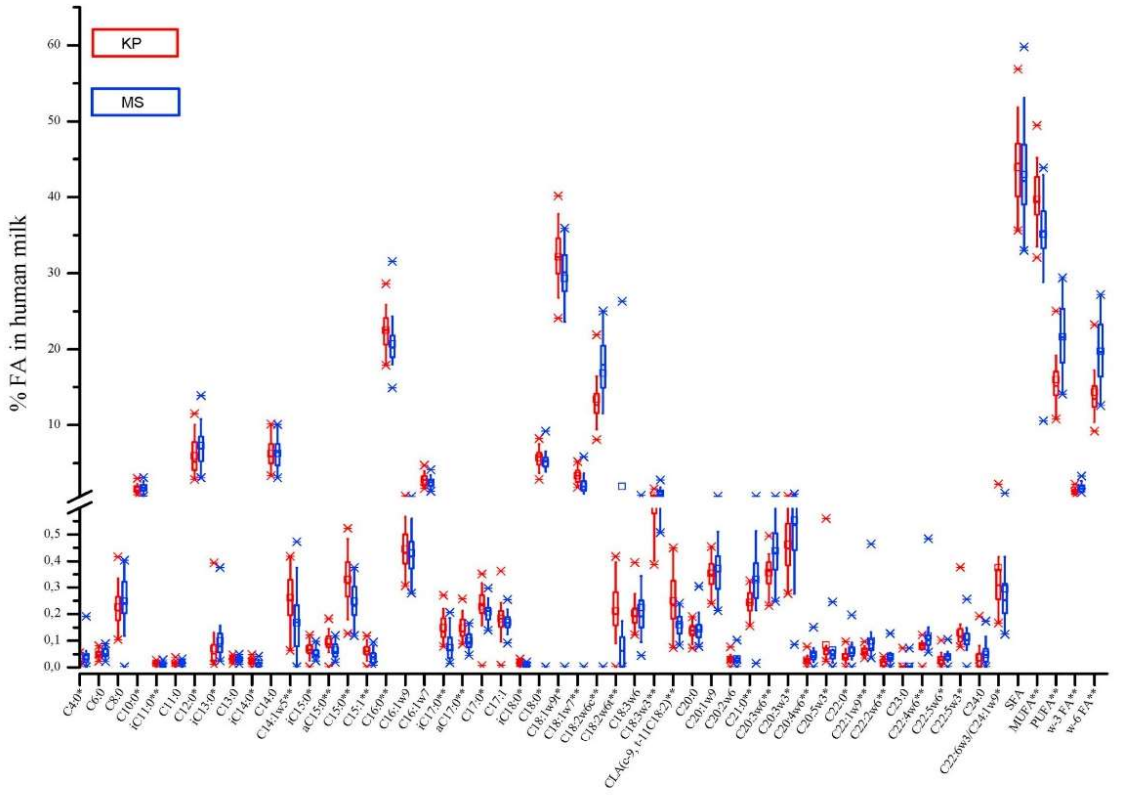


Fig. 2. FA composition of maternal milk and comparison between studied areas - coastal (KP) and inland (MS) (* two groups- KP *vs* MS are statistically significantly different ($p < 0.05$), ** two groups- KP *vs* MS are statistically significantly different ($p < 0.01$), Mann-Whitney U test) (Jagodic et al., 2020).

showed higher percentages of C18:3ω6, C20:3ω3, and SFAs in mature milk (Table S3) *vs* colostrum (Fidler et al., 2000). One reason could be the smaller number of SFAs detected in studies of the colostrum (Fidler et al., 2000). Previous studies (Chen et al., 1997; Wu et al., 2010) reported that the total amount of SFAs increases during the lactation period; the same was observed in the present study despite the lower percentage of individual SFAs in Slovenia compared to France (Guesnet et al., 1993). However, this was not necessarily observed for all individual FAs in the present study and the study by Chen et al. (1997).

Comparing the amount of MUFA in colostrum (Fidler et al., 2000) and mature milk (present study, Table S3), the levels were higher in colostrum than in mature milk, as observed for both ω-6 and ω-3 LCP and their ratio (ω-6 LCP:ω-3 LCP). This observation is in accordance with the literature (Chen et al., 1997; Guesnet et al., 1993). Similarly was observed also in the case of percentage of 20:4ω6 (Table S3) and is in accordance with the study by Chen et al. (1997).

A comparison of the percentages of 28 FAs (reported in both studies) in mature milk between different areas: KP, MS (Table S3) and Ljubljana (Benedik, 2015); revealed a lower percentage of FAs in Ljubljana *vs* KP for 13 FAs and in Ljubljana *vs* MS for 17 FAs. The data of percentage of FAs in KP and MS, as a sum or separately, were compared with literature information for Italy, a neighbouring country (Serra et al., 1997). SFAs in mature milk in Slovenia, MS (43.0%) and KP (43.8%) were similar to those in Italy (45.5%). MUFAs were lower in Slovenia, especially in MS (39.9% in KP and 35.1% in MS *vs* 42.7% in Italy). PUFAs were higher in Slovenia than Italy in (15.9% in KP and 21.6% in MS *vs* 11.8% in Italy). C18:2ω6 was higher in KP than in Italy (13.6% in KP *vs* 9.8% in Italy). Further, C18:3ω3 was higher in KP than in Italy (0.71% in KP *vs* 0.36% in Italy) (Serra et al., 1997). Although the diet of residents of the KP area, which is close to the Italian border, is considered to be similar to Italian's with frequent fresh seafood consumption, there was not much similarity of FAs % in milk between Italy and KP region, which contradicts the abovementioned general belief. Another comparison was made with a European population using the review by Koletzko et al. (1992). FA levels in mature milk from KP and MS mostly fit within the European population ranges reported in the reviewed studies for mature human milk (Koletzko et al., 1992). Difference was obtained for MS in the cases of C18:2ω6 (18.8% *vs* 6.9–16.4%) and sum of ω-6 and ω-3 PUFAs (21.5% *vs* 8.5–19.6%), which had higher percentages than reported for European population (Koletzko et al., 1992). Further, higher values for the 18:2ω6:18:3ω3 ratio were seen in both KP (20.7% *vs* 8.6–16.9%) and MS (21.3% *vs* 8.6–16.9%) compared to literature (Koletzko et al., 1992). A study by Nishimura et al. (2013) of women living far from coastal areas in Brazil indicated lower percentages of SFAs (42.7% *vs* 43.4%) and MUFAs (29.2% *vs* 37.4%) and higher percentages of ω-3 (2.1% *vs* 1.6%) and ω-6 PUFA (21.9% *vs* 17.1%) compared to Slovenia (2013). Further, Chen et al. (1995) reported that in Slovenia, total SFA (43.4% *vs* 38.5%), ω-6 PUFA (18.9% *vs* 11.4%), and ω-3 LCP (0.7% *vs* 0.3%) were higher but total ω-6 LCP (0.63% *vs* 0.83%) was lower compared to Canada; ω-3 PUFA was higher in MS (1.7% *vs* 1.5%) and lower in KP (1.4% *vs* 1.5%) compared to Canada.

3.4. Relationships between FAs and dietary habits

3.4.1. Daily intake of different foods

Studies suggested that maternal diet is a key factor influencing the FA composition of human milk. Studies of FAs with tracer techniques using stable isotopes showed that most PUFAs in maternal milk are not derived directly from the maternal diet but from tissue stores (Jensen, 1999; Kelishadi et al., 2012; Nishimura et al., 2014; Ruan et al., 1995; Sauerwald et al., 2001). However, long-term dietary intake affects the FA composition in tissue stores; therefore, the dietary habits remained the key factor (Sauerwald et al., 2001) and were considered accordingly.

Table S4 shows correlations between the FA compositions in milk samples of our study participants and daily intake of different foods, which was reported by the participants as explained in Sections 2.1 and 2.4.

Vegetable intake correlated positively and significantly with the percentage of C6:0 (Table S4). In general, a vegan/vegetarian diet is rich in C18:2ω6 and C18:3ω3 (Finley et al., 1985; Sanders and Reddy, 1992; Sauerwald et al., 2001), and vegetables are rich in C12:0, C14:0, C16:0, C18:1, C18:0, C18:2, and C18:3 with C16:0, C18:1, and C18:2 predominating (Bruchner and Peng, 2007). Increased consumption of MUFAs and PUFAs is known to be related to decreased levels of SFAs and a favourable PUFAs:SFAs ratio; many vegetables have a favourable ω-3:ω-6 ratio (Bruchner and Peng, 2007). However, the results (Table S4) did not show statistically significant correlations between vegetable intake and the predominating FAs mentioned above.

Higher fruit intake resulted in higher percentage of C20:5ω3, lower percentage of C22:5ω6, and lower ω-6:ω-3 FA ratio (Table S4). No significant correlations were seen with higher fruit intake and PUFAs or MUFAs in breast milk (Table S4). However, the questionnaire data in our study did not include different types or parts of fruits. Fruits show large interspecies differences as well as large differences within different parts of the fruit (mesocarp, kernels/seeds, and nuts or products from them). However, fruits and vegetables both generally contain more UFAs compared to SFAs (Kamel and Kakuda, 2007).

Intake of nuts positively and significantly correlated with the percentages of iC15:0, aC15:0, C15:0, C15:1, iC17:0, and aC17:0 and negatively correlated with the percentages of C20:3ω3, C22:4ω6, C22:5ω6, and sum of percentages of ω-3 LCP and all LCPs in breast milk (Table S4). Kamel and Kakuda (2007) reported that most nuts contain more than 50% fat; most of these are UFAs, with high percentages of C18:2ω6 and C18:1. In disagreement with our study the results did not show correlation with those two (C18:2ω6 and C18:1).

Intake of milk positively and significantly correlated with the percentages of C16:1ω7 and C20:2ω6 and negatively correlated with the percentages of C20:3ω3 and LCP in breast milk (Table S4). Previous studies suggested that the FA profile of milk and dairy products depends on the diet of cows. The concentrations of SFAs in milk are usually lower in summer compared to winter because of the higher intake of PUFAs from fresh forage during the summer (Chilliard et al., 2007; Collomb et al., 2008). Palmquist and Jansen (2007) reported that in milk and butter, the three major constituents in SFAs, MUFAs, and PUFAs are C16:0, C18:0, and C14:0; C18:1ω9, C16:1ω7, and C14:1ω5; and C18:2ω6, C18:2t, and C18:3ω3, respectively. Our result did not show significant correlations with the group of SFAs, MUFAs or PUFAs, but in agreement with the literature (Palmquist and Jansen (2007)) significant correlation was observed for C16:1ω7.

Higher intake of eggs resulted in higher percentages of iC14:0, iC15:0, aC15:0, C15:0, C15:1, aC17:0, and CLA and higher (iC15:0 + aC15:0):C15:0 ratio in breast milk. Poultry positively and significantly correlated with the percentages of C20:3ω3, C22:4ω6, and C22:5ω6 and the sum of ω-3 LCP and LCP and correlated negatively with C18:1ω7 (Table S4). Cantor et al. (2007) reported that of the FAs in egg, 36%, 48%, and 16% are SFAs, MUFAs, and PUFAs, respectively. The predominant FAs in egg are C16:0, C18:1, and C18:2. For light chicken muscle, the lipids comprise 37% SFAs, 33% MUFAs, and 31% PUFAs with the main representatives being C16:0, C18:0, C18:1, C16:1, C18:2, C20:4ω6, and C22:6ω3, and for dark muscle without skin, the lipids comprise 31% SFAs, 38% MUFAs, and 31% PUFAs with a similar FA profile to that of light chicken muscle. In comparison to chicken muscle, turkey muscle has less SFAs and PUFAs but more MUFAs (Cantor et al., 2007). Our results (Table S4) did not show any significant correlations between the predominant FAs in poultry and egg as reported by Cantor et al. (2007) with FAs in milk samples and their daily intake factors.

The intake of game correlated positively with the percentages of iC11:0, iC14:0, aC15:0, C15:0, aC17:0, C17:1, iC18:0, C18:2ω6t, and CLA and negatively with the percentages of C18:2ω6(t + c), sum of PUFA, ω-6 FA, ω-6:ω-3 FA ratio, and sum of ω-6 and ω-3 FA (Table S4). The intake of other meats only correlated negatively with the percentage of iC17:0 (Table S4). FAs in meat usually have medium to long chain lengths. Of the FAs in meat, 40%, 40%, and 2%–25% are SFAs, MUFAs, and PUFAs, respectively, with C18:1ω9 being the major FA (Wood et al., 2007). In contrast to the literature (Wood et al., 2007), our result did not show significant correlation between the daily intake of any meat and C18:1ω9 in breast milk.

Intake of freshwater fish correlated positively with the percentages of C4:0, C18:2ω6c, C18:2ω6(c + t), sum of PUFA, ω-6 FA, ω-6:ω-3 FA ratio, and sum of ω-6 and ω-3 FA and correlated negatively with the percentages of C15:1, iC17:0, aC17:0, C18:1ω9, C18:1ω7, C18:2ω6t, CLA, C22:6ω3/C24:1ω9, and sum of MUFA (Table S4). The results showed different correlations for each studied type of seafood with the composition of stable isotopes of FAs. Intake of fresh seafood correlated positively with higher percentages of C14:1ω5, iC15:0, aC15:0, C15:1, iC17:0, aC17:0, C18:1ω9, C18:1ω7, C18:1ω6t, C18:3ω6, CLA, C20:5ω3, C22:5ω3, and sum of MUFA (Table S4), while negatively with the percentages of C4:0, C8:0, C10:0, C11:0, C12:0, iC13:0, C18:2ω6(c + t), C20:3ω3, C22:2ω6, C22:4ω6, C22:5ω6, sum of PUFA, ω-6 FA, ω-6:ω-3 FA ratio, sum of ω-6 and ω-3 FA, ω-6 LCP, LCP, and C18:2ω6:C18:3ω3 ratio (Table S4).

Frozen seafood intake correlated positively only with the percentage of iC13:0; while canned seafood intake correlated positively only with the percentage of C18:3ω6 (Table S4) and negatively with the percentages of C22:1ω9 and C22:2ω6 (Table S4).

All seafood intake correlated positively with the percentages of iC15:0, aC15:0, C15:1, iC17:0, aC17:0, C18:1ω7, CLA, and sum of MUFA., while negatively with the percentages of C4:0, C18:2ω6(c + t), C22:2ω6, C22:4ω6, C22:5ω3, sum of PUFA, ω-6 FA, ω-6:ω-3 FA ratio, sum of ω-6 and ω-3 FA, ω-6 LCP and C18:2ω6:C18:3ω3 ratio (Table S4).

Fish and fish oil are known to be good sources of FAs such as ω-3 FA (especially 20:5ω3 and 22:6ω3) (Ackman, 2007; Boyer et al., 2005; Harris et al., 1984; Innis, 2007). Francois et al. (1998) showed that 20:5ω3 and 22:6ω3 were significantly higher after consuming menhaden oil and remained elevated in human milk longer than other FAs. They also noted that when lactating mothers consume fish regularly, their milk contains greater amounts of 20:5ω3 and 22:6ω3 for a longer period of time than the milk of mothers who eat fish only occasionally (Francois et al., 1998). Further, Benedik et al. observed that 22:6ω3 was influenced by fish intake before/during pregnancy and during lactation and with 22:6ω3 supplement intake during pregnancy and lactation (Benedik, 2015; Benedik et al., 2014). Ackman reported that some freshwater fish have FAs similar to those of seawater fish, including important ones like C20:5ω3 and C22:6ω3 FAs (Ackman, 2007). From the abovementioned literature we would expect that more ω-3 FA (especially 20:5ω3 and 22:6ω3) would be associated with higher daily intake of seafood, but results showed positive correlations only in case of fresh seafood and C18:3ω6, CLA, C20:5ω3 and C22:5ω3. Of course, it should be noted here, that 22:6ω3 was not determined separately, which means we could not distinguish it from C24:1ω9 to check the association with seafood.

Coffee and tea intake correlated positively only with the percentage of C23:0, while negatively with the percentage of C11:0 and sum of SFA. Furthermore, alcohol intake correlated negatively with the percentage of C20:1ω9 (Table S4). The latter was expected, because alcohol, tea, and coffee have low percentages of fats on a weighed or a volume basis. The main FAs in typical red wines are C16:0, C14:0, and C12:0 (Chang and Chow, 2007; Kamel and Kakuda, 2007), which did not show any significant correlations with our data.

3.4.2. Use of different oils and supplements in diet

Because certain differences in FA contents (KP *vs* MS) were not fully explainable in the discussion above, we also focused on the consumption of different oils (some of which were locally produced) and dietary supplements. In MS, participants might be consuming more locally produced food based on animal and plant fats (e.g. corn oil and sunflower oil rich in C18:2ω6 and canola oil rich in C18:3ω3) (Francois et al., 1998; Jensen, 1999). In accordance with this, higher consumption of sunflower oil (using *vs* not using) was reported in mothers from MS than from KP (55% *vs* 19%) (Table S2), but unfortunately, not all types of animal and plant fats and oils were specified to check further associations (see Table S2). Another reason for the differences between the study areas could be the intake of food supplements, although the difference in self-reported intake was not statistically significant between MS and KP (81% *vs* 75%) (Table S2) and could be questionable owing to the use of self-reported questionnaires.

The use of omega-based dietary supplements, other types of dietary supplements, olive oil/sunflower oil, butter, margarine, or other types of oil were examined using a Mann-Whitney *U* test to identify any connections between our experimental results and the self-reported information; the results are presented in Table S5.

The use of olive oil was associated with C18:1, C18:2, and C18:3, specifically with the percentages of C18:1ω9, C18:1ω7, C18:2ω6c, C18:2ω6t, C18:2ω6(c + t), C18:3ω3, and CLA as well as with those of C4:0, C12:0, iC14:0, C14:1ω5, iC15:0, aC15:0, C15:0, C15:1, iC17:0, aC17:0, C17:0, C18:1ω9, C18:1ω7, C18:2ω6c, C18:2ω6t, C18:2ω6(c + t), C18:3ω3, CLA, C21:0, C22:0, C22:1ω9, C22:2ω6, C22:5ω6, C24:0, sum of SFA, PUFA, ω-3 FA, ω-6:ω-3 FA ratio, iC13:0/C13:0, ω-6 LCP, and ω-3 LCP in milk samples (Table S5). From the literature, the FAs with highest percentages in olive oils are C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, and C20:0, with C18:1 having the highest percentage (White, 2007).

The use of sunflower oil was associated with the percentages of C12:0, C18:1ω9, C18:1ω7, C18:2ω6c, C18:2ω6t, C18:2ω6(c + t), and CLA as well as with those of C8:0, C11:0, C12:0, C15:1, iC17:0, aC17:0, C21:0, C20:3ω6, C20:3ω3, C22:0, C22:2ω6, C22:5ω6, C22:5ω3, sum of PUFA, ω-3 FA, ω-6:ω-3 FA ratio, ω-6 LCP, ω-3 LCP, and C18:2ω6:C18:3ω3 ratio (Table S5). Sunflower oil has the highest percentage of C18:2, followed by C18:1, C16:0, C18:0, C12:0, C18:3, C20:0, C14:0 and C16:1 (White, 2007).

In our study, the use of butter was associated with the percentages of C4:0, C12:0, C15:0, C16:0, iC17:0, aC17:0, C18:1ω9, C18:2ω6(c + t), and CLA as well as with those of iC15:0, aC15:0, C22:1ω9, C22:2ω6, sum of PUFA, ω-3 FA, ω-6:ω-3 FA ratio, and ω-6 LCP (Table S5). White (2007) reported FA percentages higher than 0.1% in butter fat, with C18:1 and C16:0 being the highest, followed by C18:0, C14:0, C4:0, C12:0, C18:2, C6:0, C10:0, C16:1, C15:0, C8:0, C14:1, C17:0, C18:3, C20:1, C17:1, C20:4, C11:0, and C13:0.

The use of margarine was associated with the percentages of C16:1ω9, C17:1, C18:2ω6c, C18:2ω6(c + t), sum of PUFA, ω-3 FA, ω-6:ω-3 FA ratio, iC13:0:C13:0 ratio, and ω-6 LCP (Table S5). The use of other types of oil was associated with the percentages of only C6:0 and iC13:0 (Table S5). Trans-FAs account for less than 5% of total FAs in butter, whereas they can account for up to 60% of FAs in margarine (Dhaka et al., 2011). However, in our study, no differences were seen in specific trans-FAs between butter and margarine intake (Table S5).

3.4.3. Frequency of different meals during the day

The frequency of different meals during the day (descriptive statistics is given in Table S2) had different influences on FA levels (Table S6). The frequency of lunch did not differ between participants, so no correlations were determined. The frequency of breakfast and dinner did not correlate statistically significant with the percentages of any FAs. In mothers who reported having 'morning tea' and 'afternoon tea' more often, the levels of iC11:0, iC14:0, C14:1ω5, aC15:0, C15:0, C16:0, C18:2ω6t, and CLA were higher and those of C18:2ω6c, C18:2ω6(c + t), C22:1ω9, sum of PUFA, ω-6 FA, ω-6, and ω-3 FA were lower. Further, the 'morning tea' frequency correlated positively with the percentages of iC17:0, aC17:0, C18:0, and sum of MUFA. The results suggested that mothers had eaten certain food items which had higher proportions of these FAs (iC11:0, iC14:0, C14:1ω5, aC15:0, C15:0, C16:0, C18:2ω6t, CLA, iC17:0, aC17:0, C18:0, and MUFA) during the morning and afternoon teas. 'Morning tea' correlated negatively only with C18:3ω3 and 'afternoon tea' correlated negatively with only C22:2ω6 and C24:0.

3.5. Limitations and strengths of the study

The major limitation of the present study is that the samples of milk were primarily not collected for the purpose of this particular study. Collection of samples including the sampling areas was designed within the First National HBM program with objectives to estimate overall exposure of Slovenian inhabitant to different environmental pollutants. There were 12 study areas in the First National HBM, among which two were selected for the purpose of the present study – coastal and inland. Consequently, the sample size was low and probably not entirely representative for the respective areas. Furthermore, the questionnaire was not especially designed for the FAs evaluation in breast milk. However, the study gives a good starting point for evaluating dietary habits using advanced biomarker technique because it has an advantage of two distinct geographic areas with residents having distinct dietary habits as described above.

4. Conclusions

The study presents results of FAs in mature human milk from two geographically distinct areas, KP (coastal area) and MS (inland area), in Slovenia. Differences were expected in the dietary habits and frequencies of consumption of some local food items. The food frequency questionnaire data showed that the study areas (inland *vs* coastal) differed statistically significantly in terms of the intake of game (lower in inland area), freshwater fish (higher in inland area), fresh seafood (lower in inland area), and frozen seafood (higher in inland area). The results of the FA composition of human milk showed that the mean percentages of 35 individual FAs differed statistically significantly between the study areas: they were higher in KP for 19 FAs and in MS for 16 FAs. Despite the higher intake of fresh seafood in KP, the levels of SFAs and MUFAs were higher in KP and the levels of PUFAs, ω-3, and ω-6 were lower in KP compared to those in MS. This finding was not expected and indicates a discrepancy between the measured and self-reported data. However, the study confirmed that the dietary habits of Slovenian inland and coastal primiparous women influenced the FAs composition of their milk samples. Because the self-reported data on food intake is difficult to fill in and has high uncertainty, this study should serve as a good starting point toward establishing more precise biomarkers of dietary intake.

CRediT authorship contribution statement

Marta Jagodic: Writing - original draft, Formal analysis, Investigation, Methodology, Validation. **Janja Snoj Tratnik:** Writing - review & editing. **Doris Potočnik:** Formal analysis, Investigation, Methodology, Validation. **Darja Mazej:** Conceptualization, Writing -

review & editing. **Nives Ogrinc:** Writing - review & editing. **Milena Horvat:** Conceptualization, Supervision, Writing - review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2020.111299.

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Supplements of "Dietary habits of Slovenian inland and coastal primiparous women and fatty acid composition of their human milk samples"

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Table S1: Daily intake factors and comparison between two studied areas from questionnaire data

Daily intake factor (meal/day)	KOPER (KP)					POMURJE (MS)					Total				
	N	Mean	SD	Min	Max	N	Mean	SD	Min	Max	N	Mean	SD	Min	Max
Vegetable	36	1.27	0.75	0.43	2.50	38	1.16	0.61	0.43	2.50	74	1.21	0.68	0.43	2.50
Fruit	36	1.77	0.83	0.43	2.50	38	1.63	0.90	0.43	2.50	74	1.70	0.87	0.43	2.50
Milk	36	1.58	0.85	0.43	2.50	38	1.26	0.79	0.14	2.50	74	1.42	0.83	0.14	2.50
Nuts	36	0.24	0.30	0.02	1.00	37	0.17	0.22	0.00	1.00	73	0.21	0.26	0.00	1.00
Eggs	36	0.26	0.20	0.00	0.79	38	0.26	0.23	0.02	0.79	74	0.26	0.22	0.00	0.79
Poultry	36	0.29	0.23	0.00	1.00	38	0.32	0.15	0.00	0.43	74	0.31	0.19	0.00	1.00
Game	36	0.05	0.10	0.00	0.43	38	0.02	0.03	0.00	0.14	74	0.031*	0.07	0.00	0.43
Other meat	34	0.25	0.22	0.00	0.79	37	0.27	0.21	0.00	1.00	71	0.26	0.21	0.00	1.00
Freshwater fish	36	0.02	0.02	0.00	0.07	38	0.05	0.05	0.00	0.14	74	0.03*	0.04	0.00	0.14
Fresh seafood	35	0.09	0.05	0.00	0.14	35	0.04	0.08	0.00	0.43	70	0.07*	0.07	0.00	0.43
Frozen seafood	36	0.04	0.04	0.00	0.14	38	0.06	0.04	0.00	0.14	74	0.05*	0.04	0.000	0.14
Canned seafood	35	0.09	0.18	0.00	1.00	37	0.08	0.09	0.00	0.43	72	0.08	0.14	0.000	1.00
Seafood all	34	0.22	0.19	0.06	1.14	35	0.18	0.15	0.02	0.71	69	0.20	0.17	0.020	1.14
Coffee/tea	36	0.55	0.66	0.00	2.50	38	0.73	0.73	0.00	2.50	74	0.65	0.70	0.000	2.50
Alcohol	36	0.06	0.12	0.00	0.43	38	0.04	0.05	0.00	0.14	74	0.05	0.09	0.000	0.43

* significant difference between KP and MS ($p < 0.05$), Mann-Whitney U test. N – number of cases, SD – standard deviation, Min – minimum, Max – maximum. Food intake categories from the questionnaire were converted to continuous estimated daily intake factors (from never – 0, less than once per month – 0.02, 1–3 times per month – 0.07, once per week – 0.14, 2–4 times per week – 0.43, 5–6 times per week – 0.79, once per day – 1, more than once per day – 2.5) (Valent et al., 2013).

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Table S2: Dietary habits and comparison between two studied areas from questionnaire data

		Koper (KP) (%)	Pomurje (MS) (%)	Total (%)
Frequency of breakfast	**every day**	92	95	93
	every second day	5.6	2.7	4.1
	2x per week	2.8	0	1.4
	never	0	2.7	1.4
Frequency of 'morning tea'*	**every day**	71	26	48
	every second day	8.6	29	19
	2x per week	8.6	21	15
	1x per week	8.6	7.9	8.2
	never	2.9	16	9.6
Frequency of lunch	**every day**	100	100	100
Frequency of 'afternoon tea'	**every day**	49	24	36
	every second day	26	19	22
	2x per week	17	27	22
	1x per week	5.7	14	9.7
	never	2.9	16	9.7
Frequency of dinner	**every day**	78	68	73
	every second day	11	24	18
	2x per week	11	2.6	6.8
	1x per week	0	2.6	1.4
	never	0	2.6	1.4
Use of olive oil*	**No**	2.8	55	30
	Yes	97	45	70
Use of sunflower oil*	**No**	81	45	62
	Yes	19	55	38
Use of butter	**No**	67	82	74
	Yes	33	18	26
Use of margarine	**No**	92	84	88
	Yes	8.3	16	12
Type of water supply	**public water supply**	86	74	80
	public water supply and water	5.6	0	2.7
	bottled water	8.3	18	14
Intake of food supplements	**No**	25	19	22
	Yes	75	81	78
Intake of food supplements with omega	**No**	78	82	80
	Yes	22	18	20

% - percentage; * significant difference between KP and MS ($p < 0.05$)

Supplements of "Dietary habits of Slovenian inland and coastal primiparous women and fatty acid composition of their human milk samples", Jagodic et al. 2020

Table S3: Percentage of FAs determined in the present study and comparison with literature

Reference	Presented study						Fatty acid composition of human milk in different regions of Slovenia (Fidler et al., 2000)[#]						Nutrition during pregnancy and lactation and fatty acid composition of human milk (Benedik, 2015)[**]	
Type of sample	Mature milk						Colostrum						Mature milk	
% FA	Koper (KP) (N = 36)		Pomurje (MS) (N = 38)		Total (N = 74)		Celje (N = 15)		Koper (N = 12)		Ljubljana (N = 14)		Ljubljana (N = 174)	
	Mean	SD	Mean	SD	Mean	SD	EM	SEM	EM	SEM	EM	SEM	Mean	SD
C4:0	0.02	0.01	0.04	0.04	0.03*	0.03								
C6:0	0.05	0.01	0.05	0.02	0.05	0.02								
C8:0	0.22	0.07	0.25	0.09	0.24	0.08								
C10:0	1.55	0.53	1.79	0.54	1.67*	0.54							1.16	0.28
iC11:0	0.01	0.01	0.01	0.01	0.01**	0.01								
C11:0	0.02	0.01	0.02	0.01	0.02	0.01							0.01	0.00
C12:0	5.79	2.40	7.32	2.69	6.58*	2.65							4.47	1.49
iC13:0	0.07	0.07	0.10	0.07	0.08*	0.07								
C13:0	0.03	0.01	0.03	0.01	0.03	0.01							0.03	0.01
iC14:0	0.02	0.01	0.01	0.01	0.02*	0.01								
C14:0	6.20	1.78	6.27	1.92	6.24	1.84	5.89	0.43	6.93	0.64	6.71	0.54	5.46	1.41
C14:1ω5	0.26	0.09	0.17	0.11	0.21**	0.11	0.58	0.12	0.87	0.18	0.55	0.15	0.26	0.09
iC15:0	0.07	0.02	0.05	0.02	0.06*	0.02								
aC15:0	0.10	0.04	0.06	0.03	0.08**	0.04								
C15:0	0.33	0.09	0.25	0.07	0.29**	0.09	0.89	0.10	0.76	0.15	0.61	0.13	0.36	0.10
C15:1[a]	0.06	0.03	0.04	0.02	0.05**	0.03	0.25	0.05	0.20	0.07	0.18	0.06	0.00	0.00
C16:0	22.6	2.20	20.7	2.67	21.6**	2.62	23.10	0.53	24.03	0.79	22.50	0.66	23.23	2.34
C16:1ω9	0.45	0.09	0.43	0.09	0.44	0.09								
C16:1ω7*	2.84	0.76	2.50	0.66	2.67	0.73	2.47	0.12	2.98	0.18	2.39	0.15	2.79	0.62
iC17:0	0.15	0.05	0.07	0.05	0.11**	0.06								
aC17:0	0.15	0.04	0.10	0.03	0.12**	0.04								
C17:0	0.23	0.07	0.21	0.04	0.22*	0.06	0.37	0.02	0.38	0.02	0.36	0.02	0.30	0.06
C17:1[a]	0.19	0.06	0.17	0.03	0.18	0.05	0.24	0.01	0.27	0.02	0.25	0.01		
iC18:0	0.02	0.01	0.01	0.01	0.02*	0.01								
C18:0	5.70	1.11	5.05	1.59	5.37*	1.40	6.71	0.28	6.17	0.41	5.74	0.35	6.30	1.04
C18:1ω9c/9t	32.2	3.44	29.3	5.83	30.7*	4.99								
C18:1ω9c														
C18:1ω9t							35.72	0.54	36.55	0.81	35.37	0.68		
C18:1ω7	3.47	0.93	2.01	1.38	2.72**	1.39								
C18:2ω6c*	13.4	3.00	16.8	6.07	15.1**	5.10							13.97	3.40
C18:2ω6t[c]	0.22	0.10	1.97	6.61	1.12**	4.79	14.72	0.70	12.62	1.04	17.35	0.87	0.06	0.03
C18:3ω6	0.21	0.06	0.22	0.13	0.22	0.10	0.05	0.03	0.18	0.04	0.07	0.04	0.14	0.05
C18:3ω3	0.71	0.26	1.01	0.49	0.86**	0.42	0.93	0.04	0.70	0.06	1.07	0.05	0.86	0.49
CLA(c-9, t-11C18:2)	0.25	0.08	0.16	0.04	0.20**	0.08								
C18:4ω3							0.21	0.02	0.32	0.02	0.29	0.02		
C19:0							0.01	0.00	0.01	0.00	0.01	0.00		
C20:0	0.14	0.03	0.14	0.04	0.14	0.04	0.32	0.01	0.26	0.02	0.27	0.02	0.17	0.03
C20:1ω9	0.35	0.05	0.37	0.10	0.36	0.08	1.44	0.05	1.31	0.08	1.08	0.07	0.37	0.06
C20:2ω6	0.03	0.02	0.03	0.02	0.03	0.02	1.39	0.08	1.09	0.11	1.19	0.09	0.29	0.06
C21:0	0.24	0.05	0.33	0.14	0.29**	0.11								
C20:3ω6	0.36	0.06	0.44	0.10	0.40**	0.09	0.86	0.05	0.90	0.08	0.81	0.06	0.40	0.09

Reference	Presented study						Fatty acid composition of human milk in different regions of Slovenia (Fidler et al., 2000)[¥]						Nutrition during pregnancy and lactation and fatty acid composition of human milk (Benedik, 2015)[¥¥]	
Type of sample	Mature milk						Colostrum						Mature milk	
% FA	Koper (KP) (N = 36)		Pomurje (MS) (N = 38)		Total (N = 74)		Celje (N = 15)		Koper (N = 12)		Ljubljana (N = 14)		Ljubljana (N = 174)	
	Mean	SD	Mean	SD	Mean	SD	EM	SEM	EM	SEM	EM	SEM	Mean	SD
C20:3ω3	0.46	0.09	0.55	0.17	0.51*	0.14	0.15	0.01	0.11	0.01	0.13	0.01	0.03	0.01
C20:4ω6	0.03	0.01	0.05	0.03	0.04**	0.03	1.06	0.05	1.05	0.07	0.93	0.06	0.52	0.10
C20:5ω3	0.08	0.09	0.06	0.06	0.07*	0.08	0.11	0.01	0.08	0.02	0.10	0.01	0.07	0.06
C22:0	0.04	0.02	0.06	0.04	0.05*	0.04	0.37	0.02	0.30	0.03	0.28	0.03	0.05	0.01
C22:1ω9	0.06	0.01	0.10	0.08	0.08**	0.06							0.08	0.02
C22:2ω6	0.02	0.01	0.04	0.03	0.03**	0.03	0.25	0.02	0.25	0.03	0.28	0.03	0.03	0.01
C23:0	0.00	0.00	0.00	0.01	0.00	0.01							0.00	0.00
C22:3ω3							0.23	0.05	0.22	0.07	0.21	0.06		
C22:4ω6	0.08	0.02	0.12	0.07	0.10**	0.06	0.77	0.06	0.67	0.09	0.57	0.07		
C22:5ω6	0.03	0.02	0.04	0.02	0.03*	0.02								
C22:5ω3	0.13	0.06	0.11	0.04	0.12*	0.05	0.49	0.03	0.41	0.05	0.35	0.04		
C24:0	0.05	0.06	0.05	0.04	0.05	0.05							0.04	0.01
C22:6ω3/C24:1ω9	0.37	0.33	0.30	0.18	0.33*	0.27								
C22:6ω3							0.49	0.03	0.41	0.05	0.35	0.04	0.26	0.15
C24:1ω9													0.04	0.01
SFA	43.8	4.75	43.0	5.92	43.4	5.36	37.66	0.95	38.83	1.42	36.48	1.19	41.58	4.57
MUFA	39.9	3.93	35.1	5.55	37.4**	5.36	40.70	0.57	42.18	0.86	39.82	0.72	42.22	3.81
PUFA	16.0	3.13	21.6	4.09	18.9**	4.61							16.21	3.65
ω-3 FA	1.38	0.33	1.74	0.53	1.57**	0.47	2.55	0.10	2.23	0.02	2.50	0.13	1.22	0.59
ω-6 FA	14.3	3.03	19.7	3.93	17.1**	4.42	19.09	0.78	16.77	1.17	21.20	0.98	14.98	3.49
ω-6/ω-3 FA	10.7	2.54	12.0	3.32	11.4	3.02	7.65	0.32	7.67	0.48	8.58	0.40	13.78	4.67
ω-6 + ω-3 FA	15.7	3.15	21.5	4.10	18.7**	4.65	21.64	0.85	19.00	1.26	23.70	1.06	16.21	3.65
ω-6 LCP[a]	0.54	0.10	0.71	0.19	0.63**	0.17	4.33	0.19	3.97	0.29	3.78	0.24		
ω-3 LCP[a]	0.68	0.20	0.73	0.21	0.70	0.20	1.41	0.10	1.21	0.15	1.13	0.12		
LCP[a]	1.22	0.27	1.44	0.35	1.33**	0.33	5.74	0.28	5.18	0.41	4.91	0.35		
18:2ω6 / 18:3ω3[a]	20.7	6.26	21.3	7.66	21.0	6.97	15.81	0.46	17.89	0.69	16.29	0.58		
ω-6 /ω-3 LCP[a]	0.83	0.17	1.04	0.44	0.94*	0.35	3.08	0.20	3.28	0.30	3.33	0.25		

[a] from literature C15:1 were obtained C15:1ω5; C16:1ω7 for colostrum – obtained C16:1ω7t, for C17:1 in colostrum data reported for C17:1ω7, for C18:2ω6c for Benedik: C18:2ω6cc and for C18:2ω6t for Benedik: C18:2ω6tt. For other summed FAs, we used the following calculations; * two groups- KP *vs* MS are statistically significantly different ($p<0.05$), **two groups- KP *vs* MS are statistically significantly different ($p<0.01$), Mann-Whitney U test.

ω-6 LCP = C22:5ω6; C22:4ω6; C22:2ω6; C20:4ω6; C20:3ω6; C20:2ω6

ω-3 LCP = C22:5ω3, C20:5ω3; C20:3ω3

LPC: ω-6 LCP + ω-3 LCP

C18:2ω6 = C18:2ω6 (t+c)

EM – estimated means, SEM – standard deviation of estimated means.

N – number of cases, SD – standard deviation, Min – minimum, Max – maximum

[¥] (Fidler et al., 2000)

[¥¥] (Benedik, 2015) – volunteers were mainly from Ljubljana region (85.6%)

Table S4: Spearman's correlation of FAs and dietary intake factor

Spearman's correlations for daily intake factor		Vegetable	Fruit	Milk	Nuts	Eggs	Poultry	Game	Other meat	Fresh water fish	Fresh seafood	Frozen seafood	Canned seafood	All seafood	Coffee/ tea	Alcohol
C4:0	r_s	-.042	-.031	-.211	-.159	-.008	.211	-.030	-.026	.317**	-.394**	-.109	-.041	-.311**	.041	-.023
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C6:0	r_s	.245*	.165	-.107	.041	.022	.145	-.064	-.117	.168	-.206	.045	.009	-.143	-.023	-.033
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C8:0	r_s	.186	.144	-.151	-.039	.065	.125	-.129	-.074	.131	-.293*	.001	.078	-.196	-.080	-.021
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C10:0	r_s	.150	.095	-.041	.000	-.052	.049	-.195	-.196	.077	-.264*	.121	-.001	-.141	-.146	-.099
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
iC11:0	r_s	-.050	-.045	.079	-.013	.184	.172	.236*	.069	-.030	.085	-.138	.000	.017	-.177	-.119
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C11:0	r_s	-.086	-.149	.003	-.214	.041	.047	.094	.123	.099	-.329**	.057	-.140	-.196	-.236*	-.114
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C12:0	r_s	.078	.072	-.070	.018	-.060	.059	-.154	-.207	.147	-.336**	.027	.024	-.204	-.145	-.146
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
iC13:0	r_s	.056	.152	-.118	-.030	.064	.068	.057	-.020	.219	-.299*	.294*	-.086	-.156	-.008	.037
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C13:0	r_s	.104	.060	.139	.137	.205	.106	.086	-.121	-.015	-.070	.134	.028	.019	-.200	-.227
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
iC14:0	r_s	-.117	.077	.132	.161	.257*	.153	.351**	.045	-.116	.229	-.093	.018	.180	-.106	-.101
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C14:0	r_s	.053	.043	.128	.158	.090	.027	.059	-.187	.033	-.162	-.025	.066	-.088	-.174	-.178
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C14:1ω5	r_s	.056	.179	.189	.134	.145	.019	.094	-.101	-.108	.281*	-.011	-.027	.151	-.150	-.119
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
iC15:0	r_s	.115	.092	.211	.279*	.375**	.032	.221	-.063	-.137	.247*	.090	.123	.256*	-.057	-.104
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
aC15:0	r_s	.075	.106	.194	.309**	.357**	-.022	.280*	-.093	-.162	.298*	.103	.126	.314**	-.080	-.108
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C15:0	r_s	.043	.107	.225	.328**	.342**	.003	.260*	-.101	-.191	.350**	.015	.080	.291*	-.031	-.097
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C15:1	r_s	.031	.077	.190	.345**	.372**	.025	.160	-.027	-.246*	.400**	.073	.026	.307*	.016	-.119
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C16:0	r_s	-.213	-.105	.005	.033	.107	-.117	.210	.117	-.164	.198	-.125	-.027	.131	-.152	-.066
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C16:1ω9	r_s	.062	-.054	.037	.033	.119	-.155	.063	-.040	-.074	.196	.040	-.001	.161	.100	.162
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C16:1ω7	r_s	-.052	-.077	.249*	-.128	.013	-.017	.070	.118	-.066	.159	-.037	-.091	.058	.060	-.018
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74

Spearman's correlations for daily intake factor		Vegetable	Fruit	Milk	Nuts	Eggs	Poultry	Game	Other meat	Fresh water fish	Fresh seafood	Frozen seafood	Canned seafood	All seafood	Coffee/ tea	Alcohol
iC17:0	r_s	.151	.174	.157	.367**	.172	-.144	.153	-.260*	-.282*	.680**	-.054	.115	.513**	.104	.085
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
aC17:0	r_s	.113	.155	.164	.310**	.243*	-.091	.238*	-.167	-.254*	.467**	-.051	.111	.362**	.036	.023
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C17:0	r_s	-.068	.040	.055	.200	.137	.064	.151	.084	-.139	.203	-.066	-.051	.102	.096	.007
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C17:1	r_s	-.115	.011	.028	-.004	.143	.045	.256*	.199	-.067	.140	.026	.023	.147	.057	.059
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
iC18:0	r_s	-.199	-.031	.081	.062	.057	.116	.267*	.096	-.184	.223	-.202	-.041	.076	.019	.029
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C18:0	r_s	-.191	-.095	.004	-.095	.046	.081	.161	.068	-.045	-.041	-.071	-.038	-.086	-.134	-.091
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C18:1ω9	r_s	-.005	.069	-.020	.014	-.093	-.090	.141	.047	-.270*	.266*	-.122	-.070	.132	.104	.099
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C18:1ω7	r_s	.158	.135	.127	.176	-.028	-.371**	.081	-.082	-.260*	.494**	-.133	.049	.340**	.132	.133
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C18:2ω6c	r_s	.141	-.045	-.198	-.038	-.009	.149	-.191	.005	.387**	-.234	.106	-.030	-.211	.188	.148
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C18:2ω6t	r_s	-.153	-.082	.205	.002	.050	-.119	.241*	.034	-.274*	.363**	-.197	-.024	.180	-.221	-.160
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C18:2ω6(c+t)	r_s	-.042	-.162	-.167	-.193	-.099	.117	-.292*	.084	.333**	-.320**	.142	-.044	-.276*	.102	.049
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C18:3ω6	r_s	.127	.097	-.102	.085	.166	-.121	-.164	-.101	.051	.237*	.047	.242*	.199	.194	.138
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C18:3ω3	r_s	-.210	.026	-.049	-.069	-.193	.030	-.110	.030	.131	-.014	.189	-.052	.033	-.129	-.148
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
CLA(c-9. t-11C18:2)	r_s	.042	.101	.202	.184	.260*	.003	.343**	-.118	-.252*	.453**	-.070	.011	.310**	-.065	-.128
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C20:0	r_s	-.099	-.067	-.058	-.065	-.005	.026	.133	.042	-.047	-.166	-.117	-.056	-.181	-.108	-.177
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C20:1ω9	r_s	-.169	.040	.024	-.047	-.100	-.123	.184	.110	-.188	.113	-.048	-.158	.042	-.019	-.230*
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C20:2ω6	r_s	.033	-.009	.269*	.105	-.069	.032	.070	-.069	-.200	.160	.053	-.135	.111	-.001	.025
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C21:0	r_s	-.044	.011	-.024	-.136	-.209	.102	-.159	.059	.170	-.158	.101	-.125	-.169	-.058	-.009
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C20:3ω6	r_s	.015	-.046	-.138	-.066	.171	.153	-.089	-.152	.165	-.225	.064	-.030	-.192	.056	-.002
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C20:3ω3	r_s	-.158	-.065	-.303**	-.352**	.116	.361**	-.007	.061	.202	-.305*	.013	.015	-.229	-.004	-.137
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C20:4ω6	r_s	-.210	.037	-.140	-.132	-.192	.058	.045	.020	.188	-.101	.162	.003	.022	-.103	-.104
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74

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Spearman's correlations for daily intake factor		Vegetable	Fruit	Milk	Nuts	Eggs	Poultry	Game	Other meat	Fresh water fish	Fresh seafood	Frozen seafood	Canned seafood	All seafood	Coffee/ tea	Alcohol
C20:5ω3	r_s	.091	.236*	.035	-.047	.160	-.075	.026	.021	-.182	.259*	-.074	-.033	.134	-.040	-.139
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C22:0	r_s	.053	.020	.013	-.026	.090	-.002	-.069	.092	.042	-.173	-.028	-.104	-.201	.037	-.127
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C22:1ω9	r_s	-.021	.052	-.008	.073	-.128	-.017	.003	.139	.198	-.121	-.051	-.242*	-.183	.058	-.074
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C22:2ω6	r_s	-.121	-.137	-.151	-.159	-.124	.206	-.119	.165	.151	-.360**	-.030	-.280*	-.364**	.049	-.024
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C23:0	r_s	.180	.058	.104	.097	.193	.131	.228	.071	-.007	-.070	-.133	.108	-.056	.320**	-.046
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C22:4ω6	r_s	-.084	-.148	-.064	-.247*	.081	.321**	-.075	.028	.172	-.506**	.085	-.019	-.321**	.041	-.150
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C22:5ω6	r_s	-.218	-.238*	-.060	-.232*	.139	.287*	-.041	.137	.141	-.368**	-.067	-.121	-.347**	-.002	-.141
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C22:5ω3	r_s	.025	.135	-.013	.116	.125	.169	.217	-.132	-.082	.287*	-.084	-.080	.165	.102	-.016
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C24:0	r_s	.025	-.189	.041	.191	.013	.028	-.118	-.025	-.002	.026	-.012	-.106	-.077	.156	.087
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C22:6ω3/C24:1ω9	r_s	.063	.142	-.050	-.011	.147	-.087	.027	.025	-.242*	.231	-.165	-.053	.053	.059	-.050
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
SFA	r_s	-.057	-.020	.035	.079	.074	.052	.060	-.067	.039	-.148	.009	.027	-.057	-.289*	-.149
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
MUFA	r_s	.054	.135	.068	.061	-.063	-.193	.174	.005	-.322**	.437**	-.140	-.016	.271*	.149	.119
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
PUFA	r_s	-.048	-.147	-.157	-.176	-.079	.136	-.258*	.076	.328**	-.307**	.145	-.036	-.255*	.114	.029
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
ω-3 FA	r_s	-.197	.068	-.075	-.097	-.009	.168	.014	-.004	.087	-.027	.070	-.041	-.013	-.031	-.223
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
ω-6 FA	r_s	-.043	-.162	-.167	-.189	-.087	.118	-.296*	.063	.335**	-.325**	.143	-.039	-.277*	.111	.047
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
ω-6/ω-3 FA	r_s	.062	-.242*	-.059	-.057	-.152	-.138	-.266*	.090	.265*	-.298*	.004	-.067	-.312**	.136	.219
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
iC13:0/C13:0	r_s	.049	.119	-.167	-.057	-.011	-.001	.051	.039	.217	-.231	.222	-.120	-.157	.081	.140
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
(iC15:0+aC15:0)/C15:0	r_s	.065	.056	.056	.103	.297*	.024	.135	-.026	-.012	.019	.201	.156	.154	-.164	-.125
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
ω-6 + ω-3 FA	r_s	-.047	-.145	-.161	-.177	-.084	.130	-.268*	.077	.323**	-.306**	.142	-.036	-.256*	.110	.031
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
ω-6 LCP	r_s	-.069	-.080	-.167	-.163	.094	.217	-.096	-.070	.188	-.335**	.124	-.070	-.239*	.049	-.061
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
ω-3 LCP	r_s	-.094	.019	-.221	-.247*	.171	.288*	.005	.008	.091	-.094	-.015	.006	-.095	.019	-.150
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74

Spearman's correlations for daily intake factor		Vegetable	Fruit	Milk	Nuts	Eggs	Poultry	Game	Other meat	Fresh water fish	Fresh seafood	Frozen seafood	Canned seafood	All seafood	Coffee/ tea	Alcohol
LCP	r_s	-.102	-.033	-.242*	-.235*	.122	.282*	-.078	-.039	.139	-.257*	.056	-.073	-.214	.056	-.120
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
C18:2ω6 / C18:3ω3	r_s	.143	-.198	-.056	-.070	.085	.012	-.114	.106	.170	-.267*	-.157	.006	-.320**	.148	.170
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74
ω-6 / ω-3 LCP	r_s	-.011	-.136	.027	.109	-.080	-.026	-.145	-.118	.143	-.185	.170	-.026	-.111	.016	.117
	N	74	74	74	73	74	74	74	71	74	70	74	72	69	74	74

** Correlation was significant at the 0.01 level (2-tailed). * Correlation iwas significant at the 0.05 level (2-tailed). U test. ω-6 LCP = C22:5ω6; C22:4ω6; C22:2ω6; C20:4ω6; C20:3ω6; C20:2ω6. ω-3 LCP = C22:5ω3. C20:5ω3; C20:3ω3. LPC: ω-6 LCP + ω-3 LCP. C18:2ω6=C18:2ω6 (t+c).

Table S5: Comparison of intake of oils and dietary supplements with percentage of FAs in human milk using Mann-Whitney U-test

Mann-Whitney U-test	Omega supplemnts	Supplements	Use of olive oil	Use of sunflower oil	Use of butter	Use of margarine	Use of another type of oil
C4:0	.503	.982	.019*	.115	.025*	.320	.829
C6:0	.080	.106	.081	.137	.531	.424	.011*
C8:0	.256	.330	.214	.024*	.469	.770	.077
C10:0	.811	.697	.071	.051	.227	.327	.164
iC11:0	.807	.260	.077	.388	.718	.945	.430
C11:0	.156	.279	.088	.048*	.517	.861	.868
C12:0	.670	.649	.035*	.043*	.030*	.157	.193
iC13:0	1.000	.984	.071	.482	.279	.110	.023*
C13:0	.709	.697	.484	.606	.961	.782	.125
iC14:0	.333	.855	.002*	.197	.265	.353	.468
C14:0	.842	.471	.545	.332	.171	.536	.154
C14:1ω5	.728	.982	.013*	.288	.114	.493	.198
iC15:0	.447	.611	.003*	.524	.025*	.396	.238
aC15:0	.472	.530	.001**	.203	.018*	.319	.211
C15:0	.371	.431	.001**	.173	.011*	.178	.205
C15:1	.424	.932	.000**	.041*	.213	.453	.260
C16:0	.979	.149	.079	.163	.030*	.288	.663
C16:1ω9	.185	.305	.181	.163	.103	.011*	.532
C16:1ω7	.926	.215	.712	.396	.183	.424	.701
iC17:0	.219	.401	.000**	.000**	.001**	.130	.435
aC17:0	.641	.365	.000**	.016*	.004*	.077	.377
C17:0	.770	.921	.038*	.702	.731	.143	.638
C17:1	.739	.539	.077	.434	.111	.011*	.567
iC18:0	.904	.456	.144	.682	.065	.063	.776
C18:0	.416	.077	.309	.274	.439	.515	.341
C18:1ω9	.585	.548	.016*	.025*	.031*	.063	.614
C18:1ω7	.958	.316	.012*	.001**	.284	.195	.377
C18:2ω6c	.613	.921	.012*	.016*	.160	.008*	.532
C18:2ω6t	.302	.522	.033*	.031*	.467	.935	.625
C18:2ω6(c+t)	.968	.828	.000**	.001**	.037*	.031*	.885
C18:3ω6	.831	.766	.139	.778	.093	.858	.415

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Mann-Whitney U-test	Omega supplemnts	Supplements	Use of olive oil	Use of sunflower oil	Use of butter	Use of margarine	Use of another type of oil
C18:3ω3	.256	.974	.048*	.192	.052	.660	.290
CLA(c-9, t-11C18:2)	.567	.879	.000**	.013*	.007*	.173	.252
C20:0	.576	.068	.685	.409	.873	.405	.187
C20:1ω9	.690	.393	.261	.454	.704	.092	.898
C20:2ω6	.256	.521	.729	.568	.114	.473	.532
C21:0	.279	.687	.000**	.014*	.126	.343	.377
C20:3ω6	.472	.984	.210	.032*	.722	.547	.939
C20:3ω3	.432	.658	.634	.010*	.598	.473	.543
C20:4ω6	.850	.445	.055	.624	.162	.681	.454
C20:5ω3	.123	.687	.047*	.299	.750	.935	.555
C22:0	.352	.356	.250	.036*	.134	.471	.623
C22:1ω9	.393	.756	.032*	.372	.047*	.720	.726
C22:2ω6	.521	.297	.001**	.003*	.009*	.172	.543
C22:4ω6	.661	.345	.514	.587	.319	1.000	1.000
C22:5ω6	.632	.311	.011*	.000**	.484	.684	.831
C22:5ω3	.226	.733	.227	.015*	.696	.741	.468
C24:0	.821	.602	.017*	.378	.331	.327	.602
C22:6ω3/C24:1ω9	.239	.878	.863	.202	.405	.220	.610
SFA	.071	.305	.003*	.203	.990	.614	.818
MUFA	.680	.496	.522	.591	.778	.858	.298
PUFA	.852	.787	.001**	.002*	.017*	.030*	.688
ω-3 FA	1.000	.838	.001**	.001**	.033*	.050*	.898
ω-6 FA	.267	.787	.434	.140	.075	.807	.396
ω-6/ω-3 FA	.958	.828	.001**	.001**	.044*	.034*	.885
iC13:0/C13:0	.576	.630	.005*	.274	.893	.003*	.638
(iC15:0+aC15:0)/C15:0	.894	.869	.061	.978	.454	.089	.211
ω-6 + ω-3 FA	.947	.668	.064	.916	.539	.948	.510
ω-6 LCP	1.000	.838	.001**	.001**	.030*	.044*	.844
ω-3 LCP	.523	.942	.029*	.004*	.606	.637	.871
LCP	.651	.807	.499	.087	.883	.125	.912
C18:2ω6 / C18:3ω3	.464	.900	.381	.007*	.883	.614	.701
ω-6 / ω-3 LCP	.273	.984	.634	.332	.685	.092	.224

** Difference was significant at the 0.01 level (two-tailed). * Difference was significant at the 0.05 level (two-tailed).

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Table S6: Spearman's correlations between maternal frequency of different meals during the day and FAs in human milk

Spearman's correlations		Frequency of breakfast	Frequency of 'morning tea'	Frequency of 'afternoon tea'	Frequency of dinner
C4:0	r_s	.094	-.163	-.056	-.015
	N	73	73	72	74
C6:0	r_s	.119	-.058	.080	.051
	N	73	73	72	74
C8:0	r_s	.124	-.092	.013	.052
	N	73	73	72	74
C10:0	r_s	.165	-.079	.034	.041
	N	73	73	72	74
iC11:0	r_s	.141	.241*	.257*	.004
	N	73	73	72	74
C11:0	r_s	-.110	-.122	.015	-.070
	N	73	73	72	74
C12:0	r_s	.167	-.027	-.016	.061
	N	73	73	72	74
iC13:0	r_s	-.096	-.166	-.020	.066
	N	73	73	72	74
C13:0	r_s	.147	.025	.125	.037
	N	73	73	72	74
iC14:0	r_s	.139	.348**	.279*	-.077
	N	73	73	72	74
C14:0	r_s	.202	.122	.108	.051
	N	73	73	72	74
C14:1ω5	r_s	.090	.308**	.354**	-.001
	N	73	73	72	74
iC15:0	r_s	.108	.215	.212	.076
	N	73	73	72	74
aC15:0	r_s	.120	.275*	.234*	.083
	N	73	73	72	74
C15:0	r_s	.107	.290*	.268*	.077
	N	73	73	72	74
C15:1	r_s	.136	.209	.140	.191
	N	73	73	72	74
C16:0	r_s	-.001	.240*	.321**	-.029
	N	73	73	72	74
C16:1ω9	r_s	-.103	-.045	-.005	.207
	N	73	73	72	74
C16:1ω7	r_s	.009	.052	.185	.214
	N	73	73	72	74
iC17:0	r_s	.155	.273*	.182	.181
	N	73	73	72	74
aC17:0	r_s	.076	.296*	.203	.123
	N	73	73	72	74
C17:0	r_s	.034	.152	.097	.089
	N	73	73	72	74
C17:1	r_s	.042	.055	.174	.123
	N	73	73	72	74
iC18:0	r_s	-.036	.298*	.097	.024
	N	73	73	72	74
C18:0	r_s	-.040	.157	.056	-.111
	N	73	73	72	74
C18:1ω9c/9t	r_s	-.186	.212	.198	-.084
	N	73	73	72	74
C18:1ω7	r_s	-.053	.198	.170	.116
	N	73	73	72	74
C18:2ω6c	r_s	-.094	-.324**	-.386**	.039
	N	73	73	72	74
C18:2ω6t	r_s	.136	.248*	.245*	.103
	N	73	73	72	74
C18:2ω6(c+t)	r_s	-.071	-.450**	-.435**	.007
	N	73	73	72	74
C18:3ω6	r_s	.033	.022	.002	.037
	N	73	73	72	74
C18:3ω3	r_s	.084	-.233*	-.097	-.073
	N	73	73	72	74

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Spearman's correlations		Frequency of breakfast	Frequency of 'morning tea'	Frequency of 'afternoon tea'	Frequency of dinner
CLA(c-9. t-11C18:2)	r_s	.078	.310**	.259*	.140
	N	73	73	72	74
C20:0	r_s	-.011	.077	.013	-.127
	N	73	73	72	74
C20:1ω9	r_s	.011	.036	.129	-.161
	N	73	73	72	74
C20:2ω6	r_s	.101	.204	.161	.033
	N	73	73	72	74
C21:0	r_s	-.013	-.136	-.221	-.118
	N	73	73	72	74
C20:3ω6	r_s	.017	-.054	-.146	-.002
	N	73	73	72	74
C20:3ω3	r_s	-.093	-.224	-.209	.163
	N	73	73	72	74
C20:4ω6	r_s	.097	-.133	-.157	.017
	N	73	73	72	74
C20:5ω3	r_s	-.008	-.018	.152	.019
	N	73	73	72	74
C22:0	r_s	.016	-.120	-.219	-.093
	N	73	73	72	74
C22:1ω9	r_s	.154	-.249*	-.235*	-.113
	N	73	73	72	74
C22:2ω6	r_s	.088	-.222	-.331**	.010
	N	73	73	72	74
C23:0	r_s	.073	-.043	-.040	-.046
	N	73	73	72	74
C22:4ω6	r_s	-.122	-.168	-.139	.159
	N	73	73	72	74
C22:5ω6	r_s	.038	-.106	-.148	-.013
	N	73	73	72	74
C22:5ω3	r_s	.007	.017	-.087	.095
	N	73	73	72	74
C24:0	r_s	-.096	-.198	-.344**	-.070
	N	73	73	72	74
C22:6ω3/C24:1ω9	r_s	-.031	-.125	-.124	.052
	N	73	73	72	74
SFA	r_s	.184	.125	.168	-.029
	N	73	73	72	74
MUFA	r_s	-.136	.264*	.226	-.035
	N	73	73	72	74
PUFA	r_s	-.051	-.440**	-.420**	.013
	N	73	73	72	74
ω-3 FA	r_s	.066	-.219	-.107	.034
	N	73	73	72	74
ω-6 FA	r_s	-.058	-.452**	-.437**	.006
	N	73	73	72	74
ω-6/ ω-3 FA	r_s	-.081	-.143	-.225	-.093
	N	73	73	72	74
iC13:0/C13:0	r_s	-.154	-.195	-.070	.068
	N	73	73	72	74
(iC15:0+aC15:0)/C15:0	r_s	.116	.091	.090	.039
	N	73	73	72	74
ω-6 + ω-3 FA	r_s	-.048	-.439**	-.420**	.008
	N	73	73	72	74
ω-6 LCP	r_s	.032	-.151	-.206	.030
	N	73	73	72	74
ω-3 LCP	r_s	-.074	-.228	-.191	.160
	N	73	73	72	74
LCP	r_s	-.078	-.229	-.200	.110
	N	73	73	72	74
C18:2ω6 / C18:3ω3	r_s	-.126	-.042	-.152	-.016
	N	73	73	72	74
ω-6 / ω-3 LCP	r_s	.123	-.003	-.134	-.107
	N	73	73	72	74

** Correlation was significant at the 0.01 level (two-tailed). * Correlation was significant at the 0.05 level (two-tailed).

Supplements of "Dietary habits of Slovenian inland and coastal primiparous women and fatty acid composition of their human milk samples", Jagodic et al. 2020

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3.2 Selected Elements and Fatty Acid Composition in Human Milk as Indicators of Seafood Dietary Habits

Jagodic, M., Potočnik, D., Snoj Tratnik, J., Mazej, D., Pavlin, M., Trdin, A., Eftimov, T., Kononenko, L., Ogrinc, N., Horvat, M. Selected elements and fatty acid composition in human milk as indicators of seafood dietary habits. Environmental research: multidisciplinary journal of environmental sciences, ecology, and public health, ISSN 0013-9351, 2020, vol. 180, str. 108820-1-108820-11, doi: 10.1016/j.envres.2019.108820

The present study aimed to test whether the levels of selected elements and FAs and $\delta^{13}C_{FA}$ in human milk could represent a more reliable marker of seafood dietary habits and sources of food consumed as assessed from the questionnaire data.

It is the first study to investigate stable isotopes of FAs in human milk in Slovenia. This study showed that human milk samples from coastal (KP) and inland (MS) areas had different elemental and FA compositions and different FA stable isotope profiles, which is indicative of the different dietary habits and lifestyles in the two distinct study areas of Slovenia. Therefore, the FA composition and its $\delta^{13}C_{FA}$ values together with the elemental composition in maternal milk could be used as a marker of fresh seafood intake.

Data mining approaches confirmed that using the classifier for fresh seafood intake based on the seven variables, including elemental composition, levels of FAs, and their stable isotopes in milk, enables distinguishing between seafood consumption less than once per month *vs* at least once per month fresh with 84% accuracy. Clustering could be used to divide the data sets into two with 90% accuracy. A clustering approach based on the percentage of the FAs iC17:0, C4:0, C18:2ω6t, aC17:0, CLA, C22:4ω6, and $\delta^{13}C$ of C18:1ω9c is an accurate indicator to distinguish between higher and lower fresh seafood intake. This confirmed our hypothesis that stable isotope analysis in combination with elemental analysis is an important tool to distinguish between diets, particularly for seafood. These findings suggest that in human-related studies related to the risks and benefits of seafood consumption, the analyses of FAs, their stable isotopes, and elemental compositions can significantly improve the information on seafood consumption.

The candidate's contribution to this paper consisted of literature overview, extraction of data from published papers and performing analytical and statistical analyses of this case study. The candidate prepared the tables, and drafted the manuscript in collaboration with other co-authors.

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Selected elements and fatty acid composition in human milk as indicators of seafood dietary habits☆

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ABSTRACT

The maternal diet and living environment can affect levels of chemical elements and fatty acid (FA) composition and their stable isotopes ($\delta^{13}C_{FA}$) in human milk. Information obtained from questionnaires is frequently imprecise, thus limiting proper associations between external and internal exposures as well as health effects. In this study, we focused on seafood as a source of potentially toxic and essential elements and nutritional FAs. Concentrations of selected elements in human milk (As, Cd, Cu, Mn, Pb, Se and Zn) were determined using inductively coupled plasma mass spectrometry (ICP-MS) and Hg using cold vapour atomic-absorption spectrometry (CV-AAS). The identification and quantification of FAs in maternal milk were performed by an in-situ trans-esterification method (FAMEs), and the characterization of FAMEs was performed by gas chromatography with a flame ionisation detector (GC-FID). $\delta^{13}C_{FA}$ was determined by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Seventy-four lactating Slovenian women from the coastal area of Koper (KP), with more frequent consumption of seafood, and the inland area of Pomurje (MS), with less frequent seafood consumption, were included in this study. Along with basic statistical analyses, data mining approaches (classification and clustering) were applied to investigate whether FA composition and $\delta^{13}C_{FA}$ could improve the information regarding dietary sources of potentially toxic elements. As and Hg levels in milk were found to be statistically higher in populations from KP than in those from MS, and 71% of individual FAs and 30% of individual $\delta^{13}C_{FA}$ values in milk differed statistically between the studied areas. In 19 cases, the levels of FAs in milk were higher in KP than in MS; these FAs include C20:5ω3 and C22:6ω3/C24:1ω9, which are typically contained in fish. In 16 cases, the mean percentage of FAs was higher in MS than in KP; these FAs include the PUFAs C18:2ω6, C18:3ω3, and C20:4ω6 which are important for human and infant growth. The difference in $\delta^{13}C$ levels of C10:0, C12:0, C14:0, C16:1, C16:0, C18:1ω9c, C22:6ω3, and $\delta^{13}C$ 18:0–16:0 in the study groups was statistically significant. In all seven cases where $\delta^{13}C$ of FA significantly differed between KP and MS, $\delta^{13}C$ was higher in KP, indicating a higher proportion of a marine-based diet. The data mining approaches confirmed that the percentage of selected FAs (iC17:0, C4:0, C18:2ω6t, aC17:0, CLA, and C22:4ω6) and $\delta^{13}C_{FA}$ of C18:1ω9c in human milk could be used to distinguish between high and low frequency of fresh seafood consumption.

1. Introduction

Human milk is considered an essential and irreplaceable part of a healthy diet and is known as the best nutrition source for new-borns owing to its high protein, vitamin, mineral, and fatty acid (FA) contents (Bratanič, 2018; Chu, 2013; Gura, 2014; Krawinkel, 2011; WHO, 2011). According to literature, the mother's diet and living environment can affect the levels of elements and FA compositions in human milk (Jensen, 1999;

☆ Our study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). The research protocol of the study was approved by the National Medical Ethics Committee (NMEC) of the Republic of Slovenia (number of accordance: 42/12/07 and 53/07/09). All participants provided signed informed consent prior to sampling.

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Kelishadi et al., 2012; Nishimura et al., 2013; Ruan et al., 1995; Rudge et al., 2009). FAs in human milk originate from three sources: (1) mobilisation from maternal endogenous stores of FAs owing to deficient energy intake, (2) endogenous synthesis of FAs from precursor FAs when excess energy is fed, and (3) diet composition (Francois et al., 1998; Jensen, 1999, 1996; Sauerwald et al., 2001). Francois et al. (1998) found that a single meal of specific fats could significantly affect the FA profile of human milk for 1–3 days, with maximum levels of specific FAs occurring within a 24-h period (Francois et al., 1998). Consumption of saturated fatty acids (SFAs) is associated with a higher incidence of cardiovascular diseases, whereas unsaturated FAs are beneficial for human health. For example, omega-3 polyunsaturated FAs (ω-3 PUFAs) play an essential role in the development of neurological functions in the early years of life, and conjugated linoleic acids (CLAs) have anti-diabetic, anti-atherogenic, and anti-carcinogenic health effects (Lock and Bauman, 2004; Nunes and Torres, 2010). The ω-6 and ω-3 PUFA contents can influence the optimal growth and development of the baby (Birch et al., 1992; Chen et al., 1997; Innis, 2014, 2007; Jensen, 1999; Keim et al., 2012; Koletzko et al., 1988; Ruxton et al., 2004). Apart from the beneficial components, several potentially toxic substances can also move from the mother to the child through human milk, and their levels are commonly determined in human milk as biomarkers of infant exposure (Rudge et al., 2009). Seafood is an important source of PUFAs and also of potentially toxic elements. Therefore, seafood intake should be evaluated in the context of potential risks and benefits in health-related human biomonitoring studies (HBM), especially in infants and children as they are the most susceptible groups (Andersen et al., 2000; Bravi et al., 2016; Grandjean and Landrigan, 2006; Mozaffarian, 2006; Strain et al., 2015; Tatsuta et al., 2018; Valent et al., 2013; Zeilmaker et al., 2013). One of the critical steps in determining the risks and benefits from seafood intake via human milk is to associate external and internal exposures.

In environment- and health-related studies, human exposure to selected contaminants is assessed by (1) measuring substances and their metabolites in biomarkers such as blood, hair, urine, and human milk (internal exposure) and (2) using data obtained from questionnaires (external exposure). Data obtained from questionnaires is frequently imprecise, and thus, it limits proper associations between exposure and effects. Like in many other studies, in a Slovenian-first HBM study, diet was evaluated through self-administered food frequency questionnaires (Snoj Tratnik et al., 2019). However, this approach is known to produce highly uncertain data as most people tend not to remember the exact frequency of the different types of seafood they have consumed. Oken et al. (2014) compared three methods for assessing fish intake through questionnaires via correlation with analytical results of mercury in blood and hair and plasma docosahexaenoic acid (DHA), where, for example, plasma DHA concentration correlated with fish intake assessed with a one-question screener but not with the other two. They concluded that a longer questionnaire does not provide an advantage over shorter ones in evaluating the intake of fish, DHA, and mercury compared with biomarkers (Oken et al., 2014). The alternative independent approach is to estimate seafood consumption frequency through the FA and stable isotope composition in human milk. For example, the DHA concentration in human milk is known to be directly correlated with fish consumption habits (Cohen et al., 2005; Leino et al., 2013).

Because the FA composition is known to be correlated with diet, the analysis of the stable carbon isotope ratio ($^{13}C/^{12}C$, expressed as $\delta^{13}C$ per mil, ‰) could also be used for tracking dietary habits. This is because variations in the isotopic composition of carbon ($\delta^{13}C$) occur owing to differences in the fixation pathways of CO_2, which are correlated with environmental and physiological conditions (Boschker and Middelburg, 2002). All plants in naturally synthesized organic compounds assimilate carbon isotope ^{12}C in preference to ^{13}C, resulting in different $\delta^{13}C$ owing to different enzymatic processes and sizes of metabolic carbon pools (Hayes, 1993). Thus, $\delta^{13}C$ differences were observed between different types of plants: C3 plants (tree, shrubs, temperate grasses), C4 plants (sugar cane, subtropical grasses, maize), and crassulacean acid metabolism (CAM) plants (Meier-Augenstein, 1999; Smith and Epstein, 1971; Tykot, 2006). Further, differences in $\delta^{13}C$ between human collagen from a diet based on terrestrial C3 food and a marine diet as well as terrestrial and marine sediments for saturated long-chain ω-FAs (ω-LCFs) were observed (Richards and Hedges, 1999). In general, higher $\delta^{13}C$ values in collagen are related to a marine diet. Only a few studies have measured $\delta^{13}C$ values of FAs in milk, and these were mainly related to determining the geographical origin of milk (Ehtesham et al., 2013) or the dietary habits of animals (Richter et al., 2012). Richter et al. (2012) tested the potential use of $\delta^{13}C$ values of a single FA as tracers for the transformation of FA from diet to milk with a focus on the metabolic origin of c9,t11–18:2 (Richter et al., 2012). To the best of our knowledge, Vetter et al. (2007) performed the only study determining $\delta^{13}C$ values in human milk, where $\delta^{13}C$ values of individual methyl-branched fatty acids (MBFAs) were compared to $\delta^{13}C$ values of straight-chain FAs in food. Their results illustrate that MBFAs have distinctive $\delta^{13}C$ values and must originate from other sources and/or from very different substrates (Vetter et al., 2007). Owing to the potential of this technique, we investigated whether it could be used as a tool for obtaining maternal dietary information (particularly for seafood intake) through the analysis of maternal milk samples.

Specifically, the present study aimed to test whether the levels of selected elements and indicatory FAs and their $\delta^{13}C_{FA}$ in human milk could represent a more reliable marker of dietary habits and sources of food consumed as assessed from the questionnaire data. We focused on seafood as a source of potentially toxic elements and nutritional FAs. Potentially toxic and essential elements and FA compositions and their $\delta^{13}C_{FA}$ were measured in human milk. Some of the most relevant potentially toxic and essential elements (ATSDR, 2013) were selected for the study: lead (Pb), mercury (Hg), cadmium (Cd), arsenic (As), zinc (Zn), copper (Cu) and selenium (Se), based on the selection criteria described by Snoj Tratnik et al. (2019).

However, it should be noted that the purpose of this study was not to interpret the human biomonitoring data of trace elements, as this has been discussed and published elsewhere (Snoj Tratnik et al., 2019), but to test the hypothesis that the FA composition and their $\delta^{13}C_{FA}$ in combination with elemental analysis in human milk could improve the differentiation between dietary habits. To test this hypothesis, the analytical results of % FAs, $\delta^{13}C_{FA}$, and elemental composition in human milk were statistically evaluated to discriminate between (1) mothers living in different geographical areas (coastal (KP) and inland (MS)) with significantly different seafood consumption patterns; (2) intake of terrestrial-based food and seafood, and (3) different types of seafood (fresh/canned/frozen). In addition to classical statistical approaches, the present study applied up-to-date data mining approaches which enabled better interpretation of a large-scale database and consideration of more variables simultaneously, unlike in classical statistical approaches.

2. Materials and methods

2.1. Description of study population

The study population included two groups of women (19–39 years old) in Slovenia who gave birth to a first-born singleton. One group was from the coastal area around Koper (KP) and one was from the inland area of Pomurje (MS), as shown in Fig. 1. The population groups were assessed as part of a national HBM study in Slovenia. A protocol to start the first national HBM survey was established in 2007 based on legislation for the implementation of HBM in Slovenia (Acta for Chemicals, No. 110/03). Participants from 12 study areas across Slovenia were recruited (2007–2015) and biological matrices were used to assess internal exposure. Snoj Tratnik et al. (2019) have described the methodology in detail.

2.2. Ethical considerations

HBM in Slovenia was approved by the Republic of Slovenia National

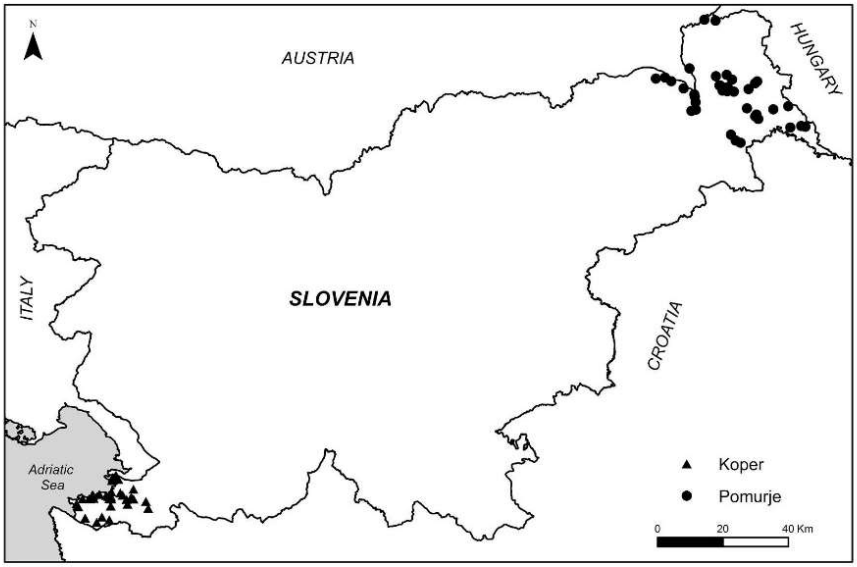


Fig. 1. Sampling areas of the present study: Koper (coastal area) and Pomurje (inland area).

Medical Ethics Committee (NMEC) with numbers of accordance 42/12/07 and 53/07/09. All participants provided informed consent and their data were pseudo-anonymised. Further, participants had the right to withdraw at any time during the study period.

2.3. Recruitment and sampling

Mothers in the KP and MS areas participated in the national HBM study. They were recruited between 2011 and 2013 through maternity hospitals, childbirth classes for parents, and/or gynaecologists. Maternal blood, urine, milk, and hair samples were collected between 5 and 11 weeks after delivery. Questionnaires were given to all participating mothers to obtain information about their age, residence history, family background, social factors, education, weight before pregnancy, parity, medical and occupational history, lifestyle, relevant paternal-/pregnancy-related information, information about the new-born child, and dietary information (frequencies of consumption of different food items and their source). The latter included the following dietary items: vegetables, fruit, nuts, milk and dietary products, eggs, meat (poultry, game, other), fresh-water fish, seafood, coffee, tea, and alcoholic beverages. Regarding seafood, participants had to mark how often they consumed (1) seafood without any conservation processing, that is, fresh seafood; (2) seafood which was frozen before consumption (for transport, storage, etc.), that is, frozen seafood; and (3) seafood which was canned for storage, that is, canned seafood, because such food processing can cause some changes in the FA profile. For example, Barrow et al. (2008) wrote about differences between freezing items and long-term storage in a freezer. In this study, seafood refers to all types of fish and shellfish, that is, any type of sea life regarded as food by humans. A total of 74 lactating women from KP (36) and MS (38) provided informed consent; filled in the self-administered questionnaires; and provided milk, urine, blood, and hair samples which were collected by trained personnel. Recruitment and sampling procedures were described in detail by Snoj Tratnik et al. (2019). All blood, urine, and milk samples were stored at −20 °C until analysis.

2.4. Sample preparation and chemical analysis

2.4.1. Levels of selected elements in maternal milk samples

For multi-elemental analysis, two sample preparation methods were followed. Briefly, for Cu, Zn, and Se, an aliquot (1 mL) of milk (0.15 g of lyophilised samples) was transferred into quartz tubes. To this, 1 mL of 65% HNO_3 (Merck, Germany, suprapure (s.p.)) and 1 mL 30% H_2O_2 (Merck, Germany, s. p.) were added and the samples were microwave-digested in a closed vessel (Microwave System Ethos 1, Milestone SN 130471) at 1500 W. Each sample was ramped to 130 °C at 10 °C/min, then to 200 °C at 10 °C/min and held for 20 min, and finally allowed to cool for 20 min. The sample was then equilibrated to room temperature. After digestion, the solution was diluted to 10 mL with Milli-Q water. External calibration was performed using an ICP Multi Element Standard solution XVI CertiPUR (Merck, Germany). For As, Cd, and Pb, an aliquot (1 mL) of milk was diluted five times with an alkaline solution containing 5 g/L of 25% ammonia (Merck, Germany, s. p.), 0.5 g/L of Triton X-100 (Sigma Aldrich, SigmaUltra), and 0.5 g/L of ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA, Fisher Scientific, analytical reagent grade) in Milli-Q water (adapted from (Barany et al., 1997)). For calibration, a standard addition procedure was used. An inductively coupled plasma mass spectrometer (ICP-MS) with an octapole reaction system (ORS) (7500ce, Agilent, Japan) was used for determining the levels of selected elements (As, Cd, Cu, Pb, Se, and Zn).

For Hg, 1 mL of sample was taken in a glass volumetric flask (30 mL), to which 2 mL of a mixture of 65% HNO_3 (Merck, Germany, p. a.)-$HClO_4$ (Merck, Germany, s. p.) (1:1, v/v) and 5 mL of 96% H_2SO_4 (Merck, Germany, s. p.) were added. For digestion, the flasks were heated at 220 °C for 20 min. After cooling, samples were diluted with Milli-Q water. The total Hg concentration in milk was determined using an automatic system based on cold vapour atomic fluorescence spectrometry (CVAFS; Tekran 2600, Tekran Instruments Corporation, Canada) (Akagi, 1997; Snoj Tratnik et al., 2019).

To ensure quality, blank samples, control samples, and reference materials were run together with the samples on a daily basis. The accuracy of the results was checked by analysing the certified reference materials: NIST 8435 (whole milk powder), NIST 1549 (non-fat milk powder), NIST 1549a (whole milk powder), BCR 150 (spiked skim milk

powder), and control materials FAPAS 07190 or 07172. For As, no suitable reference materials were available; therefore, the results of a laboratory control milk sample were checked using neutron activation analysis (Byrne, 1987; Vakselj and Byrne, 1974).

The estimated analytical precision for Cd, Pb, and As was 15%; that for Se, 10%; that for Cu and Zn, 5%; and that for Hg, 6%. The limit of detection (LOD) for Cd, Pb, As, Hg, Se, Cu, and Zn calculated as three times the standard deviation of the blank sample was 0.1, 0.2, 0.05, 0.001, 2, 6, and 35 ng/mL of milk sample, respectively, and the limit of quantification (LOQ) for Cd, Pb, As, Hg, Se, Cu, and Zn calculated as ten times the standard deviation of the blank sample was 0.3, 0.7, 0.16, 0.003, 7, 20, and 116 ng/mL of milk sample, respectively (Snoj Tratnik et al., 2019).

A more detailed description of the methodology can be found in Miklavčič et al. (2013), Potočnik et al. (2016), and Snoj Tratnik et al. (2019).

2.4.2. Composition and stable isotopes of FAs in maternal milk

The identification and quantification of FAs in milk were performed using an in-situ trans-esterification method in which human milk triacylglycerides were extracted. The total lipid content from milk samples was extracted using a mixture of dichloromethane (J. T. Baker) and 0.5 M sodium hydroxide in methanol (J. T. Baker). The solution was purged with nitrogen and heated for 10 min at 90 °C, and then, it was cooled rapidly. To complete the reaction, fatty acid methyl esters (FAMEs) were formed by adding 14% BF_3-methanol solution (Sigma Aldrich); subsequently, the solution was purged with nitrogen and reheated for 10 min at 90 °C. Once the solution was cooled rapidly, the FAMEs were extracted with hexane and transferred to GC vials. All samples were stored at −20 °C before analysis (Park and Goins, 1994). FAMEs were characterised using an Agilent 6890 N (Network GC System) gas chromatograph equipped with an Omegawax 320 capillary column (30 m × 0.32 mm × 0.25 μm, Supelco) and flame ionisation detection (FID). The temperature program was as follows: 50 °C with hold time of 2 min and ramping to 220 °C at 4 °C/min with hold time of 15 min. The carrier gas was helium at a flow rate of 1 mL/min and the makeup gases were nitrogen (45 mL/min) and hydrogen (40 mL/min) with a corresponding air flow of 450 mL/min (Nunes and Torres, 2010). The individual FAs were identified and quantified by comparing their retention times with those of a standard FAME Mix (Supelco 37 component FAME Mix) and expressed as the weight percent of total identified FAs. The standard was analysed after every 10 samples to verify the stability of the analytical system. In each set of samples, blank samples were analysed to check the data quality. The precision of the method based on replicates of real samples was up to 5%. The LOD was 0.004%.

$\delta^{13}C_{FA}$ was determined by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) using an Agilent 6890 N GC coupled to an IsoPrime GV IRMS via a combustion interface. Separation was performed using a DB-1MS (60 m × 0.32 mm × 0.25 μm, Agilent) capillary column with He as a carrier gas at a flow rate of 1 mL/min (Gams Petrišič et al., 2013). Carbon isotope measurements are expressed in delta notation (δ) relative to the Vienna-Pee Dee Belemnite (V-PDB) standard in per mil (‰) and defined as $\delta^{13}C = [(R_s - R_{ref})/R_{ref}] \times 1000$, where R is the $^{13}C/^{12}C$ ratio in sample 's' and reference material 'ref', respectively (Brand et al., 2014). The measurement precision ranged between 0.3‰ and 0.5‰ based on the replicate analysis of the C19:0 standard (methyl nanodecanoate, RESTEK Corporation). The $\delta^{13}C$ value of the FA was determined using an elemental analyser coupled to IRMS (EA-IRMS), and the obtained value was compared to that obtained using GC-C-IRMS measurements. The isotopic shift due to the carbon introduced during methylation was corrected for by mass balance (Spangenberg and Ogrinc, 2001).

2.5. Data analysis

The concentrations of elements and the percentage of FAs below the LOD were arbitrarily assigned a value of half the LOD. For parametric statistical tests, non-normally distributed data were log10-transformed.

Based on the food intake categories from the questionnaire, the daily intake (meals per day) was estimated as follows: never = 0, less than once per month = 0.02, 1–3 times per month = 0.07, once per week = 0.14, 2–4 times per week = 0.43, 5–6 times per week = 0.79, once per day = 1, more than once per day = 2.5) as described by Valent et al. (2013). For FAs, the amount of individual FA was based on the peak area. For the composition and stable isotopes of FAs, the dataset excluded variables with less than 10 determinant cases in total amount (in case of $\delta^{13}C_{FA}$ for C18:3ω6, C18:1ω9t, C22:2, C23:0, C24:1ω9, and C24:0). The percentage of combined FAs was calculated as follows: SFA = C4:0 + C6:0 + C8:0 + C10:0 + iC11:0 + C11:0 + C12:0 + iC13:0 + C13:0 + iC14:0 + C14:0 + iC15:0 + aC15:0 + C15:0 + C16:0 + iC17:0 + aC17:0 + C17:0 + iC18:0 + C18:0 + C20:0 + C21:0 + C22:0 + C23:0 + C24:0; MUFA = C14:1ω5 + C15:1 + C16:1ω9 + C16:1ω7 + C17:1 + C18:1ω9 + C18:1ω7 + C20:1ω9 + C22:1ω9; PUFA = C18:2ω6c + C18:2ω6t + C18:3ω6 + C18:3ω3 + CLAc9t11C18:2 + C20:2ω6 + C20:3ω6 + C20:3ω3 + C20:4ω6 + C20:5ω3 + C22:2ω6 + C22:4ω6 + C22:5ω6 + C22:5ω3; ω-3 FA = C18:3ω3 + C20:3ω3 + C20:5ω3 + C22:5ω3; ω-6 FA = C18:2ω6c + C18:2ω6t + C18:3ω6 + C20:2ω6 + C20:3ω6 + C20:4ω6 + C22:2ω6 + C22:4ω6 + C22:5ω6; ω-6 LCP = C22:5ω6 + C22:4ω6 + C22:2ω6 + C20:4ω6 + C20:3ω6 + C20:2ω6; ω-3 LCP = C22:5ω3 + C20:5ω3 + C20:3ω3; LPC: ω-6 LCP + ω-3 LCP; C18:2ω6 = C18:2ω6 (t + c).

Statistical analyses were performed using the statistical programs SPSS and STATA 12/SE. Graphical plots were created using OriginPro2017. Statistical significance was defined as $p < 0.05$ and marginal significance, as $0.05 < p < 0.1$. Descriptive statistics, univariate analyses, and multiple linear regression were used to examine possible associations between FA compositions, their $\delta^{13}C_{FA}$, elemental composition of maternal milk samples, and lifestyle. Univariate analyses included the Spearman test for categorical data and the Pearson test for continuous data, analysis of variance (ANOVA), chi-square tests (Pearson and Fischer's), and Mann-Whitney *U* test (Field, 2009; Košmelj, 2007).

Data mining approaches for supervised (i.e. classification) and unsupervised (i.e. clustering) learning were applied to identify fresh seafood intake (< 1 per month *vs* at least 1 per month) (Adankon and Cheriet, 2009; Eftimov et al., 2017; Granitto et al., 2006; Kaufman and Rousseeuw, 1990; Steel and Torrie, 1960). For data mining approaches, the data set for predicting fresh food intake consisted of 38 participants described by 64 variables (features). Seven out of 64 were elements in maternal milk, 50 were the composition of FAs in maternal milk, and one was the age of the baby on the day of sampling. Further, six $\delta^{13}C_{FA}$ values were added ($\delta^{13}C_{FA}$ of C10:0, C12:0, C14:0, C16:1, C16:0, and C18:1ω9c). The output label (class) consisted of 23 mothers that consumed fresh seafood less than once per month and 15 participants that consumed it at least once per month. For classifiers, support vector machines were trained and used for classification (Adankon and Cheriet, 2009). A subset of relevant variables (variable/feature selection) was selected to simplify the models to make it easier to interpret them, reduce training times, avoid dimensionality, and enhance generalization by reducing overfitting. In this case, the recursive feature elimination (RFE) method (Granitto et al., 2006) and its implementation from the R package 'caret' were used (Kuhn, 2008). After selecting relevant variables, the classifiers were learned using the 'ksvm' function with default parameters from the 'kernelab' package in the R programming language (Karatzoglou et al., 2004). Classifiers were evaluated using leave-one-out cross validation because of the small data sets. In leave-one-out cross validation, a classifier is trained on all participants except for one and a prediction is made for that participant. The average accuracy was computed and used to evaluate the classifier. However, to check the robustness of the learned classifiers, this was evaluated using bootstrapping with the traditional approach for evaluation, which involved splitting (100 times) the participants into 75% used for training and 25% used for testing. When performing splitting,

one half of the participants used for training or testing were from one prediction label and the other half, from the other. This was done to avoid an imbalanced classification problem.

Clustering was performed to understand how a sample might comprise distinct subgroups given a set of variables. The (dis)similarity between the participants was calculated using the Gower distance from the R package 'cluster' (Maechler, 2019). After calculating the distances between the participants, the 'partitioning around medoids' (PAM) algorithm was used to perform clustering (Kaufman and Rousseeuw, 1990). The data sets were the same as those used in the classification task.

3. Results and discussion

3.1. General characteristics of study population

Tables S1 and S2 list the general characteristics of the study population from the two studied areas, namely, the coastal area around Koper (KP) and the inland agricultural area Pomurje (MS), respectively. Significant differences were observed in birth length, babies' feeding, and the frequency of seafood consumption. Babies born in KP had a higher birth length than those born in MS, and 72% of children in KP were breastfed exclusively compared to 47% in MS at the time of sampling. Fresh seafood (average daily intake factor 0.09 *vs* 0.04) was more frequently consumed in KP than in MS, which was expected owing to the availability of fresh seafood in the coastal region. In contrary, fresh-water fish (average daily intake factor 0.02 *vs* 0.05) and frozen seafood (average daily intake factor 0.04 *vs* 0.06) were less frequently consumed in KP than in MS.

3.2. Selected elements in human milk

In this study, some of the most relevant toxic and potentially toxic elements, listed among the top 20 chemicals on the Priority List of Hazardous Substances (ATSDR, 2013), were measured in human milk samples as infant's first food. Infants are one of the most susceptible groups owing to their increased absorption rates and diminished ability to detoxify many exogenous compounds (Grandjean and Landrigan, 2006). It is known that several of these substances move from the mother to the child through breast milk (Röllin et al., 2009; Rudge et al., 2009). The toxic effects of elements also depend on the interactions between elements, especially when the metabolism of elements is similar. Risk assessments for selected elements have been performed by several organisations (Burtis et al., 2012). Essential elements also have a potential for toxicity, but even more important is the role of essential elements from a nutritional viewpoint. Element selection was described in Snoj Tratnik et al. (2019).

Table S3 lists the levels of selected elements (As, Hg, Cd, Pb, Se, Cu, and Zn) in maternal milk samples and the percentages of samples below or above the literature ranges according to Iyengar (1998). For human milk, reference values have not yet been established. Therefore, we referred to the values and ranges for potentially toxic and essential elements from Iyengar (1998). The concentrations of elements showed a significant difference between the study areas only in cases of As and Hg: both were higher in the coastal area compared to the inland area owing to the higher frequency of seafood consumption (Table S1). This difference between the two areas was expected as seafood is an important source of both elements for the general population, as previously confirmed for the Slovenian population (Miklavčič et al., 2013). Among the potentially toxic elements, none of the participating mothers had Cd, Pb, or Hg levels exceeding the literature range (Iyengar, 1998), whereas two mothers had As levels in their milk that exceeded values of 3 ng/mL (Iyengar, 1998), 3.50 ng/mL, and 3.70 ng/mL, respectively (Table S1).

A comparison of the results of this study with literature data showed that the Hg concentration in our study areas (mean of Hg in KP: 0.27 ± 0.20 ng/mL, mean Hg in MS: 0.20 ± 0.22 ng/mL; median in KP: 0.19 ng/mL, median in MS: 0.14 ng/mL) was lower than the reported median (min-max) in Germany (1998; 0.37 (< 0.2–6.9) ng/mL) (Drasch et al., 1998), mean concentration in Austria (2002; 1.59 ± 1.21 ng/mL) (Gundacker et al., 2010), Italy (2008) with Hg concentrations in pooled samples ranging from 2.63 to 3.53 ng/mL (Abballe et al., 2008), and Iran (2019; median (min-max), 2.8 (< 1–28.7) ng/mL) (Vahidinia et al., 2019). The Cd concentration (mean of Cd in KP: 0.06 ± 0.03 ng/mL, mean of Cd in MS: 0.07 ± 0.04 ng/mL; median in KP: 0.05 ng/mL, median in MS: 0.05 ng/mL) was lower than that reported for Sweden (2012; 0.09 ± 0.05 ng/mL) (Björklund et al., 2012), Greece (2008; 0.14 ± 0.12 ng/mL) (Leotsinidis et al., 2005), and Italy (values below LOQ of 0.5 ng/mL) (Abballe et al., 2008). The As concentration (mean of As in KP: 0.57 ± 0.85 ng/mL, mean of As in MS: 0.17 ± 0.14 ng/mL; median in KP: 0.33 ng/mL, median in MS: 0.13 ng/mL) was similar to that reported for Sweden (2012; 0.55 ± 0.70 ng/mL) (Björklund et al., 2012) and lower than that reported for Iran (2019; mean (range): 0.85 (0.50–4.00) ng/mL) (Samiee et al., 2019). The Pb concentration (mean of Pb in KP: 0.40 ± 0.29 ng/mL, mean of Pb in MS: 0.36 ± 0.50 ng/mL; median in KP: 0.34 ng/mL, median in MS: 0.23 ng/mL) was higher than that reported for Greece (2008; 0.15 ± 0.25 ng/mL) (Leotsinidis et al., 2005); lower than that reported for Sweden (2012; 1.5 ± 0.90 ng/mL) (Björklund et al., 2012), Austria (2002; 1.63 ± 1.66 ng/mL) (Gundacker et al., 2010) and Italy (2008; concentrations in pooled samples ranging from 0.971 to 1.07 ng/mL) (Abballe et al., 2008); and much lower than that reported for Iran (median: 45.7 ng/mL, mean: 96.7 ng/mL from 2-month postpartum) (Samiee et al., 2019).

Among the essential elements in human milk, Se, Cu, and Zn (Table S3) were evaluated and compared with the literature data. The deviations observed were marginal, implying that no deficiencies and excess values were present in the study population. Mičetić-Turk et al. (2000) reported the Se status in Slovenian human milk but only for the colostrum, where Se levels were 29 ± 10 ng/mL. The levels observed in our study were lower (mean of Se in KP: 12.3 ± 2.6 ng/mL, mean of Se in MS: 12.4 ± 4.0 ng/mL; median in KP and MS: 12.0 ng/mL), which was expected for mature milk because the Se content of human milk is known to decrease postpartum (Benemariya et al., 1995; Izquierdo Álvarez et al., 2007; Kosta et al., 1983). Björklund et al. (2012) reported similar concentrations to our study (13 ± 2.6 ng/mL) for milk obtained from Swedish mothers 2–3 weeks postpartum. The Cu concentration (mean of Cu in KP: 318 ± 60 ng/mL, mean of Cu in MS: 355 ± 121 ng/mL; median in KP: 313 ng/mL, median in MS: 344 ng/mL) was lower than that reported for Greece (2008; 390 ± 108 ng/mL) (Leotsinidis et al., 2005) and Sweden (2012; 471 ± 75 ng/mL) (Björklund et al., 2012) and similar to that reported for Italy (2008; concentrations in pooled samples ranging from 354 to 424 ng/mL) (Abballe et al., 2008) and Iran (mean: 350 ± 10 ng/mL) (Taravati Javad et al., 2018). The Zn concentration (mean of Zn in KP: 1935 ± 670 ng/mL, mean of Zn in MS: 2249 ± 1144 ng/mL; median in KP: 2016 ng/mL, median in MS: 2064 ng/mL) was lower than that reported for Greece (2008; 2990 ± 920 ng/mL) (Leotsinidis et al., 2005), Sweden (2012; 3471 ± 979 ng/mL) (Björklund et al., 2012), and Iran (mean: 1380 ± 1100 ng/mL) (Taravati Javad et al., 2018) and higher than that reported for Italy (2008; concentrations in pooled samples ranged from 705 to 904 ng/mL) (Abballe et al., 2008).

However, it should be noted that the element levels in milk were not normalized according to the water content (not available) and should be considered informative. From the non-normalized levels, we can conclude that the levels of potentially toxic elements do not represent a health risk for the mother-child pairs. Further, the results indicate that most babies are sufficiently supplied with the essential elements studied. A detailed interpretation of trace element levels in a larger population of the national HBM study, including KP and MS, is presented in more detail in Snoj Tratnik et al. (2019).

3.3. Composition and stable isotopes of FAs in human milk

Table S4 and Fig. 2 present the percentages of FAs in maternal milk from mothers in KP and MS. Forty-nine FAs were detected, and the

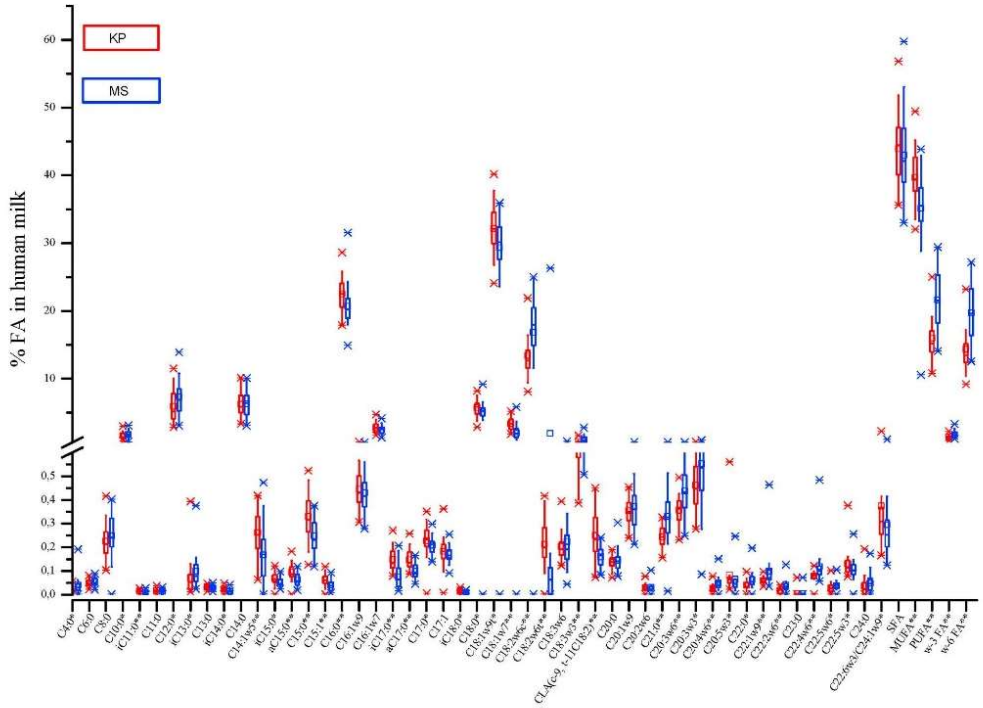


Fig. 2. FA composition of maternal milk and comparison between studied areas – coastal (KP) and inland (MS) (* two groups-KP vs MS are statistically significantly different ($p < 0.05$), ** two groups-KP vs MS are statistically significantly different ($p < 0.001$), Mann-Whitney *U* test).

mean percentages of 35 individual FAs showed a statistically significant difference between the studied areas. In 19 cases, the levels were higher in KP than in MS, including for C20:5ω3 and C22:6ω3/C24:1ω9, which are typically contained in fish (Innis, 2014, 2007; Jensen, 1999). In 16 cases, the mean percentage of FAs was higher in MS than in KP, including PUFAs important (essential) for humans and infant growth, namely, C18:2ω6, C18:3ω3, and C20:4ω6 (Boyer et al., 2005; Harris et al., 1984; Innis, 2014, 2007; Jensen, 1999; Sauerwald et al., 2001). In MS, participants may have eaten more locally produced food based on animal and plant fats rich in these FAs (for instance, corn and sunflower oil with C18:2ω6 and canola oil rich in C18:3ω3) (Francois et al., 1998; Jensen, 1999). Higher consumption of sunflower oil was reported in mothers from MS than in those from KP (55% vs 19%; data not shown); however, this is only a presumption because not all types of animal and plant fats and oils were specified in the questionnaire. Another reason could be the intake of food supplements, although the difference in self-reported intake was not statistically significant between MS and KP (81% *vs* 75%; data not shown). The mean percentages of FAs in the different study areas were statistically significant. Samples from KP had higher percentage of MUFAs, whereas PUFAs, ω-3 FAs, ω-6 FAs, iC13:0/C13:0, ω-6 + ω-3 FAs, ω-6 LCPs, ω-3 LCPs, LCPs, and ω-6/ω-3 LCPs were lower in KP than in MS.

Fig. 3 and Table S5 show the average of $\delta^{13}C_{FA}$ for individual FAs separately for both studied areas and for the total study population. The difference in $\delta^{13}C$ levels of C10:0, C12:0, C14:0, C16:1, C16:0, C18:1ω9c, C22:6ω3, and $\delta^{13}C$ 18:0–16:0 in the study groups was statistically significant. In all seven cases where $\delta^{13}C$ of FA significantly differed between KP and MS (Table S5), $\delta^{13}C$ was higher in KP, indicating a higher proportion of a marine-based diet.

The correlations between the general characteristics of the study population with the composition of FAs and stable isotopes in human milk are presented in summary in Table 1 and in detail in Tables S6 and S7. Maternal age correlated positively with aC17:0 and $\delta^{13}C_{FA}$ of C20:3w-6 and negatively with C18:2ω6, sum of PUFAs, ω-6 FAs, ω-3 FAs, their ratio (ω-6/ω-3), and their sum (ω-6 + ω-3 FAs). The baby's age at the time of sampling correlated positively with iC14:0, iC15:0, aC15:0, (iC15:0 + aC15:0)/C15:0, and $\delta^{13}C_{FA}$ of C15:1 and negatively with C16:1ω9. Pre-pregnancy body mass index correlated positively with C16:0 and C18:0 and negatively with C18:3ω6, C18:3ω3, and C24:0. Education levels correlated positively with aC17:0, C18:1ω7, C18:3ω6, and $\delta^{13}C_{FA}$ of C14:0 and negatively with C22:5ω3 (Table 1). Among these variables, the baby's age on the day of sampling was included in the data mining approach to adjust for the maturity of human milk (described in section 2.5).

Could levels of selected elements, composition, and stable isotopes of FAs in human milk be used as indicators of seafood dietary habits?

Studies suggest that maternal diet is a key factor in determining the composition of FAs in human milk (Jensen, 1999; Kelishadi et al., 2012; Nishimura et al., 2014; Ruan et al., 1995; Sauerwald et al., 2001). As stated in the Introduction, the aim of this study was to test differences in FA composition, $\delta^{13}C_{FA}$ levels, and selected element composition in two population groups with significantly different dietary habits: an inland population (MS) with low seafood consumption and a coastal population (KP) with significantly higher seafood consumption (Tables S1 and S2). In the following subchapters, we first investigate differences in FA composition in human milk in terms of differences between terrestrial-based food and seafood intake, and second, we investigate whether there are differences in FA composition in human milk between different types of seafood and frequency of consumption and how elements typical of seafood intake (As, Hg, and Se) are statistically correlated with each other.

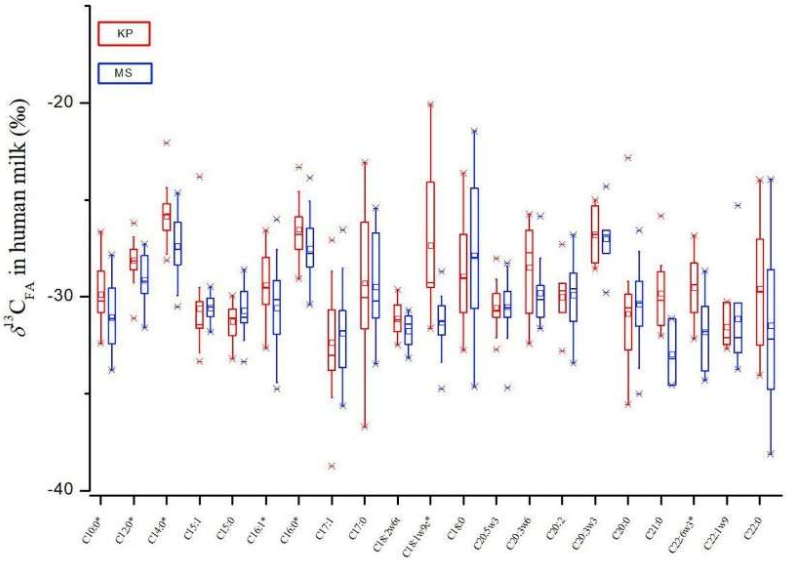


Fig. 3. Stable isotopes of FAs in maternal milk and comparison between studied areas – coastal (KP) and inland (MS). (* two groups-KP vs MS are statistically significantly different ($p < 0.05$), ** two groups-KP vs MS are statistically significantly different ($p < 0.001$), Mann-Whitney *U* test).

Table 1
Summary of statistically significant correlations ($p < 0.05$) between the general characteristics of the study population with FA composition and stable isotopes[a].

Maternal age		Baby's age	
+	–	+	–
aC17:0 $\delta^{13}C_{C20:3\omega\text{-}6}$	C18:2ω6 PUFAs ω-6 FAs ω-3 FAs ω-6/ω-3 FAs ω-6 + ω-3 FAs	iC14:0 iC15:0 aC15:0 (iC15:0 + aC15:0)/C15:0 $\delta^{13}C_{C15:1}$	C16:1ω9
Pre-pregnancy BMI		**Education level**	
+	-	+	-
C16:0 C18:0	C18:3ω6 C18:3ω3 C24:0	aC17:0 C18:1ω7 C18:3ω6 $\delta^{13}C_{C14:0}$	C22:5ω3

[a] For the specific correlations, refer to Tables S6 and S7.

3.3.1. Difference in FA composition between terrestrial-based food and seafood

The questionnaire data in Table S1 confirmed that more fresh-water fish were consumed in MS than in KP and more fresh and frozen seafood were consumed in KP than in MS. A comparison of the FA composition reveals a closer characteristic of maternal milk for higher seafood intake (C20:5ω3, C22:5ω3, and C22:6ω3) in KP than in MS. For different FA groups, statistically significant differences were observed for MUFAs (higher in KP), PUFAs (higher in MS), and ω-3 and ω-6 FAs (both higher in MS), which is inconsistent with the knowledge that ω-3 and PUFAs are typical of seafood. By contrast, ω-6 FAs are typical of vegetable oils and margarines, mayonnaise, and meat. Differences in FA compositions were observed in relation to other terrestrial-based food items; however, a discussion of the same is beyond the scope of this paper and will be presented in a separate paper.

3.3.2. FA composition and intake of different types of seafood (fresh/canned/frozen)

Fish, fish oil, and seafood are known to be good sources of FAs such as ω-3 FAs (especially of 20:5ω3 and 22:6ω3) (Innis, 2014, 2007; Strain et al., 2015). Francois et al. (1998) showed that 20:5ω3 and 22:6ω3 were significantly higher after consuming menhaden oil and remained elevated in human milk longer than did other FAs. The authors also stated that the milk of a lactating mother who consumes fish regularly contains greater amounts of 20:5ω3 and 22:6ω3 for a longer period of time than that of a mother who eats fish only occasionally (Francois et al., 1998). Furthermore, in the Slovenian study 'My-milk', Benedik (2015) found that 22:6ω3 was influenced by fish intake before/during pregnancy and during lactation and with 22:6ω3 supplement intake during pregnancy and lactation.

In the present study, seafood intake reported by participating women was provided for the three basic types: fresh, frozen, and canned seafood. Based on this data, the sum of total seafood intake was calculated. To compare the (1) results of FA composition and stable isotopes in maternal milk with (2) questionnaire-based data on seafood intake, different categorizations of variables of seafood intake were tested. Similar results (correlations) were obtained using different categorizations, and the daily intake estimates were used in further evaluations with basic statistical methods. Different correlations between seafood intake and composition of FAs and their stable isotopes were revealed depending on the type of seafood.

Table S8 shows the correlations between composition and $\delta^{13}C_{FA}$ with dietary daily intake factors of the different types of seafood. Daily fresh seafood intake correlated with 24 individual FAs: 11 negatively and 13 positively. Thirteen of these FAs also correlated with total seafood intake; among these, canned seafood intake correlated only with C22:2ω6. Among groups of FAs, MUFA, PUFA, ω-6, ω-6/ω-3 FA ratio and sum, ω-6 LCP, LCP, and C18:2ω6/C18:3ω3 ratio correlated with fresh seafood daily intake and seafood intake. Higher intake of fresh seafood was associated with significantly higher values of $\delta^{13}C_{FA}$ for C12:0, C14:0, C16:0, C18:1ω9c, and C22:6ω3. These results suggest that during processing (freezing/defrosting, conservation processing) of frozen and canned seafood, FA composition and $\delta^{13}C_{FA}$ can change

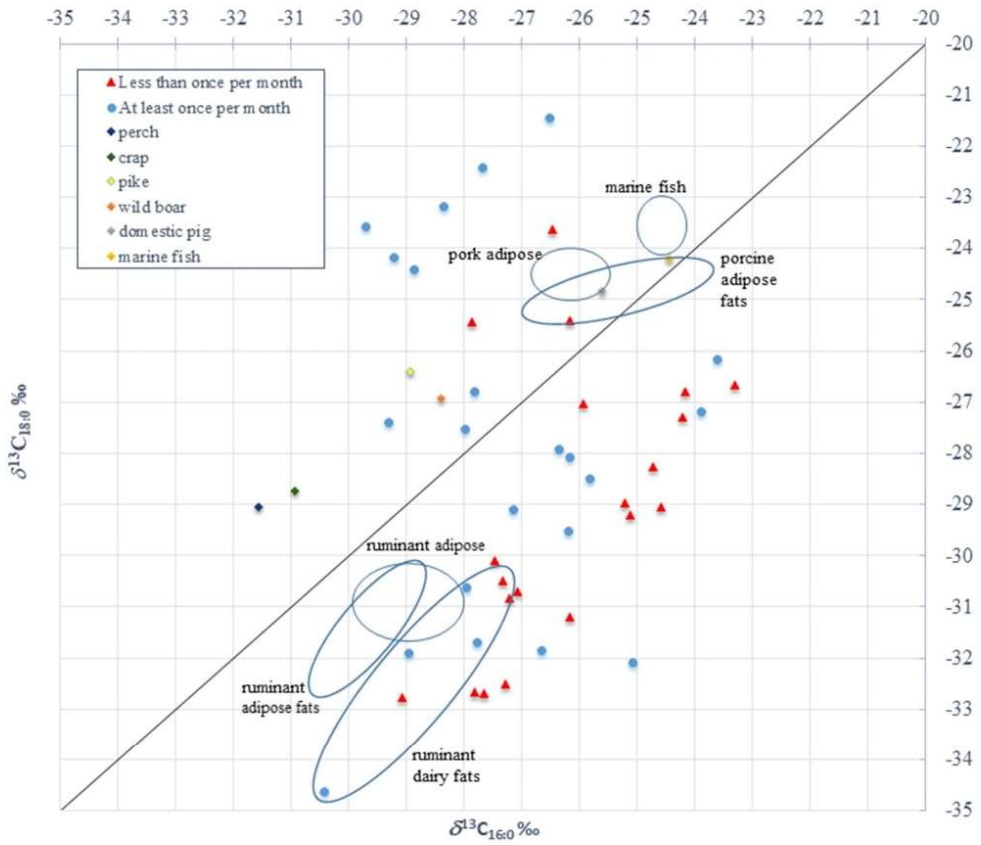


Fig. 4. Carbon isotope composition of $\delta^{13}C_{16:0}$ *vs* $\delta^{13}C_{18:0}$ relationship in human samples of mothers eating fresh seafood less than once per month vs at least once per month. Together with the data for selected commodities (fish, pork, …) from the literature (Regert, 2011; Spangenberg et al., 2006; Steele et al., 2010).

(Table S8). For example, between the 13 individual FAs that positively correlated with fresh seafood intake, only C18:3ω6 positively correlated with canned seafood intake and among the 11 FAs which negatively correlated with fresh seafood intake, only C22:2ω6 correlated negatively with canned food. Among these, iC13:0 correlated positively with frozen seafood. Accordingly, only fresh seafood was considered in further analysis.

The sources of fat in milk can be further evaluated using the $\delta^{13}C_{16:0}$ and $\delta^{13}C_{18:0}$ relationship (Fig. 4). Fat samples of modern animals that have been fed exclusively on C3 forage grasses are also included for comparison. These reference fats include adipose samples of pig, cattle, lamb, deer, and fish; milk fat samples of cow, goat, and sheep are also included (Regert, 2011; Spangenberg et al., 2006; Steele et al., 2010). The $\delta^{13}C$ values of the major fatty acids in oils from C3 plants vary from −36.5 to −27.5‰ and plot near the 1:1 line in the $\delta^{13}C_{16:0}$ vs. $\delta^{13}C_{18:0}$ diagram (Spangenberg and Ogrinc, 2001). It is seen that most of our data plot below the 1:1 line, indicating fats of different origins and mixtures between ruminant (cow) and non-ruminant origin (pork). None of the samples plot in the region of fat originating from marine fish. Thus, it is evident that $\delta^{13}C_{FA}$ values solely cannot be used as an indicator of seafood dietary intake because the mother ate fish only once per month. This consumption is too low to be detected by the isotope composition of relevant FAs.

3.3.3. Levels of trace elements, FA composition, and seafood intake frequency

According to literature, higher seafood intake can increase the ω-3 PUFA content (Chen et al., 1997; Kelishadi et al., 2012; Wu et al., 2010), which was also observed in our study for some ω-3 PUFAs (positive correlation between percentage of C20:5ω3 and C22:5ω3 with fresh seafood intake). As, Hg, and Se levels in blood, hair, and milk samples are also known to be positively associated with seafood consumption (Hughes, 2006; Miklavčič et al., 2013; Reilly, 1996; UNEP Chemicals Branch, 2008). The results of this study are consistent with the literature and show positive correlations between fresh seafood intake and As, Se, and Hg levels in maternal blood (data not shown); As and Hg in maternal milk; and Hg in maternal hair samples (data not shown), indicating that fresh seafood is an important source of As, Se, and Hg (Snoj Tratnik et al., 2019).

In the study population, fresh seafood intake and As and Hg in milk samples correlated positively and significantly with the percentage of FAs: MUFAs (and with Hg and As in milk), iC17:0 (and with As in milk), aC17:0 (and with As in milk), C18:1ω7 (and with As and Hg in milk), C18:3ω6 (and with As in milk), C22:5ω3 (and with As in milk), CLA (and with As in milk), C20:5ω3 (and with As in milk), and C18:2ω6t (and with As in milk). Other FAs (C14:1ω5, iC15:0, aC15:0, C15:0, C15:1, and C18:1ω9) also correlated with fresh seafood intake but not with As and/or Hg in human milk. Some FAs (iC14:0, C16:0, C17:0, iC18:0, and

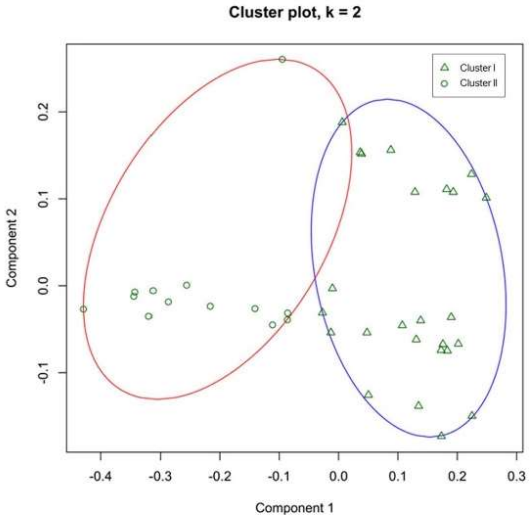


Fig. 5. Clustering of 38 participants described with seven variables for fresh seafood intake. These two components explain 51.9% of the point variability.

C22:6ω3/C24:1ω9) were only marginally ($0.05 < p < 0.1$) positively correlated with fresh seafood intake. Among them, only C22:6ω3/C24:1ω9 correlated with As and/or Hg in human milk, which could be due to unseparated peaks for these two FAs. Related data are presented in Tables S8 and S7.

Among the 19 individual FAs (in case of C22:6ω3 and C24:1ω9, both together) that marginally correlated with daily intake of fresh seafood, nine showed a positive correlation with As and/or Hg. If we consider ω-3 PUFAs, which correlated with fresh seafood intake, then all FAs correlated with As and/or Hg in human milk (Tables S8 and S7).

Furthermore, among $\delta^{13}C_{FA}$ values which (at least marginally) positively correlated with fresh seafood intake (Table S8), three $\delta^{13}C_{FA}$ values also positively correlated with As and/or Hg in human milk (Table S7): C18:1ω9c (and with Hg in milk), C12:0 (and with As in milk), and C14:0 (and with As in milk). This suggests that the composition and stable isotopes of FAs together with the elemental composition in maternal milk represent a good and reliable marker of fresh seafood intake.

For further confirmation of our results, data mining was performed for fresh seafood intake (< 1 per month *vs* at least 1 per month). For classification using the RFE method, the following seven variables were selected (the method and process of selection are described in more detail in Section 2.5): percentage of the FAs iC17:0, C4:0, C18:2ω6t, aC17:0, CLA, C22:4ω6, and $\delta^{13}C_{FA}$ of C18:1ω9c. Using leave-one-out cross validation, the accuracy of distinguishing between fresh seafood intake frequencies was 92%, whereas bootstrapping gave 84% accuracy.

For clustering, we used the same data set of 38 participants described with the same seven variables as in the classification task for fresh seafood intake. We omitted the variable for fresh seafood intake, and by using the Silhouette curve, we estimated that the best splitting would be into two clusters (Fig. S1). The average silhouette width defines how well each instance lies within its cluster. A high average silhouette width indicates good clustering. The curve presents different values of the average silhouette width for different numbers of clusters, from which the optimal number is the one that maximizes the average silhouette over the range of all possible values (Kaufman and Rousseeuw, 1990). By using the PAM method, participants were clustered into two clusters (I: < 1 per month – 1, at least 1 per month – 12; II: < 1 per month – 22, at least 1 per month – 3) (Fig. 5). From this, it is clear that by using these seven variables, we can distinguish between the selected frequencies of fresh seafood intake even if the variable for fresh seafood intake is not included in the clustering.

Among the variables which were selected as potential indicators of fresh seafood intake using the above-mentioned method, C4:0, aC17:0, and iC17:0 are usually present in butter, milk, and other dairy products; CLA is present in dairy products and red meat; C18:2ω6t is present in vegan and vegetarian diets; and C22:4ω6 is present in vegetable oils. $\delta^{13}C_{FA}$ of C18:1ω9c could be linked through food intake as one of the main representatives of MUFA, and it is found in vegetable oils such as olive oils and canola, peanut, hazelnut, almond, and avocado oils. All these variables show an FA profile indicative of fresh seafood intake.

4. Conclusions

The present study's results are unique because only a few studies have reported the composition or/and stable isotopes of FAs and selected elements in human milk worldwide, and the present study is the first to investigate stable isotopes of FAs in human milk in Slovenia. This study showed that human milk samples from coastal (KP) and inland (MS) areas had different elemental and FA compositions and different FA stable isotope profiles, which is indicative of the different dietary habits and lifestyles in the two distinct study areas of Slovenia. Therefore, the FA composition and its $\delta^{13}C_{FA}$ values together with the elemental composition in maternal milk could be used as a marker of fresh seafood intake.

Data mining approaches confirmed that using the classifier for fresh seafood intake based on the seven variables, including elemental composition, levels of FAs, and their stable isotopes in milk, enables distinguishing between seafood consumption less than once per month *vs* at least once per month fresh with 84% accuracy. Clustering could be used to divide the data sets into two with 90% accuracy. A clustering approach based on the percentage of the FAs iC17:0, C4:0, C18:2ω6t, aC17:0, CLA, C22:4ω6, and $\delta^{13}C$ of C18:1ω9c is an accurate indicator to distinguish between higher and lower fresh seafood intake. This confirmed our hypothesis that stable isotope analysis in combination with elemental analysis is an important tool to distinguish between diets, particularly for seafood. These findings suggest that in human-related studies related to the risks and benefits of seafood consumption, the analyses of FAs, their stable isotopes, and elemental compositions can significantly improve the information on seafood consumption.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2019.108820.

Declaration of interest

None.

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Supplements: Selected elements and fatty acid composition in human milk as indicators of seafood dietary habits

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Table S1. General characteristics of the studied population

	KOPER (KP)					POMURJE (MS)					Total				
	N	Mean	SD	Min	Max	N	Mean	SD	Min	Max	N	Mean	SD	Min	Max
Maternal age [years]	36	29.2	3.72	23.0	36.0	38	28.8	3.52	20.0	38.0	74	29.0	3.60	20.0	38.0
Pre-pregnancy BMI [kg/m^2]	35	23.3	3.63	17.9	35.2	38	23.3	4.26	17.7	35.1	73	23.3	3.94	17.7	35.2
N of amalgam filings	28	3.96	3.80	0	12	28	3.96	2.73	0	10	56	3.96	3.28	0	12
Daily intake of water [L]	35	2.21	0.67	1.5	4.0	38	2.00	0.58	1.00	3.00	73	2.10	0.63	1.0	4.0
Estimated gestational age	33	39.4	1.75	34.3	41.6	37	39.8	0.78	37.4	41.0	70	39.6	1.33	34.3	41.6
Birth weight [kg]	36	3.39	0.52	2.3	4.4	38	3.26	0.38	2.28	3.95	74	3.33	0.46	2.3	4.4
Birth length [cm]	36	53.6	2.46	47.0	59.0	38	50.7	1.43	48.0	54.0	74	52.1**	2.46	47.0	59.0
Baby weight on the sampling day [kg]	31	4.29	0.76	3.10	6.30	33	4.51	0.62	2.79	5.50	64	4.41	0.69	2.79	6.30
Baby age on the day of sampling [month]	36	1.76	0.19	1.43	2.47	38	1.70	0.25	1.30	2.43	74	1.73	0.23	1.30	2.47
Daily intake factor[#]															
Fresh-water fish	36	0.02	0.02	0.00	0.07	38	0.05	0.05	0.00	0.14	74	0.03*	0.04	0.00	0.14
Fresh seafood	35	0.09	0.05	0.00	0.14	35	0.04	0.08	0.00	0.43	70	0.07*	0.07	0.00	0.43
Frozen seafood	36	0.04	0.04	0.00	0.14	38	0.06	0.04	0.00	0.14	74	0.05*	0.04	0.00	0.14
Canned seafood	35	0.09	0.18	0.00	1.00	37	0.08	0.09	0.00	0.43	72	0.08	0.14	0.00	1.00
Seafood all	34	0.22	0.19	0.06	1.14	35	0.18	0.15	0.02	0.71	69	0.20	0.17	0.02	1.14

* two groups-KP *vs* MS were statistically significantly different ($p < 0.05$), ** two groups-KP *vs* MS were statistically significantly different ($p < 0.001$), Mann-Whitney U test.

N – number of cases, SD – standard deviation, Min – minimum, Max – maximum, BMI – Body mass index.

[#]Food intake categories from the questionnaire were converted to continuous estimated daily intake factors (for never – 0, less than once per month – 0.02, 1-3 times per month – 0.07, once time per week – 0.14, 2-4 times per week – 0.43, 5-6 times per week – 0.79, once per day – 1, more than 1 per day – 2.5) (Valent et al., 2013).

Table S2. General categorical characteristics of the studied population

		KOPER (KP) (N = 36)	POMURJE (MS) (N = 38)	Total (N = 74)
		%	%	%
Education	Primary	12	5	9
	Secondary	33	49	41
	University	55	46	50
Smoking	No	89	84	87
	Yes	11	16	14
Passive smoking	No	86	68	77
	Yes	14	32	23
Smoking during pregnancy	No	100	97	99
	Yes	0	3	1
Baby's sex	Male	50	55	53
	Female	50	45	47
Birth weight	<3.1 kg	31	32	31
	3.11–3.66 kg	33	47	41
	≥3.67 kg	36	21	28
Baby's feeding*	Exclusively breastfeeding	72	47	60
	Between only and prevailing breastfeeding	0	3	1
	Prevailing breastfeeding	28	24	26
	Breastfeeding is only addition	0	26	14

* The pattern of responses in the two areas (KP and MS) was statistically significantly different, ($p < 0.05$, Fisher's Exact test or Pearson chi-square test). N – Number of cases, % - percentage of cases.

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Table S3. Levels of selected elements in human milk and a comparison between the two study areas and their respective literature ranges

	KOPER (KP) (N = 36)					POMURJE (MS) (N = 38)					Total (N = 74)							
	Mean (ng/mL)	SD (ng/mL)	GM (ng/mL)	Min (ng/mL)	Max (ng/mL)	Mean (ng/mL)	SD (ng/mL)	GM (ng/mL)	Min (ng/mL)	Max (ng/mL)	Mean (ng/mL)	SD (ng/mL)	GM (ng/mL)	Min (ng/mL)	Max (ng/mL)	LR# (ng/mL)	% < LR	% > LR
Cu	318	60.4	312	167	430	355	120.9	336	179	621	337	97.5	324	167	621	200 - 400	4.05	21.6
Zn	1935	670	1817	710	3663	2249	1144	1991	610	5860	2096	950	1904	610	5860	1000 – 2000	6.76	51.4
As	0.57	0.845	0.34	0.08	3.70	0.17	0.143	0.14	0.03	0.91	0.36**	0.629	0.21	0.03	3.70	< 3	-	2.70
Se	12.3	2.60	12.0	7.39	18.6	12.4	3.97	11.8	6.42	26.7	12.3	3.35	11.9	6.42	26.7	10 – 25	21.6	1.35
Cd	0.06	0.028	0.05	0.05	0.18	0.07	0.039	0.06	0.05	0.22	0.06	0.035	0.06	0.05	0.22	< 1	-	0
Pb	0.40	0.291	0.30	0.10	1.07	0.36	0.500	0.23	0.10	2.97	0.38	0.409	0.26	0.10	2.97	< 5	-	0
Hg	0.27	0.203	0.20	0.03	0.83	0.20	0.223	0.11	0.001	1.01	0.23*	0.215	0.15	0.001	1.01	< 3	-	0

* two groups-KP *vs* MS were statistically significantly different ($p < 0.05$), ** two groups-KP *vs* MS were statistically significantly different ($p < 0.001$), Mann-Whitney U test

N – number of cases, SD – standard deviation, Min – minimum, Max – maximum; GM – geometric mean, LR-literature range.

#(Iyengar, 1998)

Table S4. FA composition of human milk and comparison between study areas

	KOPER (KP) (N = 36)				POMURJE (MS) (N = 38)				Total (N = 74)				
% FA in maternal milk	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	N < LOD
C4:0	0.02	0.01	0.00	0.06	0.04	0.04	0.00	0.19	0.03*	0.03	0.00	0.19	4
C6:0	0.05	0.01	0.02	0.08	0.05	0.02	0.02	0.09	0.05	0.02	0.02	0.09	0
C8:0	0.22	0.07	0.10	0.42	0.25	0.09	0.00	0.40	0.24	0.08	0.00	0.42	1
C10:0	1.55	0.53	0.84	3.00	1.79	0.54	0.72	3.12	1.67*	0.54	0.72	3.12	0
iC11:0	0.01	0.01	0.00	0.03	0.01	0.01	0.00	0.03	0.01**	0.01	0.00	0.03	18
C11:0	0.02	0.01	0.00	0.04	0.02	0.01	0.00	0.03	0.02	0.01	0.00	0.04	6
C12:0	5.79	2.40	2.64	11.5	7.32	2.69	3.10	13.9	6.58*	2.65	2.64	13.9	0
iC13:0	0.07	0.07	0.01	0.39	0.10	0.07	0.02	0.38	0.08*	0.07	0.01	0.39	0
C13:0	0.03	0.01	0.01	0.05	0.03	0.01	0.01	0.05	0.03	0.01	0.01	0.05	0
iC14:0	0.02	0.01	0.00	0.05	0.01	0.01	0.00	0.04	0.02*	0.01	0.00	0.05	14
C14:0	6.20	1.78	3.36	10.1	6.27	1.92	3.11	10.1	6.24	1.84	3.11	10.1	0
C14:1ω5	0.26	0.09	0.06	0.42	0.17	0.11	0.00	0.47	0.21**	0.11	0.00	0.47	2
iC15:0	0.07	0.02	0.00	0.12	0.05	0.02	0.02	0.10	0.06*	0.02	0.00	0.12	1
aC15:0	0.10	0.04	0.00	0.18	0.06	0.03	0.02	0.12	0.08**	0.04	0.00	0.18	1
C15:0	0.33	0.09	0.13	0.52	0.25	0.07	0.12	0.38	0.29**	0.09	0.12	0.52	0
C15:1	0.06	0.03	0.00	0.12	0.04	0.02	0.01	0.09	0.05**	0.03	0.00	0.12	1
C16:0	22.6	2.20	17.9	28.6	20.7	2.67	14.9	31.5	21.6**	2.62	14.9	31.5	0
C16:1ω9	0.45	0.09	0.31	0.69	0.43	0.09	0.28	0.64	0.44	0.09	0.28	0.69	0
C16:1ω7	2.84	0.76	1.68	4.73	2.50	0.66	1.31	4.14	2.67	0.73	1.31	4.73	0
iC17:0	0.15	0.05	0.08	0.27	0.07	0.05	0.02	0.21	0.11**	0.06	0.02	0.27	0
aC17:0	0.15	0.04	0.09	0.26	0.10	0.03	0.05	0.17	0.12**	0.04	0.05	0.26	0
C17:0	0.23	0.07	0.01	0.35	0.21	0.04	0.14	0.30	0.22*	0.06	0.01	0.35	0
C17:1	0.19	0.06	0.01	0.36	0.17	0.03	0.09	0.25	0.18	0.05	0.01	0.36	0
iC18:0	0.02	0.01	0.00	0.03	0.01	0.01	0.00	0.02	0.02*	0.01	0.00	0.03	21
C18:0	5.70	1.11	2.88	8.24	5.05	1.59	0.00	9.21	5.37*	1.40	0.00	9.21	1
C18:1ω9	32.2	3.44	24.1	40.2	29.3	5.83	0.00	35.9	30.7*	4.99	0.00	40.2	1
C18:1ω7	3.47	0.93	1.85	5.23	2.01	1.38	0.00	5.83	2.72**	1.39	0.00	5.83	7
C18:2ω6c	13.4	3.00	8.09	21.9	16.8	6.07	0.00	25.0	15.1**	5.10	0.00	25.0	3
C18:2ω6t	0.22	0.10	0.00	0.42	1.97	6.61	0.00	26.3	1.12**	4.79	0.00	26.3	15
C18:2ω6(c+t)	13.6	2.99	8.42	22.2	18.8	3.83	11.7	26.3	16.3**	4.31	8.42	26.3	0
C18:3ω6	0.21	0.06	0.12	0.39	0.22	0.13	0.04	0.77	0.22	0.10	0.04	0.77	0
C18:3ω3	0.71	0.26	0.39	1.62	1.01	0.49	0.51	2.77	0.86**	0.42	0.39	2.77	0
CLA(c-9, t-11C18:2)	0.25	0.08	0.07	0.45	0.16	0.04	0.08	0.24	0.20**	0.08	0.07	0.45	0
C20:0	0.14	0.03	0.07	0.19	0.14	0.04	0.08	0.30	0.14	0.04	0.07	0.30	0
C20:1ω9	0.35	0.05	0.24	0.45	0.37	0.10	0.21	0.65	0.36	0.08	0.21	0.65	0
C20:2ω6	0.03	0.02	0.00	0.08	0.03	0.02	0.00	0.10	0.03	0.02	0.00	0.10	7
C21:0	0.24	0.05	0.16	0.33	0.33	0.14	0.02	0.68	0.29**	0.11	0.02	0.68	0
C20:3ω6	0.36	0.06	0.23	0.50	0.44	0.10	0.25	0.67	0.40**	0.09	0.23	0.67	0
C20:3ω3	0.46	0.09	0.28	0.69	0.55	0.17	0.09	0.99	0.51*	0.14	0.09	0.99	0
C20:4ω6	0.03	0.01	0.00	0.08	0.05	0.03	0.00	0.15	0.04**	0.03	0.00	0.15	6
C20:5ω3	0.08	0.09	0.02	0.56	0.06	0.06	0.00	0.25	0.07*	0.08	0.00	0.56	1

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	KOPER (KP) (N = 36)				POMURJE (MS) (N = 38)				Total (N = 74)				
% FA in maternal milk	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	N < LOD
C22:0	0.04	0.02	0.00	0.10	0.06	0.04	0.00	0.20	0.05*	0.04	0.00	0.20	6
C22:1ω9	0.06	0.01	0.04	0.10	0.10	0.08	0.04	0.46	0.08**	0.06	0.04	0.46	0
C22:2ω6	0.02	0.01	0.00	0.04	0.04	0.03	0.00	0.13	0.03**	0.03	0.00	0.13	13
C23:0	0.00	0.00	0.00	0.03	0.00	0.01	0.00	0.07	0.00	0.01	0.00	0.07	69
C22:4ω6	0.08	0.02	0.00	0.12	0.12	0.07	0.06	0.48	0.10**	0.06	0.00	0.48	1
C22:5ω6	0.03	0.02	0.00	0.10	0.04	0.02	0.00	0.11	0.03*	0.02	0.00	0.11	14
C22:5ω3	0.13	0.06	0.08	0.38	0.11	0.04	0.00	0.26	0.12*	0.05	0.00	0.38	1
C24:0	0.05	0.06	0.00	0.19	0.05	0.04	0.00	0.17	0.05	0.05	0.00	0.19	21
C22:6ω3/C24:1ω9	0.37	0.33	0.17	2.25	0.30	0.18	0.12	1.07	0.33*	0.27	0.12	2.25	0
SFA	43.8	4.75	35.6	56.9	43.0	5.92	33.0	59.8	43.4	5.36	33.0	59.8	-
MUFA	39.9	3.93	32.1	49.5	35.1	5.55	10.6	43.9	37.4**	5.36	10.6	49.5	-
PUFA	16.0	3.13	10.8	25.0	21.6	4.09	14.1	29.4	18.9**	4.61	10.8	29.4	-
ω-3 FA	1.38	0.33	0.89	2.24	1.74	0.53	1.15	3.32	1.57**	0.47	0.89	3.32	-
ω-6 FA	14.3	3.03	9.23	23.2	19.7	3.93	12.6	27.2	17.1**	4.42	9.23	27.2	-
ω-6/ω-3 FA	10.7	2.54	5.69	16.0	12.0	3.32	4.13	20.1	11.4	3.02	4.13	20.1	-
iC13:0/C13:0	2.42	2.55	0.47	15.1	3.52	2.40	0.80	11.6	2.98*	2.52	0.47	15.1	-
(iC15:0+aC15:0)/C15:0	0.48	0.10	0.03	0.65	0.45	0.07	0.33	0.59	0.47	0.09	0.03	0.65	-
ω-6 + ω-3 FA	15.7	3.15	10.5	24.7	21.5	4.10	13.8	29.2	18.7**	4.65	10.5	29.2	-
ω-6 LCP	0.54	0.10	0.31	0.86	0.71	0.19	0.41	1.33	0.63**	0.17	0.31	1.33	-
ω-3 LCP	0.68	0.20	0.42	1.63	0.73	0.21	0.29	1.26	0.70	0.20	0.29	1.63	-
LCP	1.22	0.27	0.83	2.49	1.44	0.35	0.75	2.59	1.33**	0.33	0.75	2.59	-
C18:2ω6 / C18:3ω3	20.7	6.26	7.19	35.2	21.3	7.66	4.74	44.2	21.0	6.97	4.74	44.2	-
ω-6 / ω-3 LCP	0.83	0.17	0.43	1.27	1.04	0.44	0.54	3.38	0.94*	0.35	0.43	3.38	-
* two groups-KP *vs* MS were statistically significantly different ($p < 0.05$). ** two groups-KP *vs* MS were statistically significantly different ($p < 0.001$). Mann-Whitney U test. ω-6 LCP = C22:5ω6; C22:4ω6; C22:2ω6; C20:4ω6; C20:3ω6; C20:2ω6. ω-3 LCP = C22:5ω3, C20:5ω3; C20:3ω3. LPC: ω-6 LCP + ω-3 LCP. C18:2ω6 = C18:2ω6 (t+c).														
N – number of cases, SD – standard deviation, Min – minimum, Max – maximum, LOD – limit of detection.													

Table S5. Stable isotopes of FAs in human milk and comparison between study areas

	KOPER (KP)					POMURJE (MS)					Total				
	N	Mean	SD	Min	Max	N	Mean	SD	Min	Max	N	Mean	SD	Min	Max
δ^{13}C C10:0	32	-29.9	1.44	-32.4	-26.7	38	-31.1	1.61	-33.8	-27.8	70	-30.5*	1.63	-33.8	-26.7
δ^{13}C C12:0	36	-28.1	0.93	-31.1	-26.2	38	-29.1	1.20	-31.6	-27.3	74	-28.7**	1.18	-31.6	-26.2
δ^{13}C C14:0	36	-25.9	1.28	-28.1	-22.1	38	-27.4	1.46	-30.5	-24.7	74	-26.6**	1.57	-30.5	-22.1
δ^{13}C C15:1	9	-30.6	2.80	-33.3	-23.8	6	-30.6	0.81	-31.8	-29.5	15	-30.6	2.17	-33.3	-23.8
δ^{13}C C15:0	19	-31.3	0.92	-33.2	-30.0	13	-30.7	1.38	-33.4	-28.6	32	-31.1	1.14	-33.4	-28.6
δ^{13}C C16:1	36	-29.4	1.51	-32.7	-26.6	37	-30.6	2.07	-34.8	-26.0	73	-30.0*	1.89	-34.8	-26.0
δ^{13}C C16:0	36	-26.5	1.40	-29.1	-23.3	37	-27.5	1.45	-30.4	-23.9	73	-27.0*	1.50	-30.4	-23.3
δ^{13}C C17:1	23	-32.4	2.47	-38.8	-27.1	15	-31.9	2.58	-35.7	-26.6	38	-32.2	2.49	-38.8	-26.6
δ^{13}C C17:0	23	-29.3	3.55	-36.7	-23.1	16	-29.5	2.45	-33.5	-25.4	39	-29.4	3.11	-36.7	-23.1
δ^{13}C C18:2ω6t	10	-31.1	0.89	-32.5	-29.7	13	-31.8	0.91	-33.2	-30.7	23	-31.5	0.94	-33.2	-29.7
δ^{13}C C18:1ω9c	13	-27.4	3.88	-31.6	-20.1	28	-31.3	1.20	-34.8	-28.7	41	-30.1**	3.00	-34.8	-20.1
δ^{13}C C18:0	21	-28.9	2.72	-32.8	-23.6	22	-27.9	3.54	-34.6	-21.5	43	-28.4	3.18	-34.6	-21.5
δ^{13}C C20:5ω3	25	-30.6	1.08	-32.7	-28.0	16	-30.5	1.52	-34.7	-28.3	41	-30.5	1.25	-34.7	-28.0
δ^{13}C C20:3ω6	11	-28.5	2.28	-32.4	-25.8	14	-29.8	1.69	-31.6	-25.9	25	-29.2	2.04	-32.4	-25.8
δ^{13}C C20:2	11	-30.0	1.75	-32.8	-27.3	10	-29.9	1.88	-33.4	-26.8	21	-30.0	1.76	-33.4	-26.8
δ^{13}C C20:3ω3	4	-26.8	1.74	-28.6	-25.0	6	-27.0	1.79	-29.8	-24.3	10	-29.0	1.67	-29.8	-24.3
δ^{13}C C20:0	24	-30.9	2.73	-35.6	-22.8	18	-30.4	2.25	-35.0	-26.6	42	-30.7	2.52	-35.6	-22.8
δ^{13}C C21:0	8	-29.8	2.05	-32.0	-25.8	3	-33.0	1.73	-34.6	-31.1	11	-30.7	2.38	-34.6	-25.8
δ^{13}C C22:6ω3	19	-29.5	1.53	-32.2	-26.9	11	-31.8	1.97	-34.3	-28.7	30	-30.4*	2.02	-34.3	-26.9
δ^{13}C C22:1ω9	5	-31.6	1.19	-32.7	-30.3	9	-31.1	2.53	-33.8	-25.3	14	-31.3	2.10	-33.8	-25.3
δ^{13}C C22:0	19	-29.6	3.32	-34.1	-24.0	14	-31.5	4.07	-38.1	-23.9	33	-30.4	3.72	-38.1	-23.9
δ^{13}C C18:0 - δ^{13}C C16:0	21	-2.83	2.27	-5.22	2.83	21	-0.29	4.06	-7.00	6.14	42	-1.56*	3.49	-7.00	6.14
* two groups-KP *vs* MS were statistically significantly different ($p < 0.05$). ** two groups-KP *vs* MS were statistically significantly different ($p < 0.001$). Mann-Whitney U test															
N – number of detected cases. SD – standard deviation. Min – minimum. Max – maximum															

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Table S6. Correlations of maternal lifestyle with composition and stable isotopes of FAs

Spearman correlations		Maternal age	Pre-pregnancy BMI	Education levels
C4:0	r_s	-.012	.001	-.080
	N	74	73	70
C6:0	r_s	-.182	-.261*	-.005
	N	74	73	70
C8:0	r_s	.053	-.080	-.001
	N	74	73	70
C10:0	r_s	.029	-.155	-.014
	N	74	73	70
iC11:0	r_s	-.056	.061	.067
	N	74	73	70
C11:0	r_s	-.072	.023	-.056
	N	74	73	70
C12:0	r_s	.092	-.052	-.076
	N	74	73	70
iC13:0	r_s	-.150	-.123	-.116
	N	74	73	70
C13:0	r_s	.184	-.040	.070
	N	74	73	70
iC14:0	r_s	.122	.049	.142
	N	74	73	70
C14:0	r_s	.178	.032	.013
	N	74	73	70
C14:1ω5	r_s	.152	-.062	.138
	N	74	73	70
iC15:0	r_s	.183	-.045	.166
	N	74	73	70
aC15:0	r_s	.198	-.009	.196
	N	74	73	70
C15:0	r_s	.223	.035	.188
	N	74	73	70
C15:1	r_s	.155	.007	.084
	N	74	73	70
C16:0	r_s	.075	.392**	-.107
	N	74	73	70
C16:1ω9	r_s	.135	-.030	-.032
	N	74	73	70
C16:1ω7	r_s	.032	.001	-.081
	N	74	73	70
iC17:0	r_s	.208	-.148	.207
	N	74	73	70
aC17:0	r_s	.249*	-.041	.241*
	N	74	73	70
C17:0	r_s	.174	.137	.128
	N	74	73	70
C17:1	r_s	.100	.016	.049
	N	74	73	70
iC18:0	r_s	.129	.098	.105
	N	74	73	70
C18:0	r_s	.007	.275*	-.072
	N	74	73	70
C18:1ω9c/9t	r_s	-.023	.089	-.052
	N	74	73	70
C18:1ω7	r_s	.161	.032	.245*
	N	74	73	70
C18:2ω6c	r_s	-.187	-.222	.134
	N	74	73	70
C18:2ω6t	r_s	.008	.129	.019
	N	74	73	70
C18:2ω6(c+t)	r_s	-.277*	-.201	.022
	N	74	73	70
C18:3ω6	r_s	.095	-.253*	.355**
	N	74	73	70
C18:3ω3	r_s	-.178	-.254*	.083
	N	74	73	70
CLA(c-9, t-11C18:2)	r_s	.192	.005	.154

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Spearman correlations		Maternal age	Pre-pregnancy BMI	Education levels
	N	74	73	70
C20:0	r_s	-.028	.053	-.166
	N	74	73	70
C20:1ω9	r_s	-.032	.153	-.192
	N	74	73	70
C20:2ω6	r_s	.062	.000	-.030
	N	74	73	70
C21:0	r_s	-.195	.065	-.004
	N	74	73	70
C20:3ω6	r_s	.011	.070	-.069
	N	74	73	70
C20:3ω3	r_s	.021	.013	-.134
	N	74	73	70
C20:4ω6	r_s	-.131	-.023	.040
	N	74	73	70
C20:5ω3	r_s	.154	-.041	.097
	N	74	73	70
C22:0	r_s	-.112	-.094	-.034
	N	74	73	70
C22:1ω9	r_s	-.001	-.030	.005
	N	74	73	70
C22:2ω6	r_s	-.133	.012	-.164
	N	74	73	70
C23:0	r_s	.018	.186	-.121
	N	74	73	70
C22:4ω6	r_s	-.037	.059	-.141
	N	74	73	70
C22:5ω6	r_s	-.116	.091	-.311**
	N	74	73	70
C22:5ω3	r_s	.219	-.147	.009
	N	74	73	70
C24:0	r_s	-.024	-.300**	-.092
	N	74	73	70
C22:6ω3/C24:1ω9	r_s	.224	.129	.014
	N	74	73	70
SFA	r_s	.145	.188	-.073
	N	74	73	70
MUFA	r_s	.083	.076	.055
	N	74	73	70
PUFA	r_s	-.264*	-.197	.034
	N	74	73	70
ω-3 FA	r_s	-.044	-.148	.029
	N	74	73	70
ω-6 FA	r_s	-.274*	-.192	.018
	N	74	73	70
ω-6/ ω-3 FA	r_s	-.304**	-.001	-.056
	N	74	73	70
iC13:0/C13:0	r_s	-.191	-.070	-.143
	N	74	73	70
(iC15:0+aC15:0)/C15:0	r_s	.018	-.087	.049
	N	74	73	70
ω-6 + ω-3 FA	r_s	-.261*	-.201	.033
	N	74	73	70
ω-6 LCP	r_s	-.023	.045	-.140
	N	74	73	70
ω-3 LCP	r_s	.124	-.030	-.092
	N	74	73	70
LCP	r_s	.049	-.032	-.126
	N	74	73	70
C18:2ω6 / C18:3ω3	r_s	-.090	.215	-.108
	N	74	73	70
ω-6 / ω-3 LCP	r_s	-.133	.063	-.069
	N	74	73	70
δ^{13}C C10:0	r_s	-.037	.129	.142
	N	70	69	66

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Spearman correlations		Maternal age	Pre-pregnancy BMI	Education levels
δ^{13}C C12:0	r_s	.027	.060	.132
	N	74	73	70
δ^{13}C C14:0	r_s	.020	.118	.240*
	N	74	73	70
δ^{13}C C15:1	r_s	.099	.177	.202
	N	15	15	15
δ^{13}C C15:0	r_s	.219	.308	.010
	N	32	31	30
δ^{13}C C16:1	r_s	-.096	.079	.163
	N	73	72	69
δ^{13}C C16:0	r_s	.110	.170	.153
	N	73	72	69
δ^{13}C C17:1	r_s	.057	.282	-.064
	N	38	37	36
δ^{13}C C17:0	r_s	-.121	.253	.005
	N	39	38	37
δ^{13}C C18:2ω6t	r_s	.173	.240	.044
	N	23	23	22
δ^{13}C C18:1ω9c	r_s	.245	.224	.002
	N	41	41	39
δ^{13}C C18:0	r_s	.296	-.253	.187
	N	43	42	41
δ^{13}C C20:5ω3	r_s	-.253	-.099	-.137
	N	41	40	39
δ^{13}C C20:3ω6	r_s	.487*	.265	.234
	N	25	25	24
δ^{13}C C20:2	r_s	-.195	.164	-.375
	N	21	21	20
δ^{13}C C20:3ω3	r_s	.125	.355	-.378
	N	10	10	9
δ^{13}C C20:0	r_s	-.086	.191	.046
	N	42	41	40
δ^{13}C C21:0	r_s	-.046	-.114	-.046
	N	11	10	10
δ^{13}C C22:6ω3	r_s	.263	-.017	.248
	N	30	29	28
δ^{13}C C22:1ω9	r_s	-.288	-.358	.171
	N	14	14	13
δ^{13}C C22:0	r_s	.074	-.004	.155
	N	33	32	31

r_s – Spearman correlation coefficient. N – number. * two variables correlated statistically significantly ($p < 0.05$). ** two variables correlated statistically significantly ($p < 0.001$).

Table S7. Correlations between composition and stable isotopes of FAs in human milk and elemental composition and age of babies on the day of sampling

Correlations		Baby`s age	Log10 Cu	Log10 Zn	Log10 As	Log10 Se	Log10 Cd	Log10 Pb	Log10 Hg
Log10 C4:0	r_p	-.019	-.254*	-.193	-.090	-.001	-.184	-.186	-.083
	N	74	74	74	74	74	74	74	74
C6:0	r_p	.068	-.080	-.166	.016	-.150	.092	-.135	.092
	N	74	74	74	74	74	74	74	74
C8:0	r_p	.099	-.046	-.170	-.044	.128	.050	-.267*	-.027
	N	74	74	74	74	74	74	74	74
C10:0	r_p	.032	-.066	-.232*	-.069	.203	.196	-.216	.054
	N	74	74	74	74	74	74	74	74
iC11:0	r_s	.215	-.061	-.106	.106	.030	-.093	-.139	.001
	N	74	74	74	74	74	74	74	74
C11:0	r_p	.082	-.058	-.042	.064	-.212	-.206	.070	-.229*
	N	74	74	74	74	74	74	74	74
C12:0	r_p	.086	-.124	-.233*	-.134	.208	.277*	-.259*	.072
	N	74	74	74	74	74	74	74	74
Log10 iC13:0	r_p	.013	-.014	-.117	-.120	-.141	.010	-.120	-.004
	N	74	74	74	74	74	74	74	74
C13:0	r_p	.179	-.160	-.092	-.030	.011	.002	-.152	-.216
	N	74	74	74	74	74	74	74	74

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Correlations		Baby's age	Log10 Cu	Log10 Zn	Log10 As	Log10 Se	Log10 Cd	Log10 Pb	Log10 Hg
iC14:0	r_s	.273*	-.050	.115	.110	.039	-.163	-.061	-.063
	N	74	74	74	74	74	74	74	74
C14:0	r_p	.196	-.191	-.199	-.015	.153	.153	-.274*	.079
	N	74	74	74	74	74	74	74	74
C14:1ω5	r_p	.144	-.033	-.068	.163	.166	.048	.020	.067
	N	74	74	74	74	74	74	74	74
iC15:0	r_p	.244*	-.100	-.085	.134	-.042	-.066	-.018	-.070
	N	74	74	74	74	74	74	74	74
aC15:0	r_p	.271*	-.139	-.078	.150	-.052	-.087	-.005	-.045
	N	74	74	74	74	74	74	74	74
C15:0	r_p	.191	-.122	.006	.183	-.028	-.082	.019	-.063
	N	74	74	74	74	74	74	74	74
C15:1	r_p	.141	-.262*	-.114	.064	-.049	-.073	.123	-.053
	N	74	74	74	74	74	74	74	74
Log10 C16:0	r_p	-.037	-.067	.118	.109	-.171	-.163	.050	-.124
	N	74	74	74	74	74	74	74	74
C16:1ω9	r_p	-.266*	-.145	.208	.185	.052	-.080	.175	-.063
	N	74	74	74	74	74	74	74	74
C16:1ω7	r_p	-.132	-.094	.099	.129	.067	-.020	.137	.018
	N	74	74	74	74	74	74	74	74
iC17:0	r_p	.107	-.187	-.133	.367**	-.052	-.030	.081	.185
	N	74	74	74	74	74	74	74	74
aC17:0	r_p	.167	-.212	-.037	.263*	-.105	-.117	.076	-.014
	N	74	74	74	74	74	74	74	74
C17:0	r_p	.056	-.273*	-.068	-.057	-.182	-.095	.254*	-.159
	N	74	74	74	74	74	74	74	74
C17:1	r_s	.089	-.149	.146	.134	-.011	-.218	.221	-.177
	N	74	74	74	74	74	74	74	74
iC18:0	r_s	.197	-.073	-.068	.197	.014	-.139	.038	-.035
	N	74	74	74	74	74	74	74	74
C18:0	r_p	.093	-.033	-.131	.064	-.019	-.184	.035	-.134
	N	74	74	74	74	74	74	74	74
C18:1ω9c/9t	r_s	-.021	.127	.154	.185	-.008	-.212	.088	.106
	N	74	74	74	74	74	74	74	74
C18:1ω7	r_p	.014	-.221	-.166	.388**	.002	.009	.232*	.240*
	N	74	74	74	74	74	74	74	74
C18:2ω6c	r_p	-.190	.091	-.089	-.185	-.064	.137	-.029	.054
	N	74	74	74	74	74	74	74	74
C18:2ω6t	r_s	.188	-.113	.011	.251*	-.039	-.088	.149	.113
	N	74	74	74	74	74	74	74	74
C18:2ω6(c+t)	r_p	-.130	.069	.031	-.320**	-.164	.068	-.016	-.217
	N	74	74	74	74	74	74	74	74
C18:3ω6	r_p	.036	.029	.017	.007	-.146	.137	.072	-.106
	N	74	74	74	74	74	74	74	74
Log10 C18:3ω3	r_p	.018	.011	.066	-.263*	-.147	-.081	-.055	-.127
	N	74	74	74	74	74	74	74	74
Log10 CLA(c-9. t-11C18:2)	r_p	.152	-.213	-.071	.229*	-.023	-.087	.099	.023
	N	74	74	74	74	74	74	74	74
C20:0	r_p	.098	-.110	-.058	-.149	-.345**	-.083	.060	-.208
	N	74	74	74	74	74	74	74	74
C20:1ω9	r_p	-.191	-.176	.124	.073	.020	-.190	.183	-.155
	N	74	74	74	74	74	74	74	74
C20:2ω6	r_p	-.018	-.107	.186	.035	-.138	.018	-.066	-.289*
	N	74	74	74	74	74	74	74	74
C21:0	r_p	-.135	.303**	.319**	-.259*	-.018	.097	-.001	-.198
	N	74	74	74	74	74	74	74	74
C20:3ω6	r_p	-.197	.120	.236*	-.262*	.011	.163	-.030	-.318**
	N	74	74	74	74	74	74	74	74
C20:3ω3	r_p	-.078	.017	.220	-.076	.316**	-.029	.006	-.251*
	N	74	74	74	74	74	74	74	74
Log10 C20:4ω6	r_p	-.102	.084	.167	-.177	-.068	-.100	.077	-.185
	N	74	74	74	74	74	74	74	74
Log10 C20:5ω3	r_p	.033	-.067	.045	.343**	.121	-.015	.123	-.017
	N	74	74	74	74	74	74	74	74
Log10 C22:0	r_p	.039	-.081	-.060	-.067	-.179	-.177	.134	-.062

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Correlations		Baby`s age	Log10 Cu	Log10 Zn	Log10 As	Log10 Se	Log10 Cd	Log10 Pb	Log10 Hg
	N	74	74	74	74	74	74	74	74
Log10 C22:1ω9	r_p	-.172	-.152	.098	-.151	-.141	-.146	.010	-.354**
	N	74	74	74	74	74	74	74	74
Log10 C22:2ω6	r_p	-.083	-.012	.019	-.233*	.071	-.044	-.090	-.006
	N	74	74	74	74	74	74	74	74
C23:0	r_s	.030	.008	.125	-.088	-.033	-.112	.020	-.080
	N	74	74	74	74	74	74	74	74
Log10 C22:4ω6	r_p	-.079	.104	.154	-.225	.253*	.083	-.093	-.136
	N	74	74	74	74	74	74	74	74
Log10 C22:5ω6	r_p	-.083	.025	.066	-.066	.111	.087	-.065	-.196
	N	74	74	74	74	74	74	74	74
C22:5ω3	r_s	-.059	-.047	.199	.316**	.234*	.024	.250*	.066
	N	74	74	74	74	74	74	74	74
C24:0	r_s	-.145	-.025	-.088	.024	-.036	.003	.013	.021
	N	74	74	74	74	74	74	74	74
Log10 C22:6ω3/C24:1ω9	r_p	-.129	-.038	.126	.490**	.278*	-.020	.176	.050
	N	74	74	74	74	74	74	74	74
SFA	r_p	.129	-.197	-.193	-.007	.073	.077	-.217	-.041
	N	74	74	74	74	74	74	74	74
MUFA	r_p	-.022	.143	.141	.257*	.046	-.130	.218	.245*
	N	74	74	74	74	74	74	74	74
Log10 PUFA	r_p	-.133	.075	.047	-.319**	-.143	.061	-.014	-.230*
	N	74	74	74	74	74	74	74	74
Log10 ω-3 FA	r_p	-.006	-.011	.156	-.143	.024	-.063	.008	-.194
	N	74	74	74	74	74	74	74	74
Log10 ω-6 FA	r_p	-.146	.086	.033	-.319**	-.146	.073	-.017	-.220
	N	74	74	74	74	74	74	74	74
ω-6/ω-3 FA	r_p	-.095	.080	-.120	-.167	-.185	.134	-.032	-.008
	N	74	74	74	74	74	74	74	74
Log10 iC13:0/C13:0	r_p	-.068	.072	-.073	-.120	-.157	.015	-.051	.082
	N	74	74	74	74	74	74	74	74
(iC15:0+aC15:0)/C15:0	r_p	.328**	-.097	-.236*	.045	-.042	-.016	-.005	-.038
	N	74	74	74	74	74	74	74	74
Log10 ω-6 + ω-3 FA	r_p	-.135	.079	.048	-.319**	-.140	.063	-.018	-.228
	N	74	74	74	74	74	74	74	74
Log10 ω-6 LCP	r_p	-.198	.035	.223	-.303**	.107	.156	-.026	-.312**
	N	74	74	74	74	74	74	74	74
ω-3 LCP	r_p	-.067	-.030	.218	.212	.274*	-.032	.136	-.210
	N	74	74	74	74	74	74	74	74
LCP	r_p	-.166	-.017	.250*	-.012	.230*	.048	.090	-.296*
	N	74	74	74	74	74	74	74	74
C18:2ω6 / C18:3ω3	r_p	-.083	.051	-.059	.017	.038	.152	.034	.006
	N	74	74	74	74	74	74	74	74
Log10 ω -6/ ω -3 LCP	r_p	-.121	.047	.000	-.444**	-.193	.187	-.130	-.086
	N	74	74	74	74	74	74	74	74
δ^{13}C C10:0	r_p	.077	-.030	.072	.138	.035	-.098	.151	-.040
	N	70	70	70	70	70	70	70	70
δ^{13}C C12:0	r_p	.047	.153	.168	.308**	.156	-.073	.062	.111
	N	74	74	74	74	74	74	74	74
δ^{13}C C14:0	r_p	.166	.084	.097	.251*	.045	-.082	.061	-.001
	N	74	74	74	74	74	74	74	74
δ^{13}C C15:1	r_p	.563*	.266	-.248	.384	.358	-.110	.026	-.068
	N	15	15	15	15	15	15	15	15
δ^{13}C C15:0	r_p	-.176	-.013	.157	-.143	-.157	.008	-.152	-.368*
	N	32	32	32	32	32	32	32	32
δ^{13}C C16:1	r_p	-.019	.119	.037	.026	-.007	-.059	.119	-.037
	N	73	73	73	73	73	73	73	73
δ^{13}C C16:0	r_p	.063	.021	.215	.038	.054	-.011	.148	-.077
	N	73	73	73	73	73	73	73	73
δ^{13}C C17:1	r_p	-.152	.045	.025	-.051	-.091	-.082	-.293	-.252
	N	38	38	38	38	38	38	38	38
δ^{13}C C17:0	r_p	.265	-.155	-.150	.001	-.156	-.185	-.308	-.046
	N	39	39	39	39	39	39	39	39
	r_p	.167	-.047	-.060	.163	.262	-.120	.103	-.052

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Correlations		Baby's age	Log10 Cu	Log10 Zn	Log10 As	Log10 Se	Log10 Cd	Log10 Pb	Log10 Hg
δ^{13}C C18:2ω6t	N	23	23	23	23	23	23	23	23
δ^{13}C C18:1ω9c	r_s	-.016	.012	.020	.269	.373*	.097	.145	.416**
	N	41	41	41	41	41	41	41	41
δ^{13}C C18:0	r_p	.052	-.026	-.061	-.200	.056	.226	-.065	-.018
	N	43	43	43	43	43	43	43	43
δ^{13}C C20:5ω3	r_p	-.213	-.109	.054	-.079	-.102	-.010	.038	.002
	N	41	41	41	41	41	41	41	41
δ^{13}C C20:3ω6	r_s	-.075	.068	-.093	.152	.029	-.091	-.230	.103
	N	25	25	25	25	25	25	25	25
δ^{13}C C20:2	r_p	-.337	-.098	.109	-.061	-.205	-.011	-.096	-.007
	N	21	21	21	21	21	21	21	21
δ^{13}C C20:3ω3	r_p	-.568	-.012	-.045	.102	.130	.386	.570	-.069
	N	10	10	10	10	10	10	10	10
δ^{13}C C20:0	r_p	-.201	.221	.226	.022	.008	.219	-.002	-.166
	N	42	42	42	42	42	42	42	42
δ^{13}C C21:0	r_p	.259	-.127	-.143	.288	-.149	.[c]	-.454	-.240
	N	11	11	11	11	11	11	11	11
δ^{13}C C22:6ω3	r_p	.039	.001	-.261	.312	.076	-.124	.107	.261
	N	30	30	30	30	30	30	30	30
δ^{13}C C22:1ω9	r_p	-.287	.260	.216	.233	.194	.819**	.580*	.085
	N	14	14	14	14	14	14	14	14
δ^{13}C C22:0	r_p	-.005	-.030	-.127	.310	.416*	.221	.279	.042
	N	33	33	33	33	33	33	33	33
δ^{13}C C18:0-δ^{13}C C16:0	r_p	.084	-.023	-.174	-.269	.025	.179	-.167	-.019
	N	42	42	42	42	42	42	42	42

r_p –correlation coefficient. N – number. * two variables correlated statistically significantly ($p < 0.05$). ** two variables correlated statistically significantly ($p < 0.001$).

Table S8. Correlations between composition and stable isotopes of FAs and dietary habits

Spearman's correlations for daily intake factor		Fresh-water fish	Fresh seafood	Frozen seafood	Canned seafood	Seafood all
C4:0	r_s	.317**	-.394**	-.109	-.041	-.311**
	N	74	70	74	72	69
C6:0	r_s	.168	-.206	.045	.009	-.143
	N	74	70	74	72	69
C8:0	r_s	.131	-.293*	.001	.078	-.196
	N	74	70	74	72	69
C10:0	r_s	.077	-.264*	.121	-.001	-.141
	N	74	70	74	72	69
iC11:0	r_s	-.030	.085	-.138	.000	.017
	N	74	70	74	72	69
C11:0	r_s	.099	-.329**	.057	-.140	-.196
	N	74	70	74	72	69
C12:0	r_s	.147	-.336**	.027	.024	-.204
	N	74	70	74	72	69
iC13:0	r_s	.219	-.299*	.294*	-.086	-.156
	N	74	70	74	72	69
C13:0	r_s	-.015	-.070	.134	.028	.019
	N	74	70	74	72	69
iC14:0	r_s	-.116	.229	-.093	.018	.180
	N	74	70	74	72	69

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Spearman's correlations for daily intake factor		Fresh-water fish	Fresh seafood	Frozen seafood	Canned seafood	Seafood all
C14:0	r_s	.033	-.162	-.025	.066	-.088
	N	74	70	74	72	69
C14:1ω5	r_s	-.108	.281*	-.011	-.027	.151
	N	74	70	74	72	69
iC15:0	r_s	-.137	.247*	.090	.123	.256*
	N	74	70	74	72	69
aC15:0	r_s	-.162	.298*	.103	.126	.314**
	N	74	70	74	72	69
C15:0	r_s	-.191	.350**	.015	.080	.291*
	N	74	70	74	72	69
C15:1	r_s	-.246*	.400**	.073	.026	.307*
	N	74	70	74	72	69
C16:0	r_s	-.164	.198	-.125	-.027	.131
	N	74	70	74	72	69
C16:1ω9	r_s	-.074	.196	.040	-.001	.161
	N	74	70	74	72	69
C16:1ω7	r_s	-.066	.159	-.037	-.091	.058
	N	74	70	74	72	69
iC17:0	r_s	-.282*	.680**	-.054	.115	.513**
	N	74	70	74	72	69
aC17:0	r_s	-.254*	.467**	-.051	.111	.362**
	N	74	70	74	72	69
C17:0	r_s	-.139	.203	-.066	-.051	.102
	N	74	70	74	72	69
C17:1	r_s	-.067	.140	.026	.023	.147
	N	74	70	74	72	69
iC18:0	r_s	-.184	.223	-.202	-.041	.076
	N	74	70	74	72	69
C18:0	r_s	-.045	-.041	-.071	-.038	-.086
	N	74	70	74	72	69
C18:1ω9	r_s	-.270*	.266*	-.122	-.070	.132
	N	74	70	74	72	69
C18:1ω7	r_s	-.260*	.494**	-.133	.049	.340**
	N	74	70	74	72	69
C18:2ω6c	r_s	.387**	-.234	.106	-.030	-.211
	N	74	70	74	72	69
C18:2ω6t	r_s	-.274*	.363**	-.197	-.024	.180
	N	74	70	74	72	69
	r_s	.333**	-.320**	.142	-.044	-.276*

Supplements: Selected elements and fatty acid composition in human milk as indicators of seafood dietary habits - Jagodic et al. 2020

Spearman's correlations for daily intake factor		Fresh-water fish	Fresh seafood	Frozen seafood	Canned seafood	Seafood all
C18:2ω6(c+t)	N	74	70	74	72	69
C18:3ω6	r_s	.051	.237*	.047	.242*	.199
	N	74	70	74	72	69
C18:3ω3	r_s	.131	-.014	.189	-.052	.033
	N	74	70	74	72	69
CLA(c-9, t-11C18:2)	r_s	-.252*	.453**	-.070	.011	.310**
	N	74	70	74	72	69
C20:0	r_s	-.047	-.166	-.117	-.056	-.181
	N	74	70	74	72	69
C20:1ω9	r_s	-.188	.113	-.048	-.158	.042
	N	74	70	74	72	69
C20:2ω6	r_s	-.200	.160	.053	-.135	.111
	N	74	70	74	72	69
C21:0	r_s	.170	-.158	.101	-.125	-.169
	N	74	70	74	72	69
C20:3ω6	r_s	.165	-.225	.064	-.030	-.192
	N	74	70	74	72	69
C20:3ω3	r_s	.202	-.305*	.013	.015	-.229
	N	74	70	74	72	69
C20:4ω6	r_s	.188	-.101	.162	.003	.022
	N	74	70	74	72	69
C20:5ω3	r_s	-.182	.259*	-.074	-.033	.134
	N	74	70	74	72	69
C22:0	r_s	.042	-.173	-.028	-.104	-.201
	N	74	70	74	72	69
C22:1ω9	r_s	.198	-.121	-.051	-.242*	-.183
	N	74	70	74	72	69
C22:2ω6	r_s	.151	-.360**	-.030	-.280*	-.364**
	N	74	70	74	72	69
C23:0	r_s	-.007	-.070	-.133	.108	-.056
	N	74	70	74	72	69
C22:4ω6	r_s	.172	-.506**	.085	-.019	-.321**
	N	74	70	74	72	69
C22:5ω6	r_s	.141	-.368**	-.067	-.121	-.347**
	N	74	70	74	72	69
C22:5ω3	r_s	-.082	.287*	-.084	-.080	.165
	N	74	70	74	72	69
C24:0	r_s	-.002	.026	-.012	-.106	-.077
	N	74	70	74	72	69

Supplements: Selected elements and fatty acid composition in human milk as indicators of seafood dietary habits - Jagodic et al. 2020

Spearman's correlations for daily intake factor		Fresh-water fish	Fresh seafood	Frozen seafood	Canned seafood	Seafood all
C22:6ω3/C24:1ω9	r_s	-.242*	.231	-.165	-.053	.053
	N	74	70	74	72	69
SFA	r_s	.039	-.148	.009	.027	-.057
	N	74	70	74	72	69
MUFA	r_s	-.322**	.437**	-.140	-.016	.271*
	N	74	70	74	72	69
PUFA	r_s	.328**	-.307**	.145	-.036	-.255*
	N	74	70	74	72	69
ω-3 FA	r_s	.087	-.027	.070	-.041	-.013
	N	74	70	74	72	69
ω-6 FA	r_s	.335**	-.325**	.143	-.039	-.277*
	N	74	70	74	72	69
ω-6/ω-3 FA	r_s	.265*	-.298*	.004	-.067	-.312**
	N	74	70	74	72	69
iC13:0/C13:0	r_s	.217	-.231	.222	-.120	-.157
	N	74	70	74	72	69
(iC15:0+aC15:0)/C15:0	r_s	-.012	.019	.201	.156	.154
	N	74	70	74	72	69
ω-6 + ω-3 FA	r_s	.323**	-.306**	.142	-.036	-.256*
	N	74	70	74	72	69
ω-6 LCP	r_s	.188	-.335**	.124	-.070	-.239*
	N	74	70	74	72	69
ω-3 LCP	r_s	.091	-.094	-.015	.006	-.095
	N	74	70	74	72	69
LCP	r_s	.139	-.257*	.056	-.073	-.214
	N	74	70	74	72	69
C18:2ω6 / C18:3ω3	r_s	.170	-.267*	-.157	.006	-.320**
	N	74	70	74	72	69
ω-6 / ω-3 LCP	r_s	.143	-.185	.170	-.026	-.111
	N	74	70	74	72	69
δ^{13}C C10:0	r_s	-.078	.168	-.142	-.002	.007
	N	70	66	70	69	66
δ^{13}C C12:0	r_s	-.212	.285*	-.143	-.080	.068
	N	74	70	74	72	69
δ^{13}C C14:0	r_s	-.204	.318**	-.073	-.008	.147
	N	74	70	74	72	69
δ^{13}C C15:1	r_s	-.049	-.523	-.282	-.128	-.504
	N	15	14	15	14	14
	r_s	.213	-.136	.069	.085	.053

Supplements: Selected elements and fatty acid composition in human milk as indicators of seafood dietary habits - Jagodic et al. 2020

Spearman's correlations for daily intake factor		Fresh-water fish	Fresh seafood	Frozen seafood	Canned seafood	Seafood all
δ^{13}C C15:0	N	32	31	32	31	30
δ^{13}C C16:1	r_s	-.066	.167	-.014	-.104	.002
	N	73	70	73	71	69
δ^{13}C C16:0	r_s	-.121	.259*	-.102	-.018	.139
	N	73	69	73	71	68
δ^{13}C C17:1	r_s	.013	-.102	-.022	-.181	-.044
	N	38	36	38	36	35
δ^{13}C C17:0	r_s	.077	.052	.184	-.020	.181
	N	39	37	39	37	36
δ^{13}C C18:2ω6t	r_s	-.109	-.278	.022	.341	-.130
	N	23	21	23	23	21
δ^{13}C C18:1ω9c	r_s	-.241	.449**	-.295	-.126	.124
	N	41	39	41	40	39
δ^{13}C C18:0	r_s	-.092	-.030	.050	-.032	.029
	N	43	41	43	42	41
δ^{13}C C20:5ω3	r_s	.116	-.106	-.264	.082	-.159
	N	41	40	41	39	39
δ^{13}C C20:3ω6	r_s	-.368	.176	-.238	-.108	-.079
	N	25	24	25	24	24
δ^{13}C C20:2	r_s	.136	-.175	-.208	-.059	-.267
	N	21	20	21	20	20
δ^{13}C C20:3ω3	r_s	.143	-.296	-.474	-.067	-.516
	N	10	10	10	10	10
δ^{13}C C20:0	r_s	.101	-.144	-.018	.114	-.006
	N	42	40	42	40	39
δ^{13}C C21:0	r_s	-.255	.250	-.100	.448	.312
	N	11	10	11	10	10
δ^{13}C C22:6ω3	r_s	-.396*	.423*	-.221	.045	.145
	N	30	29	30	29	28
δ^{13}C C22:1ω9	r_s	-.037	-.127	.083	.201	-.011
	N	14	14	14	13	13
δ^{13}C C22:0	r_s	-.067	.113	-.243	.022	.036
	N	33	31	33	31	30

* two variables correlated statistically significantly ($p < 0.05$). ** two variables correlated statistically significantly ($p < 0.001$). N – number of cases, r_s – Spearman's correlation coefficient.

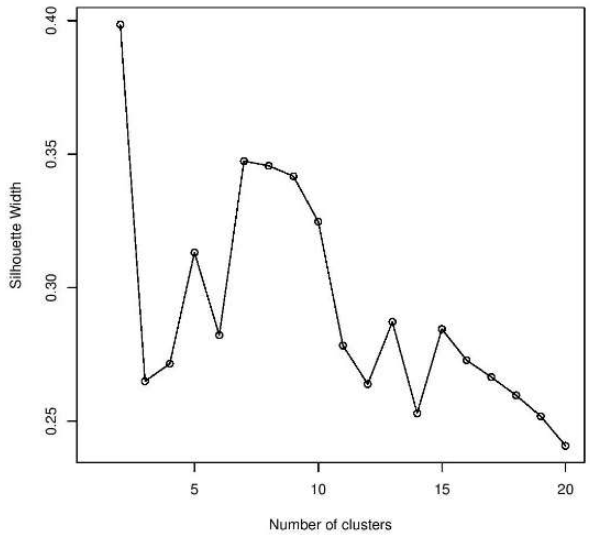


Figure S1: Silhouette curve for estimating the number of clusters for fresh seafood intake

Reference:

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3.3 Birth Weight in Relation to Maternal Blood Levels of Selected Elements in Slovenian Populations: A Cross-Sectional Study

Jagodic, M., Snoj Tratnik, J., Mazej, D., Stajnko, A., Pavlin, M., Krsnik, M., Kobal, A. B., Kononenko, L., Odland J. Ø., Horvat, M. Journal of health science, ISSN 2328-7136, 2017, vol. 5, str. 95-106, doi: 10.17265/2328-7136/2017.02.005.

In this paper, the authors presented relations between babies' birth weight and maternal blood levels of selected trace elements in Slovenian populations. Since birth weight is recognized as an important indicator of a child's health status at delivery and also later in their life, the objective of the present study was to evaluate the relation between maternal blood levels of selected toxic and potentially toxic elements (Mn, Cu, Zn, As, Se, Cd, Pb, Hg) and birth weight of their newborns in a Slovenian population, taking into account maternal socio-demographic characteristics and dietary habits. Attention was devoted to exposure to essential and potentially toxic elements during pregnancy and the breastfeeding period, as they are recognized as being potentially responsible for adverse development and health effects in children.

The study populations included 535 first-time mothers who gave birth to a living child from 12 regions of Slovenia, including three urban areas (140 women), three rural areas (109 women) and six potentially contaminated areas due to past industrial activity (245 women). However, 41 participants were excluded due to missing blood samples, gestational diabetes, diabetes type I and II, delivery of twins, lifestyle or occupational exposure and missing information on baby birth weight. The mothers were recruited between 2007 and 2014 through selected maternity hospitals, parenting schools and gynecologists. Maternal blood was collected at 1.5 months after birth. Associations between birth weight and a) predictors obtained through the questionnaires and b) levels of selected elements were tested using bivariate tests and multiple linear regression. Multiple regression models revealed maternal age as an additional predictor for birth weight and confirmed pre-pregnancy body mass, estimated gestational age and gender of the child as the main predictors for birth weight.

Mn in maternal blood was significantly and positively associated with birth weight. The positive association observed between birth weight and Mn in maternal blood could be explained by the essentiality of Mn in fetal development as an important cofactor in enzyme reactions in bone formation and in metabolic regulation for amino acid, lipid, protein and carbohydrate levels.

The candidate's contribution to this paper consisted of literature overview, extraction of data from published papers and performing analytical and statistical analyses of this case study. The candidate prepared the tables, and drafted the manuscript in collaboration with other co-authors.

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Birth Weight in Relation to Maternal Blood Levels of Selected Elements in Slovenian Populations: A Cross-sectional Study

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Abstract: The objective of the present study was to evaluate the relation between maternal blood levels of selected toxic and potentially toxic elements (manganese (Mn), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), cadmium (Cd), lead (Pb) and mercury (Hg)) and birth weight of their new-borns in a Slovenian population, taking into account maternal socio-demographic characteristics and dietary habits. 535 women from 12 regions of Slovenia were recruited at delivery. Maternal blood was collected at 1.5 months after birth. Associations between birth weight and a) predictors obtained through the questionnaires and b) levels of selected elements were tested using bivariate tests and multiple linear regression. Multiple regression models revealed maternal age as an additional predictor for birth weight and confirmed pre-pregnancy body mass, estimated gestational age and gender of the baby as the main predictors for birth weight. Mn in maternal blood was significantly and positively associated with birth weight. The positive association observed between birth weight and Mn in maternal blood could be explained by the essentiality of Mn in foetal development as an important cofactor in enzyme reactions in bone formation and in metabolic regulation for amino acid, lipid, protein and carbohydrate levels.

Key words: Birth weight, toxic elements, essential elements, maternal whole blood, preventive health, Slovenia.

1. Introduction

Concern over problems from exposure to environmental influences on health impairment of the new-born child requires continuous monitoring of the exposure of a population to environmental agents [1]. In response, many developed countries and international bodies have established large-scale human biomonitoring surveys (HBM) [2], which show the concentrations of chemicals and their metabolites in body tissues including, for example, blood, serum, urine, and breast milk. The levels of environmental chemicals in a person's body fluid and/or tissue are indicative of exposure, but not necessarily the cause of a disease or an adverse effect [3, 4].

In this study, some of the most relevant toxic and potentially toxic elements, listed among the 20 top chemicals on the Priority list of Hazardous Substances [5], were addressed. Among the most susceptible groups are pregnant women and children, the latter because of their increased exposure to mixtures of chemicals, increased absorption rates and a diminished ability to detoxify many exogenous compounds, all

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relative to those of adults [6]. The developing foetus is particularly susceptible due to partial lack of the blood-brain barrier [7] and to the rapid and tightly controlled growth of the brain during the pregnancy period [6]. The potential risk to the developing foetus is reflected in the level of contaminants in the maternal blood. It is known that several of these substances are passed from mother to foetus through the umbilical cord and later on to the child through breast milk [8, 9]. Of concern are the long-term, subtle effects that could influence reproductive health, pregnancy outcomes (e.g., birth weight, length, gestational age), reduced resistance against diseases, mental development of the child, or the increased risk of cancer [10]. The toxicity, metabolism and absorption of certain elements are dependent mainly on the chemical species and/or oxidation state. For example, dietary exposure to organic arsenic is unlikely to cause a health risk, but inorganic arsenic is recognized as genotoxic and is a known human carcinogen [11].

Essential elements also have a potential for toxicity and risk assessments for their essential versus toxic effects have been carried out [12, 13]. Trace element deficiencies have been reported in about 40% of the world's population, mostly from developing countries. This is probably a result of poor diet and can lead to endocrine, cardiovascular, skeletal, liver, gastrointestinal, kidney, genetic, and ophthalmologic disorders [14]. Furthermore, effects of toxic elements depend on the interaction between toxic and essential elements, especially when the metabolism of a toxic element is similar to that of an essential element. Exposure to toxic elements is also influenced by lifestyle factors [15].

Birth weight is the main factor influencing infant mortality rates, health of babies and a healthy later life. Birth weight is associated with the gender of the baby, the anthropometry of the mother (e.g., poor pre-pregnancy weight, weight gain during pregnancy, shorter gestational age), parity, multiple birth, complicated pregnancy (e.g., maternal anaemia, pregnancy diabetes, poor antenatal care, problems with placenta, maternal infections), restricted foetal growth, poor nutritional intake of women, and deprived socio-economic conditions [10, 16-19]. Effects are also associated with physically demanding work during pregnancy, environmental tobacco smoke, intensity and duration of cigarette smoking during pregnancy, taking illicit drugs and consuming alcohol [10, 16-20]. In addition, maternal health in relation to pregnancy, the mother's genetic background, age, location of residence and education also influence birth weight [17, 18].

Various associations between trace elements and birth weight have been observed. Studies have shown significant direct correlations between birth weight and maternal levels of zinc (Zn) and copper (Cu) in serum [21]. As for manganese (Mn), it has been suggested that Mn levels in maternal whole blood influence birth weight [22, 23], while the absence of a correlation between birth weight and Mn, Cu, Zn and selenium (Se) in maternal blood has also been reported [24-26]. Levels of Mn in maternal blood have been found to increase throughout pregnancy [27, 28], while lower or higher Mn concentrations in maternal blood has been associated with low birth weight (U-shaped relationship) [29, 30]. Toxic elements (cadmium (Cd), lead (Pb), arsenic (As) and mercury (Hg)) may have negative effect on birth weight at relatively low levels of exposure. Xu et al. suggested that the presence of As in maternal blood has a negative influence on birth weight and on gestational age in boys [31]. Studies investigating the effects of Cd exposure during pregnancy have been in conclusive: some studies [32-34] report negative associations, some positive [35], and others no association at all [36, 37]. Exposure to Pb was found to be associated with birth weight either negatively [37, 38], or insignificantly [34-36]. Non-significant correlations have been observed for Hg in maternal blood [35, 36, 39].

The National HBM program was implemented in Slovenia, according to the Chemicals Act in 2003 and

performed in the period from 2007 to 2014 for the first time to estimate the exposure of inhabitants of different are as to selected environmental chemicals and to provide data for an environmental health risk assessment [40]. Attention was on exposure to essential and toxic substances during pregnancy and the breastfeeding period, since these are recognized as being potentially responsible for adverse development and health in children. The main objective of the present study was to examine the relation between birth weight and maternal blood levels of toxic and potentially toxic elements determined within the Slovenian HBM program and taking into account the socio-demographic characteristics and diet of the mothers.

2. Materials and Methods

2.1 Description of Study Population

The study populations included first-time mothers who gave birth to a living baby from 12 different areas across Slovenia, including three urban areas (140 women), three rural areas (109 women) and six potentially contaminated areas due to past industrial activity (245 women) (Fig. 1). Areas of Jesenice, Celje, Idrija with Posočje, Savinjsko-posavska and Zasavje are known to be contaminated with metals, while the area of Bela krajina with polychlorinated biphenyls (PCBs).

2.2 Recruitment and Sampling

The mothers were recruited between 2007 and 2014 through selected maternity hospitals, parenting schools and gynaecologists. Maternal blood, urine, hair and milk samples were collected between 1 to 3 months after delivery. Questionnaires were given to the mothers to obtain information about their lifestyle, living environment, socio-economic status, nutritional habits (frequency of intake), health status of the mother-child pair and breastfeeding status. A total of 535 women signed informed consent, however, 41 women were excluded due to missing blood samples, gestational diabetes, diabetes type I and II, delivery of twins, lifestyle or occupational exposure and missing information on birth weight. The final database contains data from 494 women.

2.3 Sample Preparation and Chemical Analysis of Whole Blood

Venous blood samples were collected using vacutainer tubes containing K_2EDTA (BD Vacutainer® Blood Collection Tubes for Trace Element Testing, Becton Dickinson, US), following regular laboratory procedures,

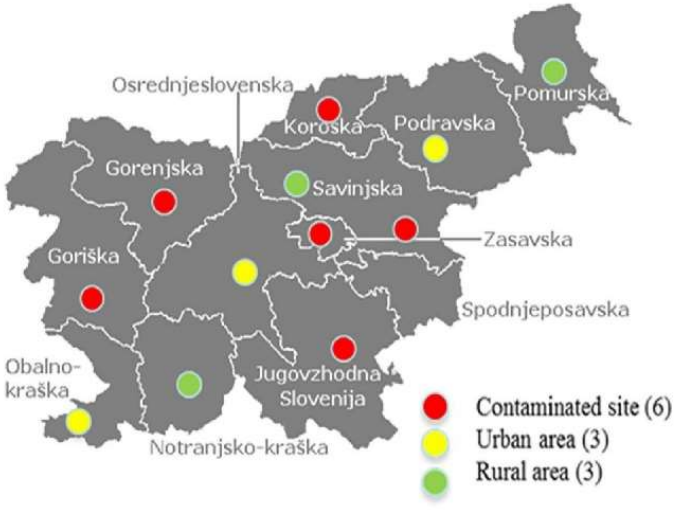


Fig. 1 Sampling locations in Slovenia [40].

and stored at -20 °C until analysis. Selected toxic and potentially toxic elements in the maternal whole blood samples were analysed at the Jožef Stefan Institute, Slovenia.

Whole blood sample is generally recognized as an appropriate matrix for assessment of exposure to Mn, Se, Cu, As, Cd, Pb and Hg. However, in the case of Zn and Cu, serum is a more indicative matrix for their status in the body [41]. Due to cost effectiveness and the limited blood volume available from the mothers, elements were determined in whole blood. For the same reason sample size varied for some elements on account of an insufficient amount of sample and this is the reason why Mn was not determined in participants from three of the selected study areas (pilot phase including one urban, one rural and one potentially contaminated area). To allow comparability with other studies, all values have been converted to ng/mL, using a factor of 1.053 g/mL for women [42].

2.3.1 Determination of As, Cd, Cu, Mn, Pb, Se and Zn in Whole Blood

As, Cd, Cu, Mn, Pb, Se and Zn in whole blood were analysed using an Octapole Reaction System (ORS) Inductively Coupled Plasma Mass Spectrometer (ICP-MS, 7500 ce, Agilent, Tokyo, Japan) equipped with an ASX-510 Autosampler (Cetac), a Babington nebuliser and a Scott-type spray chamber. The ORS was operated in helium gas mode to avoid interference.

Aliquots of 0.3 mL of whole blood sample werediluted ten times with an alkaline solution containing Triton X-100 and disodium ethylene diamine tetraacetic acid (EDTA) in contamination free tubes [43]. An aliquot of an internal standard solution containing Scandium (Sc), Gallium (Ga), Yttrium (Y) and Gadolinium (Gd) was added to each tube and standard addition was used for calibration. The following isotopes were monitored ^{45}Sc, ^{55}Mn, ^{63}Cu, ^{66}Zn, ^{69}Ga, ^{75}As, ^{78}Se, ^{89}Y, ^{111}Cd, ^{114}Cd, ^{157}Gd, ^{206}Pb, ^{207}Pb, ^{208}Pb. The instrument was tuned daily using a solution containing Li, Mg, Y, Ce, Tl and Co. On all isotopes were quantified using one central point of the spectral peaks and three repetitions.

Analytical precision was 5%, for Pb, Mn, Cu, Zn and Se, 10% for As and 20% for Cd. Limits of detection (LOD), calculated as three times the standard deviations of the blank sample, were 0.2, 0.4, 0.1, 8, 7, 30, 0.4 ng/mL for Cd, Pb, As, Se, Cu, Zn and Mn, respectively. The accuracy of the results was checked against the certified material Seronorm Trace Elements Whole Blood L-1 (SERO AS, Norway).

2.3.2 Determination of Total Hg in Whole Blood

The concentration of total Hg in whole blood was determined using a Direct Mercury Analyser (DMA-80, Milestone Srl, Italy). A known amount of blood (0.1-0.2 mL) was weighed out into a quartz boat. The sample was first dried and then thermally and chemically decomposed at 650 °C. An oxygen stream then transfers the decomposition products through an amalgamator that selectively traps gaseous mercury, which is subsequently desorbed for quantification. The resulting mercury vapour was analysed using atomic absorption spectrophotometry at 254 nm [44].

The analytical precision was 5% and with a LOD of 0.2 ng/mL. The reference material, Seronorm Trace Elements Whole Blood L-1 (SERO AS, Norway), was used to check the accuracy of the results.

2.4 Statistical Analysis

Statistical analysis included descriptive statistics (means, minimum (min), maximum (max), geometric means (GM), confidential intervals (CI) of GM, and percentage), bivariate analysis (Spearman for categorical data and Pearson for continuous), analysis of between-group effects (Mann-Whitney U, Kruskall-Wallis test or ANOVA) and multiple linear regression. Non-normally distributed data (concentrations of elements, baby birth length) were log10-transformed. Concentrations of elements below the LOD were assigned arbitrarily the value of ½ LOD. Multiple linear regression (using the enter all method) was used to evaluate the association between birth weight and all covariates that correlate with the

dependant variable with a level of significance $P < 0.25$. Highly correlated variables were not included in the same model to avoid the effect of multi-collinearity. Predefined confounders were fixed into the models (age of mother, education, pre-pregnancy body mass index (BMI) and residence). P-values < 0.05 were considered significant. All statistical analyses were performed using IBM SPSS Statistic Version 23 and STATA/SE 12.1.

3. Results and Discussion

Table 1 gives the characteristics of the study population. The mean age of the mothers was 29 years, the mean pre-pregnancy BMI was 23.1 kg/m^2, the mean estimated gestational length was 39.7 weeks, 50% of women have at least university education, 53.8% of babies were males, and the mean birth length for all babies was 51.4 cm. The mean birth weight of the babies in our study was comparable with those reported elsewhere [23, 30, 35, 38, 45, 46]. A total of 3.4% of the new-borns were below the criterion for "low birth weight" of 2,500 g [17], and 0.2% of new-borns were below 1,500 g, considered as a "very low birth weight" [47]. "High birth weights" (> 4,000 g) [48] were reported for 8.3% of the new-borns.

Table 2 gives the geometric mean concentrations of selected elements in maternal whole blood samples. In general, the level of exposure to toxic elements was low and did not pose a health risk for either the mothers or their babies (Table 2). The percentage of individuals having blood concentrations of Hg, Pb and Cd above the established reference values were 0.81, 0.40, and 3.64%, respectively (Table 2) [49, 51-53]. The GM of Cd in non-smokers was 0.34 ng/mL, 0.41 ng/mL in pre-pregnancy smokers (12%) and 0.63 ng/mL in pregnancy smokers (1.2%). As observed from correlations between blood levels and questionnaire variables, Hg was significantly associated with consumption of fresh sea fish ($r_s = 0.269$, $P < 0.001$) and canned sea fish ($r_s = 0.237$, $P < 0.001$) (data not shown), hence the weak correlations. Furthermore, no relationship was observed between Hg and amalgam fillings ($r_s = 0.017$, $P = 0.739$). Based on the questionnaire data, 41.1% of women reported eating fresh seafood at least once per month (on average less than once per month); 17.2% of mothers reported having noamalgam fillings and 23.3% did not answer this question. On average women had 4.36 amalgam fillings (data not shown).

In the case of Cu, Zn and Se, 2.0, 0.2 and 0.4% of individuals exhibited levels below their lower reference values, while 3.2, 1.4 and 4.9% of individuals had levels of Mn, Cu, Zn and Se above the upper references levels. In contrast, 75.7% of women exceeded the reference levels for Mn [49-51]. The relatively high levels of Mn in the study population were similar to those previously reported in postpartum mothers form Norway [28], in mothers before delivery in South

Table 1 General characteristics of the Slovenian study population.

Characteristics of study population		N	Mean (min-max)	% of participants
Women	Age at delivery (years)	493	29.0 (19-39)	
	Pre-pregnancy body mass index (kg/m^2)	483	23.1 (16.6-44.8)	
	Estimated gestational length (weeks)	473	39.7 (28-42)	
	Education: primary	35		7.4
	secondary	200		42.6
	university	235		50.0
Children	Gender: male	266		53.8
	female	228		46.2
	Birth weight (kg)	494	3.38 (1.40-5.17)	
	Birth length (cm)	493	51.4 (44-59)	

N = number of samples, min = minimum level, max = maximum level.

Table 2 Concentrations of elements (ng/mL) in maternal whole blood of the study population and respective reference values.

Elements	N	GM	95% CI of GM	Range (min-max)	Reference value/range	% below reference value	% above reference value
Mn (ng/mL)	370	**17.2**	16.7-17.8	5.8-35.2	**4-14** [27]	0.00	75.7
Cu (ng/mL)	494	**1,077**	1,057-1,084	657-2,004	**800-1,400** [51]	2.02	3.24
Zn (ng/mL)	494	**6,731**	6,644-6,819	3,010-11,733	**3,500-9,100** [50]	0.20	1.42
As (ng/mL)	494	**0.929**	0.866-0.997	0.201-16.798	**< 20** [51]	-	0.00
Se (ng/mL)	494	**94.7**	93.2-96.3	53.9-175.9	**60-130** [49]	0.40	4.86
Cd (ng/mL)	493	**0.346**	0.328-0.365	0.20-3.084	**< 1.0** [53]	-	3.64
Pb (ng/mL)	494	**16.8**	16.2-17.4	4.2-71.9	**< 70** [53]	-	0.40
Hg (ng/mL)	493	**1.11**	1.03-1.19	0.20-10.17	**< 5.8** [55]	-	0.81

CI = confidence interval, GM = geometric mean, min = minimum level, max = maximum level, N = number of samples.

Table 3 Correlation between birth weight and selected variables.

Correlations with baby birth weight (kg)			
Variable	r_s	***P*-value**	**N**
Residential location*	-	0.140	448
Age of mother (years)-groups	-0.043	0.346	493
Pre-pregnancy body mass index-groups	0.092	0.042	483
Education-groups	-0.033	0.469	470
Estimated gestational age (week)-groups	0.325	0.000	473
Gender of baby	-	0.033	494
	r_p	***P*-value**	**N**
Log10 Mn (ng/mL)	0.178	0.001	370
Log10 Cu (ng/mL)	-0.074	0.102	494
Log10 Zn (ng/mL)	0.004	0.938	494
Log10 As (ng/mL)	0.018	0.689	494
Log10 Se (ng/mL)	-0.042	0.347	494
Log10 Cd (ng/mL)	0.053	0.239	493
Log10 Pb (ng/mL)	0.076	0.091	494
Log10 Hg (ng/mL)	-0.040	0.376	493
Baby length (cm)	0.717	0.000	493

*potentially contaminated, rural, and urban. GM = geometric mean, *P*-value shows the level of significance, N = number of samples.

African coastal communities (from part "Rural 2") [54], in pregnant woman from Southwest Quebec [46], in the Mother and Children's Environmental Health birth cohort study in Seoul, in Cheonan, and Ulsan in the Republic of Korea at delivery [30] and in pregnant women from Costa Rica [25]. The concentrations of essential elements at 6 weeks postpartum may have changed according to physiological and metabolic changes during pregnancy and during the postpartum/lactation period, as was demonstrated by Hansen et al. who found that concentrations of Mn, Se, Zn and As increased postpartum [28].

Of the variables tested, pre-pregnancy BMI, estimated gestational age, gender of the baby and baby length were associated significantly with birth weight (Table 3). Among the selected elements, significant correlations were observed between the birth weight and Mn levels, and marginally significant correlations between Pb and Cu levels in the maternal blood samples (Table 3). No statistically significant correlations were observed between birth weight and consumption of different food items.

A positive association between Mn in maternal blood and birth weight was confirmed by the linear regression model, in which the Mn levels were adjusted for the relevant predictors (Table 4). Among

Table 4 Multiple linear regression between birth weight and selected variables.

Model						
Dependent variable	**N of observations**	**R^2**	**Adjusted R^2**	***P*-value**	**95.0% Confidence Interval for β**	
Baby birth weight (kg)	319	0.280	0.224	0.000		
Independents variable:		**β coefficient**		***P*-value**	**Lower Bound**	**Upper Bound**
Residential location	**Rural (A)**	Reference				
	Urban (B)	0.167		0.028	0.018	0.316
	Potentially contaminated (C)	0.098		0.144	-0.034	0.230
Age (years)	**< 25**	Reference				
	25-29	-0.170		0.054	-0.343	0.003
	≥ 30	-0.180		0.052	-0.362	0.001
Pre-pregnancy BMI	**under weight**	Reference				
	normal weight	0.264		0.021	0.040	0.488
	over weight	0.362		0.003	0.123	0.602
Education	**Primary**	Reference				
	Secondary	-0.071		0.520	-0.286	0.145
	Tertiary or more	-0.058		0.599	-0.276	0.159
Estimated gestational age (weeks)	**< 37**	Reference				
	37-39	0.764		0.000	0.540	0.988
	≥ 40	0.880		0.000	0.658	1.101
Intake coffee, tea	**< 1 × per month**	Reference				
	≥ 1 × per month	-0.030		0.662	-0.164	0.104
	≥ 1 × per day	-0.008		0.893	-0.126	0.110
Intake of alcohol	**never**	Reference				
	< 1 × per month	0.147		0.013	0.031	0.263
	≥ 1 × per month	0.158		0.024	0.021	0.295
Intake of tinned sea food	**< 1 × per month**	Reference				
	1 - 3 × per month	-0.009		0.874	-0.122	0.103
	≥ 1 × per week	0.051		0.451	-0.081	0.183
Intake of eggs	**< 1 × per week**	Reference				
	1 × per week	-0.017		0.796	-0.144	0.111
	> per week	0.049		0.462	-0.082	0.180
Gender of the baby	**male**	Reference				
	female	-0.117		0.021	-0.215	-0.018
Log10 Mn (ng/mL)		0.523		0.014	0.107	0.939
Log10 Cu (ng/mL)		-0.296		0.480	-1.120	0.527
Log10 Cd (ng/mL)		-0.047		0.665	-0.258	0.165
Log10 Pb (ng/mL)		-0.055		0.711	-0.344	0.235
(Constant)		2.677		0.041	0.106	5.247

N = number of samples, *P*-value shows the level of significance, R^2 = R Square = gradient of the regression line and the strength of the relationship between a predictor and the outcome; β coefficient = gradient of the regression line and the strength of the relationship between a predictor and the outcome, BMI = body mass index.

the predictors tested, the regression model revealed statistically significant correlations between birth weight and pre-pregnancy BMI, intake of alcohol before pregnancy, the baby's gender, as well as a marginally significant correlation between birth weight and the age of the mother. A higher pre-pregnancy BMI was associated with a higher birth weight, which agrees with the findings of other studies [16, 17, 56]. Furthermore, the differences in gender-related birth weight (boys were heavier) is also in accordance with

an earlier report [17]. Teenagers and young women have been reported to have lighter babies [17, 18], but in the present study the opposite was observed. The reason for this could be the narrower age interval (19-39 years) included in the present study. The model also shows that babies from urban areas had higher birth weights than those from rural areas (Table 4).

The positive association observed between birth weight and Mn in maternal blood could be explained by the essential role of Mn in foetal development. Mn is an important cofactor in enzyme reactions in bone formation and in metabolic regulation of levels of amino acid, lipid, protein and carbohydrate [25, 27]. However, in contrast to the present study, only non-significant or non-linear correlations between birth weight and Mn have been reported [22, 23, 25, 26].

In contrast with the literature [20], non-significant correlations between maternal smoking and birth weight were observed in the present study, probably due to the low frequency of smoking among the participating mothers (1.2% of women smoked during pregnancy and 12.4% before pregnancy). Although it is known that some food items appear to be beneficial for an appropriate birth weight [57, 58], our study failed to reveal any significant associations between birth weight and the food items that were included in the study questionnaire. The only association was between birth weight and alcohol intake before the pregnancy, but it was positive and also questionable due to the low levels of alcohol intake in the study population (average consumption was less than once per month).

4. Limitations

A disadvantage of the Slovenian study presented here is that the gestational age was estimated from the information obtained from the mothers, i.e., the estimated gestational age was calculated from the date of birth and predicted date of birth reported by the mothers, since clinical information from the maternity hospitals was not available. Another limitation of the study is the time-period in which blood samples were collected (1.5 months postpartum). Since literature data suggest that food intake during pregnancy can affect the levels of elements in maternal blood during and after pregnancy [59], the levels of selected elements in postpartum maternal blood samples could be used for estimation of pregnancy exposures.

5. Conclusions

The present study is the first of its kind to examine exposure to such a wide range of trace elements in relation to infant birth weight while taking into account the socio-demographic characteristics and dietary habits of the mothers. Exposure to toxic elements was low and comparable to that in other populations. Most mothers did not exceed the given reference levels. The birth weight was in agreement with other literature studies. The results of this study also show that maternal age, pre-pregnancy BMI, estimated gestational age, length of baby and marginally the gender of baby all influence the birth weight. Despite low, environmentally relevant, exposure to the selected potentially toxic elements, a significant and positive correlation was observed between birth weight and the levels Mn in blood from Slovenian mothers. Additional evaluations and further studies are needed to focus on this specific and other potential associations.

6. List of Abbreviations

AMAP: Arctic Monitoring and Assessment Program; ANOVA: Analysis of variance; As: Arsenic; ATSDR: Agency for Toxic Substances and Disease Registry; BMI: Body mass index; Cd: Cadmium; CDC: Centres for Disease Control and Prevention; Cu: Copper; LOD: Limit of detection; DMA: Direct Mercury Analyser; EDTA: Ethylenediaminetetraacetic acid; Ga: Gallium; Gd: Gadolinium; GM: Geometric mean; HBM: Human biomonitoring study; Hg: Mercury; ICP-MS: Inductively Coupled Plasma Mass Spectrometer; K: Potassium; max: Maximum level; min: Minimum level; Mn: Manganese; N: number of samples; NMEC:

National Medical Ethics Committee; ORS: Octapole Reaction System; *P*: *P*-value shows the level of significance; Pb: Lead; PCBs: Polychlorinated biphenyls; R^2: R Square = gradient of the regression line and the strength of the relationship between a predictor and the outcome; r_p: Pearson's correlation coefficient; r_s: Spearman's correlation coefficient; Sc: Scandium; Se: Selenium; WHO: World Health Organization; Y: Yttrium; Zn: Zinc; β: β coefficient = gradient of the regression line and the strength of the relationship between a predictor and the outcome.

7. Declarations

7.1 Ethics Approval and Consent to Participate

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The human biomonitoring study was approved by the Republic of Slovenia National Medical Ethics Committee (NMEC) with numbers of accordance 42/12/07 and 53/07/09. Participation of women was voluntary; informed consent was obtained from all individual participants included in the study. All samples and related information were coded and anonymised. The participants had the right to withdraw from the study at all stages of the study period.

7.2 Consent for Publication

Not applicable.

7.3 Availability of Data and Materials

The dataset used in this article was obtained from the National Human Biomonitoring Program in Slovenia. The complete dataset has not yet been published but is expected to be available within five years.

7.4 Competing Interests

The authors declare that they have no competing interests.

7.5 Funding

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7.6 Authors' Contributions

MJ and DM measured concentrations of selected elements in maternal blood samples by ICP-MS. MP, AS, JST and MJ carried out measurements of total mercury in maternal blood samples. MJ performed the statistical analysis. MJ, JST, DM, MH, ABK, LK, MK and JØO conceived the study, and participated in its design and coordination as well as helping to draft the manuscript. All authors read and approved the final manuscript.

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Chapter 4

Conclusions

The proposed dissertation represents an important contribution to current HBM studies in Slovenia and worldwide, because it complements the methods for assessing exposure to selected elements through determination of indicatory FA and their $\delta^{13}C_{FA}$ in human milk samples. Through their use we can improve the interpretation of results, especially in the case of seafood intake. It is an added value to the ongoing studies performed in the frame of public health studies.

Attention of the dissertation was devoted to the exposure to essential and toxic elements and other nutritionally rich components, such as FAs during pregnancy and the breastfeeding period, because they are recognized in case of an insufficient nutrients status or the presence of a too high exposure to potentially toxic substances as being potentially responsible for adverse development and health effects in children. Since in case of breastfeeding the quality of maternal milk influences the development of a child, the main objective was to obtain and evaluate the composition and stable carbon isotope ratios of FAs in human milk and their sources in connection with mother's dietary habits and living place. The purpose was a) to estimate the association between the contribution of selected elements in the body through seafood consumption and their concentration in human milk samples, and b) to improve the methods for accessing exposure to metals and metalloids through determination of indicatory FA and their $\delta^{13}C_{FA}$ in human milk samples as an indicator of dietary habits, especially for seafood *vs* territorial food. This was investigated since specific FAs are important for the optimal children's development and seafood contains many of them. The nutritional status of mothers is expressed in her body liquids (blood, milk). Since a mother's dietary habits during pregnancy are influencing the child directly in the womb and indirectly after birth via maternal milk, pregnant and lactating women are regarded as one of the most vulnerable groups. However, dietary recommendations for this group are inconsistent regarding seafood intake. Seafood, besides the beneficial ingredients (FAs, Se, etc.), contains also potentially toxic elements (As, Hg, PCBs, etc.). Information about seafood intake obtained from questionnaires is frequently imprecise, because participants do not necessarily know the precise answer to what kind of seafood, which species they consume, thus limiting proper associations between external and internal exposures as well as health effects. Thus, the purpose was to find a biomarker, which would give more precise information on dietary habits. Specific analyses of FAs and their $\delta^{13}C_{FA}$ in the human milk as an indicator of the consumption of seafood were applied. The study was used to confirm or reject the first and second hypothesis stated that: "Fatty acid composition of maternal milk and their $\delta^{13}C_{FA}$ could be used as an indicator of dietary habits, especially for seafood *vs* territorial food"; and "The methods for assessing the source of exposure to metals and metalloids could be improved through determination of

indicatory FAs and their $\delta^{13}C_{FA}$ in human milk samples as an indicator of dietary habits, especially for seafood."

In the first part of the dissertation, the study results of FAs and their $\delta^{13}C_{FA}$ in mature human milk from two geographically distinct areas, KP (coastal area) and MS (inland area), in Slovenia are presented. The results of the present study are unique because only a few studies have reported the composition or/and stable carbon isotope ratios of FAs and selected elements in human milk worldwide, and the present study is the first to investigate stable carbon isotope ratios of FAs ($\delta^{13}C_{FA}$) in human milk in Slovenia. First, only FAs differences between areas were evaluated. Differences were expected in the dietary habits and the frequency of consumption of some local food items. The questionnaire data of dietary habits showed that the study areas (inland *vs* coastal) differed statistically significantly in terms of the intake of game (lower in inland area), freshwater fish (higher in inland area), fresh seafood (lower in inland area), and frozen seafood (higher in inland area). The results of the FA composition of human milk showed that the mean percentages of 35 individual FAs differed statistically significantly between the study areas: 19 FAs have higher abundance in KP, while in MS only 16 FAs reflect higher levels. Despite the higher intake of fresh seafood in KP region, the levels of saturated and monounsaturated FAs were higher and the levels of PUFAs, ω-3, and ω-6 were lower compared to those in MS. This finding was not expected and indicates a discrepancy between the measured and self-reported data. Moreover, the sample size was low and probably not representative for the inland area as it was biased towards women with a higher education level, which usually correlates with a more balanced diet, possibly supported with dietary supplements for pregnant and nursing women. However, mothers' dietary habits were associated with the percentages of FAs. Further, literature-based comparisons were made for FAs, which correlated with the intake of specific dietary species. The study confirmed that the dietary habits of Slovenian inland and coastal primiparous women influenced the FAs composition of their milk samples. Because the self-reported data on food intake is difficult to fill in and has high uncertainty, this study should serve as a good starting point toward establishing more precise biomarkers of dietary intake. The next step was to include beside FAs also their $\delta^{13}C_{FA}$ values. The results showed that human milk samples from coastal (KP) and inland (MS) areas had different elemental and FA compositions and different FA stable isotope profiles, which is indicative of the different dietary habits and lifestyles in the two distinct study areas of Slovenia. Therefore, the FA composition and its $\delta^{13}C_{FA}$ values together with the elemental composition in maternal milk could be used as a marker of fresh seafood intake.

Data mining approaches confirmed that using the classifier for fresh seafood intake based on the seven variables, including elemental composition, levels of FAs, and $\delta^{13}C_{FA}$ values in milk, enables distinguishing between seafood consumption less than once per month *vs* at least once per month fresh with 84% accuracy. Clustering could be used to divide the data sets into two with 90% accuracy. A clustering approach based on the percentage of the FAs iC17:0, C4:0, C18:2ω6t, aC17:0, CLA, C22:4ω6, and $\delta^{13}C$ of C18:1ω9c is an accurate indicator to distinguish between higher and lower fresh seafood intake. This confirmed our hypothesis that stable isotope analysis in combination with elemental analysis is an important tool to distinguish between diets, particularly for seafood. These findings suggest that in human-related studies related to the risks and benefits of seafood consumption, the analyses of FAs, $\delta^{13}C_{FA}$ values, and elemental compositions can significantly improve the information on seafood consumption.

The last part of the dissertation investigates the relation between the child's birth weight and maternal blood levels of selected toxic and potentially toxic elements and socio-demographic characteristics and maternal diet and related to the last hypothesis: "Maternal dietary and other lifestyle habits are associated with the status of trace elements

in her body and can therefore influence child's health as indicated through birthweight." The main studied population was Slovenian lactating women in their childbearing years from the first Slovenian HBM. To the best of our knowledge it was the first study of its kind to examine the exposure to such a wide range of trace elements in relation to a child's birth weight while taking into account the socio-demographic characteristics and dietary habits of the mothers. Results showed that exposure to toxic elements was low and comparable to that in other populations. Most mothers did not exceed the given reference levels. The birth weight was in agreement with other literature studies. The results show that maternal age, pre-pregnancy body mass index, estimated gestational age, length of a child and marginally the gender of a child all influence the birth weight. Despite low, environmentally relevant exposure to the selected potentially toxic elements, a significant and positive correlation was observed between the birth weight and the Mn levels in blood from Slovenian mothers. Additional evaluations and further studies are needed to focus on this specific correlation and other potential associations.

The overall conclusion of this dissertation was that the routine HBM studies intended for spatial and time trends exposure to potentially toxic chemicals of population can benefit greatly from additional biomarker analysis, including compound-specific isotope signatures, which are useful for source apportionment of nutritional and toxic compounds. Moreover, data mining approaches may further improve data interpretation beyond classical statistical treatment of data in HBM studies.

In view of **future research activities**, I would suggest:

- For the investigation of FAs and $\delta^{13}C_{FA}$ values in human milk samples: firstly, to optimize the analytical method to obtain good chromatographic separation for long-chain FAs; secondly, to improve the method for the determination of $\delta^{13}C_{FA}$ using appropriate certificate reference materials for normalization; and thirdly, to increase a number of sample within selected population. This analytical approach would expand the dataset and thus, allow confirmation of the current and identify possible new biomarkers of fresh seafood consumption using the same data mining approaches.

- For the investigation of child's birth weight and selected elements in maternal blood samples, we suggest to follow-up the levels of selected elements also during the pregnancy (1-3 times), and to collect more complete questionnaire data on dietary habits with a focus on pre-pregnancy and changes during pregnancy dietary habits. Specific questions on dietary supplements should also be collected.

- An alternative way of assessing the maternal nutritional status during pregnancy, including FAs, which are available to the child during prenatal life, would be measuring FAs composition in maternal blood samples during pregnancy. That would give us a better fingerprint on the nutritional status of a mother. This will improve the possible use of data mining approaches, confirm the results of the present study and identify new possible biomarkers of specific dietary habits.

- However, among the limitations we cannot overlook the fact that FAs have very different metabolic pathways. The time period of breast milk sampling within the lactation period should be carefully defined. Moreover, is it feasible to get a specific finger-print of FAs and $\delta^{13}C_{FA}$ in human milk for different dietary habits? Selection of an appropriate biomarker for a specific purpose is crucial. For better understanding of the status of FAs before/during/after pregnancy in maternal milk and/or blood samples, their impact on maternal and child health, there should be more focused studies in this field with a different design and targeted aims.

- Using $\delta^{13}C_{FA}$ in such studies and investigation of new possible biomarkers in light of food intake is a step in the right way. The $\delta^{13}C_{FA}$ can have an important contribution to ongoing HBM studies and can significantly improve the reliability of the results and their interpretation, which has a further significance for the nutritional status and public health.

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Biography

The author of this thesis Marta Jagodic was born on June 7th, 1988 in Ljubljana, Slovenia. She finished primary school Davorin Jenko in Cerklje na Gorenjskem, Slovenia, and high school at the Diocesan Classical Gymnasium Ljubljana, Slovenia. In 2007, she enrolled in the undergraduate studies of "Chemistry" at the Faculty of Chemistry and Chemical Technology at the University of Ljubljana from where she obtained her BSc degree in chemistry with the defense entitled "Binding of ions to the unstructured fragment of the antitoxin CcdA from *Escherichia coli*" in 2012. In 2013, she started her PhD studies at the Jožef Stefan International Postgraduate School, Ljubljana, Slovenia, under the supervision of Prof. Dr. Milena Horvat.

The majority of experimental work related to her PhD was conducted at the Department of Environmental Sciences, Jožef Stefan Institute. The focus of her PhD research is the investigation of the relationship between the fatty acid composition, their stable isotopes ($\delta^{13}C_{FA}$) and trace element composition in human milk and mother's dietary habits and living area. The underlying purpose of the first objective was to improve the understanding of exposure to selected elements through determination of indicatory fatty acids and their compound-specific stable isotope signatures. The second objective of the dissertation was to investigate the associations between the levels of selected elements in maternal blood samples, maternal dietary and other life-style habits and the child's birth weight.

During her studies, she attended various workshops and national and international conferences related to her work. In 2015, she held a three-month internship at the research department of Stavanger University Hospital in cooperation with UIT the Arctic University of Norway, Tromsø. The exchange has improved her knowledge of biostatistics in human biomonitoring studies related to environmental pollution. In 2019, she undertook a one-month traineeship at the Department of Physical and Analytical Chemistry of the University of Oviedo, Spain, where she broadened her theoretical and practical knowledge in the field of analytical chemistry and isotope dilution mass spectrometry, which will play an important role in her future research work.