博士論文

Oxidative stress in indigenous populations under modernization: Investigation of the determinants and the consequences in Northern Laos

（近代化の途上にあるラオス北部少数民族集団の酸化ストレス：その決定要因および健康影響）

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Abbreviations

8-OHdG	8-hydroxy-2′-deoxyguanosine
ANOVA	analysis of variance
As	arsenic
BMI	body mass index
Cd	cadmium
CVD	cardiovascular disease
DBP	diastolic blood pressure
DBS	dried blood spot
ELISA	enzyme-linked immunosorbent assay
GPx	glutathione peroxidase
ICC	intraclass correlation coefficient
ICP-MS	inductively coupled plasma mass spectrometry
IPCS	international programme on chemical safety
LC-MS/MS	liquid chromatography tandem mass spectrometer
NCD	non-communicable disease
NIH	national institutes of health
Pb	lead
qPCR	quantitative polimerase chain reaction
ROS	reactive oxygen species
SBP	systolic blood pressure
Se	selenium

Abstract

Oxidative stress is a known risk factor for non-communicable diseases. Modernization-induced changes in subsistence and dietary patterns in a marginal population are expected to affect oxidative stress. I conducted a case study among indigenous populations in mountainous areas of Northern Laos to investigate determinants and health consequences of oxidative stress during modernization.

This study was conducted among 380 adults in three villages of Northern Laos with different levels of modernization.

In Chapter 1, I investigated the impact of modernization on trace element exposure (arsenic, cadmium, lead, and selenium) by measuring trace element concentrations in the participants' urine. The participants in more modernized villages had higher arsenic, lead, and selenium and lower cadmium concentrations in urine.

In Chapter 2, I investigated impact of modernization on oxidative stress which was assessed measuring three oxidative stress-related biomarkers (urinary 8-hydroxy-2´-deoxyguanosine [8-OHdG] and 8-isoprostane concentrations and blood telomere length). The participants in more modernized villages had higher urinary 8-OHdG concentration and shorter telomeres, while urinary 8-isoprostane concentration did not differ significantly among villages.

In Chapter 3, I investigated determinants and consequences of oxidative stress. The findings imply that exposure to arsenic and cadmium causes oxidative DNA and lipid damage, respectively, in the participants. Selenium may attenuate such oxidative damage. I have little evidence regarding the health consequences of oxidative stress.

In conclusion, specific experiences to indigenous populations of Northern Laos during modernization might result in the population-specific oxidative stress.

General introduction

According to the "health transition" theory (Riley, 2001), the disease burden shifts from communicable to non-communicable diseases (NCDs) when a population experiences modernization. I define modernization as the process of adopting the technologies, lifestyles, and social norms of economically developed populations. Anthropometric measures and risk factors of cardiovascular diseases are the most frequently studied biological traits in connection to modernization: studies in Samoan adults reported that blood pressure and the prevalence of obesity were higher in more modernized communities (Bindon & Baker, 1985; McGarvey & Baker, 1979); and in Tsimane' adults of lowland Bolivia, household expenditure on market foods was associated with higher body mass index (BMI), weight, percent body fat, and the prevalence of overweight/obesity (Rosinger et al., 2013).

Health transition process varies among populations even with similar degrees of modernization. For example, the prevalence of female obesity differed greatly among countries with similar gross national products (Monteiro et al., 2004). Mortality from cardiovascular diseases (CVDs) and cancer was high in rural areas of Bangladesh, despite the absence of significant modernization (Ahsan Karar et al., 2009). This interpopulation variability in susceptibility to NCDs during modernization has conventionally been explained by population-level differences in genetic traits, dietary habits, and/or physical activity patterns (Enas & Mehta, 1995; Hales & Barker, 1992; Lee et al., 2012; Nazroo, 1998; Popkin et al., 2009).

Oxidative stress is an imbalance between oxidation and antioxidant systems in the body (Yoshikawa & Naito, 2002). It is a known risk factor for NCDs (Maritim et al., 2003; Reuter et al., 2010). Recent studies suggested that individuals' oxidative stress levels can fluctuate with exposure to chemicals (e.g., toxic heavy metals and metalloids [hereafter

referred to collectively as “metals”]), dietary composition, physical activity, and lifestyle factors, such as smoking and drinking alcohol (Aseervatham et al., 2013). Furthermore, these factors are generally affected by modernization. Importantly, some of such changes (e.g., more agrochemical exposure and animal protein consumption) might increase oxidative stress, while others (e.g., lower smoking frequency and improved nutritional status) could decrease oxidative stress. Therefore, the impact of modernization on oxidative stress is population specific. The interpopulation variability in susceptibility to NCDs during modernization might be explained, at least in part, by population-specific oxidative stress changes resulting from modernization.

In mountainous areas of Northern Laos, where the study population of the present study resides, indigenous subsistence living was maintained until recent times because of the geographic remoteness of the areas. However, the people in these areas have begun to experience changes caused by modernization: swidden cultivation has been replaced by paddy cultivation; villagers collect wild edible plants less frequently than before; more people grow vegetable crops for money; and the consumption of animal meat and energy-dense Chinese-style food has increased gradually. These changes were driven primarily by economic investment from Chinese companies and rural development projects implemented by the Lao government (Thongmanivong & Fujita, 2006). Consequently, these areas are in the process of health transition. The proportion of deaths in Laos related to NCDs increased from 39.8% in 2000 to 65.3% in 2019 (World Health Organization, 2020). Similar trends are anticipated in mountainous areas of Northern Laos. Therefore, it would be worthwhile to study oxidative stress in the indigenous populations of Northern Laos undergoing modernization because such study may provide further insight into the roles of oxidative stress in their specific health transition.

This is a case study in the study field of human biology in indigenous populations inhabiting three villages in mountainous areas of Northern Laos with different levels of modernization. This study aimed to clarify how oxidative stress affects the health transition process in the study populations. This thesis consists of three chapters: in Chapter 1, I explored impact of modernization on trace element exposure, which could be one of the important determinants of health status; in Chapter 2, I examined the impact of modernization on oxidative stress by measuring three oxidative stress-related biomarkers; finally, in Chapter 3, I investigated determinants and consequences of oxidative stress, paying special attention to trace element exposure and dietary patterns.

Chapter 1

1. Impact of modernization on trace element exposure

1.1. Introduction

Modernization is one of most important factors explaining individual variation in biological traits seen in people in indigenous societies in the studies of human biology. In contrast, associations between modernization and exposure to environmental contaminants, such as heavy metals or persistent organic pollutants, have attracted less attention, probably because of the belief that people in indigenous societies, who usually inhabit nature-rich rural areas, are free from exposure to environmental contaminants. This is ironic because modernization has been driven by the use of various metals/chemicals.

Some heavy metals are major public health concerns because of their toxicity to humans, including the carcinogenicity of arsenic (As) and cadmium (Cd) (Ahsan et al., 2006; Chen et al., 2019; Pershagen, 1981), the nephrotoxicity of Cd and lead (Pb) (Friberg, 1984; Gonick, 2008; Goyer, 1989), and the neurotoxicity of Pb (Sanders et al., 2009). Furthermore, previous studies suggested that even in non-contaminated populations, exposure to these toxic heavy metals is associated with negative health outcomes such as high levels of oxidative stress (Ahamed & Siddiqui, 2007; Kippler et al., 2012; Kordas et al., 2018) and CVDs (Alissa & Ferns, 2011). In contrast, selenium (Se) is essential for physiological and biochemical processes in humans, and Se deficiency has been shown to cause congestive cardiomyopathy (known as Keshan disease) (Loscalzo, 2014). In addition, Se may have a protective effect against toxic heavy metals, especially As and Cd (Zwolak, 2020).

Each element has a specific distribution pattern on the Earth that is determined largely by its biogeochemical cycle; however, these patterns have been significantly modulated by anthropogenic activities, such as mining, transportation, and disposal of elements (Callender,

2014; International Programme on Chemical Safety [IPCS], 1977, 1981, 1992; Johnson, Fordyce, & Rayman, 2010). The amounts of elements that enter the human body are determined by the amounts circulating in the local ecosystem, and more importantly by the behavioral patterns of resident individuals. For example, consumption of rice is associated with higher levels of As and Cd exposure because rice absorbs As and Cd from the soil more efficiently than do other crops (Zhao & Wang, 2020); smoking also results in greater exposure to Cd and Pb (Pappas, 2011; Pinto et al., 2017). With regard to essential trace elements, animal-based foods generally contain more Se than do plant-based foods (National Institutes of Health [NIH], 2021).

Most biological monitoring of heavy metals in human populations has been conducted in industrially contaminated areas or natural "hotspots" (Ezaki et al., 2003; Komaromy-Hiller et al., 2000; Nakajima et al., 2005; Nordberg et al., 2005; Schell et al., 2003; Watanabe et al., 2001). There has been little biomonitoring of heavy metals in non-contaminated populations, especially in subsistence societies, even though such monitoring is potentially important, considering the ubiquity of the elements and the adverse health effects of low-level chronic exposure. The Se status of subsistence societies is also unknown. The exceptions are studies of Canadian Inuit that revealed high exposure to polychlorinated biphenyls from their carnivorous diet (Hild, 1998; Van Oostdam et al., 2005).

Rapid economic development has recently occurred in mountainous areas of Southeast Asia that are inhabited by ethnic-minority indigenous populations who depend on subsistence activities (Fox & Vogler, 2005; Rerkasem & Rerkasem, 1995; Rubiyanto & Hirota, 2021). Their indigenous diet consisted of plant-based foods that may not be rich in Se (Broegaard et al., 2017; NIH, 2021; Ogle, Hung, & Tuyet, 2001), while animal-based foods (e.g., meat and fish), which can be major sources of Se (Miyazaki et al., 2004; Yoshita et al., 1998), have come to be consumed more commonly (Delgado, 2003; York & Gossard, 2004).

Changes in lifestyle and diet can affect exposure to toxic heavy metals and Se intake (Almerud et al., 2021; Davis et al., 2017; Miyazaki et al., 2004; Pappas, 2011; Pinto et al., 2017; Seo et al., 2016; Song et al., 2017; Yoshita et al., 1998; Zhao & Wang, 2020). In addition, the introduction of agrochemicals, electronic devices, and construction materials may influence exposure to heavy metals (Anyanwu et al., 2018; IPCS, 1977, 1981, 1992). Interindividual variations in exposure to heavy metals and intake of Se are thought to increase in parallel with the increases in behavioral variation and economic inequality among individuals.

In this study, human biological monitoring of trace elements (As, Cd, Pb, and Se) was conducted using urine samples from indigenous populations of mountainous areas of Northern Laos. Associated factors were explored, with special attention paid to individual variations in the extent of modernization (considered as the transition from a subsistence- to market-oriented lifestyle). The objectives of this study were to characterize the trace element exposures among residents in marginal areas of Northern Laos, and to determine whether modernization-related variables can explain interindividual variations in the exposure. The findings will help to clarify the impacts of modernization on trace element exposures (toxic heavy metal exposure and Se intake) in marginal societies undergoing rapid economic development.

1.2. Methods

1.2.1. Study population and sampling

This study was conducted in three villages in Oudomxay Province in Northern Laos: Nam Nyon, Na Savang, and Na Lae (Figure 1-1 and Table 1-1). Nam Nyon is in a remote mountainous region, where the inhabitants practice shifting cultivation and gather wild edible

plants (subsistence-oriented economy). The village is not accessible by road during the rainy season. Na Savang is in a flat basin, and the main subsistence activity is irrigated wet rice farming. Residents have better access to the commercial center in Namo (district capital) than do those in Nam Nyon. Na Lae is a village in the provincial capital (Oudomxay), where people purchase most of their food from markets and shops (market-oriented economy). This study was conducted as a part of the project investigating the health transition among indigenous populations in mountainous areas of Northern Laos. I participated in the research group that consists of researchers with various backgrounds (e.g., anthropology, microbiology, analytical chemistry, etc.) and primarily worked on biomarker measurements to assess chemical exposure and health status using techniques in analytical chemistry.

The survey was conducted in August 2018 (rainy season) in Na Lae, and in March 2019 (dry season) in Nam Nyon and Na Savang. We should note that the samples were collected in rainy season in one village and in dry season in two villages, which means that the village difference in outcome variables can reflect not only the impact of modernization but also that of seasonality. However, Na Lae, where samples were collected in dry season, was the most modernized village and the seasonal changes in behavioral and dietary patterns did not seem to be remarkable; most of the participants worked in urban areas and purchased foods in local market or shops throughout the year. Thus, I assumed that the samples collected in Na Lae in dry season comparable with those collected in other two villages in rainy season.

I invited people aged 18 years or older in the target villages to participate in the study, provided they had neither a high fever nor diarrhea on the survey days. Prior to the survey, the heads of the villages explained the purpose of and need for the survey to the other villagers. The participation rate could not be calculated because the reliable lists of residents were not available in the target villages. Biological samples were collected in public

facilities, such as community centers. Height, body weight, and blood pressure (systolic and diastolic blood pressure [SBP and DBP]) were measured using a mobile stadiometer (Holtain Ltd., Crosswell, UK), a digital scale (Tanita, Tokyo, Japan), and a digital sphygmomanometer (Omron, Kyoto, Japan), respectively.

Face-to-face interviews were conducted to determine participants' sex, year of birth (to determine age at the time of the survey), smoking status (smoker/non-smoker), drinking habit (yes/no), roofing materials of their house (concrete, galvanized iron, wooden board, or cogon grass [Imperata cylindrica]), and major possessions (cell phone, tractor, motorcycle, car, truck, television, refrigerator). Each household was assigned an ID number. The possessions index was calculated by counting the number of possessions (range: 0–7). In addition, I also asked the participants about their weekly meat consumption (Appendix 1).

The participants were asked to collect spot urine samples using a urine cup (As One Co. Ltd., Osaka, Japan). Approximately 8 mL of urine was dispensed into two screw-capped polypropylene tubes (4 mL/tube) that were thoroughly washed with 15% HNO_3 (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) and rinsed with ultrapure water (Milli-Q; Merck KGaA, Darmstadt, Germany) prior to sampling. The collected urine was stored at −80°C until analysis. I obtained urine samples, anthropometric data, and completed questionnaires from 341 of the initial 380 participants.

Written informed consent was obtained from each participant before the survey. This study was approved by the research ethics committee of the Graduate School of Medicine, The University of Tokyo (approval number: 12033-(3)), and the National Ethics Committee for Health Research, Ministry of Health, Lao PDR (approval number: 2018.22.MP) (Appendices 2–6).

1.2.2. Measurement of urinary trace element concentrations

Urinary concentrations of As, Cd, Pb, and Se were measured by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7500ce; Agilent Technologies, Santa Clara, CA, USA) with a collision/reaction cell. I quantified total As in urine without speciation analysis. Urinary total As concentration was assumed to approximately reflect the exposure to toxic inorganic As of the study participants, because they rarely consumed saltwater fish or other seafood containing organic As compounds (which are less toxic than inorganic As). I simultaneously measured urinary molybdenum concentration, and a mathematical method was applied to correct for the effect of interference by molybdenum oxide on Cd (Mizuno et al., 2018). To prepare for ICP-MS analysis, 10-fold dilution with 0.5% HNO_3 (ultrapure nitric acid; Kanto Chemical Co., Inc., Tokyo, Japan) was performed. Acetic acid was added at 4% (ultrapure acetic acid; Kanto Chemical) as a carbon source to cancel carbon-related matrix effects on the As and Se analyses (Nakazawa et al., 2014) for each diluted urine sample. Then, diluted samples were filtered through a disposable cellulose acetate membrane filter (Sartorius, Göttingen, Germany). ICP multi-element standard solution (XSTC-622; SPEX, Metuchen, NJ, USA) was used to prepare working solutions at concentrations of 1 and 10 μg/kg (with the addition of 100 μg/kg standard solution for molybdenum).

The detection limits were sufficiently low for measurements of all urinary trace element concentrations. Seronorm Trace Elements Urine L-1 and -2 (SERO AS, Billingstad, Norway) were used for analytical quality assurance. The observed trace element concentrations of the reference materials were within the uncertainty range of the reference values, with small standard deviations, indicating that the analyses were accurate (Appendices 7 and 8).

Urine specific gravity was determined using a pocket refractometer (ATAGO Co., Ltd., Tokyo, Japan). In addition, a commercial kit (Lab Assay Creatinine Kit; FUJIFILM Wako Pure Chemical Corp.) was used to measure urinary creatinine concentrations based on Jaffe's reaction. I used urine specific gravity to adjust for urinary dilution, because it has been suggested to be more appropriate than adjustment for creatinine in populations with large interindividual variations in muscle mass and meat intake (Suwazono et al., 2005), as in the present study population.

Creatinine-adjusted urinary concentrations of trace elements were also calculated to allow comparison of the current results with previous reports. I compared the urinary concentrations of the four trace elements against the values reported in general populations (defined as those with no known severe exposure to toxic heavy metals or excessive/deficient Se intake) in Asian countries (e.g., China and Japan), the USA, and European countries (e.g., Spain and German), those in populations in contaminated areas, which were defined as populations known to have been severely exposed to toxic heavy metals (due to contaminated ground water, drinking water, foods, and air, occupational exposure, etc.) in Bangladesh, China, and Japan, and those in populations with Se intake suspected to be deficient in China, Poland, and India.

1.2.3. Urinary concentrations of As, Cd, Pb, and Se: suitability as biomarkers of exposure/intake

The most commonly used short-term biomarker of As exposure is the urinary concentration (Hughes, 2006; Marchiset-Ferlay et al., 2012), while the serum/plasma concentration is a short-term biomarker of Se intake (Ashton et al., 2009). Long-term exposure/intake is assessed based on urinary concentration for Cd (Borjesson et al., 1997;

Vacchi-Suzzi et al., 2016) and whole blood concentration for Pb (Sommar et al., 2014) and Se (Hambidge, 2003). Therefore, it should be noted that urinary concentrations are not commonly used biomarkers of Pb exposure and Se intake. However, as urinary Pb concentration is significantly correlated with whole blood concentration, especially in occupationally exposed populations (Bergdahl et al., 1997; Fukui et al., 1999; Gulson et al., 1998; Shimbo et al., 2000; Sommar et al., 2014), it can also reflect interindividual variation in exposure in high-exposure population. As urinary Se concentration was shown to be significantly correlated with plasma concentration or intake (Alaejos & Romero, 1993; Longnecker et al., 1996; Wąsowicz & Zachara, 1987), it can also be used as a short-term biomarker of intake.

1.2.4. Modernization-related variables

To explore relationships between modernization and urinary trace element concentrations, I used the following four modernization-related variables, which are thought to reflect the transition from subsistence- to market-oriented lifestyles.

First, village (Nam Nyon, Na Savang, and Na Lae) was taken as the population-level variable of modernization; of the three villages, Nam Nyon was the least modernized and Na Lae was the most modernized; Na Savang was intermediate.

Second, roofing material was used as a household-level indicator of the degree of dependence on the cash economy. As building a concrete roof takes a relatively long time (several years) and has a high cost (> USD $1,000), only individuals with a substantial cash income can build such houses. I assumed that the lifestyle of participants inhabiting a house with a concrete roof was more modernized than that of those inhabiting a house constructed with other roofing materials.

The possessions index was also used as an indicator of modernization. It was generally expected that participants with a higher income would have more possessions. Unlike a house with a concrete roof, the possessions of interest can be purchased at reasonably low cost (e.g., a motorcycle costs around USD $300), so I assumed that the possessions index would be informative regarding earlier stages of modernization.

Finally, I took the BMI, calculated as body weight (kg) divided by height squared (m^2), as a marker of modernization-induced nutritional changes (Bu et al., 2021; Xu et al., 2016).

1.2.5. Statistical analysis

Urinary trace element concentrations were log-transformed to approximate a normal distribution. I divided the four types of house roofing material into two groups: "concrete" and "others." The chi-square test was used to determine differences in sex, smoking status, drinking habit, roofing material, and possessions among the villages. I examined the differences in age and possessions index among the villages using the Kruskal–Wallis test, while differences in BMI, blood pressure, and urinary concentrations of As, Cd, Pb, and Se were examined using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test for urinary trace element concentrations. Following these bivariate analyses, I performed multilevel analyses with household as a random effect to examine the associations of the modernization-related variables (village, roofing material, possessions index, and BMI) with urinary concentrations of As, Cd, Pb, and Se after adjusting for the covariates (sex, age, and smoking status). The covariates were selected based on previous studies: sex (Berglund et al., 2011; Jin et al., 2000; Olsson et al., 2002), age (Berglund et al., 2011; Inoue et al., 2014; Rodríguez Rodríguez et al., 1995), and smoking status (Pappas,

2011; Pinto et al., 2017).The least square means of urinary trace element concentrations were also calculated using these multilevel models. All analyses were performed using R software (ver. 4.1.0; R Foundation for Statistical Computing, Vienna, Austria).

1.3. Results

Table 1-2 shows the general characteristics of the participants. The study population comprised 108 residents from 43 households in Nam Nyon, 150 from 97 households in Na Savang, and 83 from 70 households in Na Lae. There were significant differences among the villages in age, smoking status, BMI, blood pressure, roofing material, possessions (except truck), and the possessions index ($p < 0.05$), which confirmed that Na Lae and Nam Nyon were the most and least modernized villages, respectively.

Table 1-3 shows geometric mean (GM) and geometric standard deviation (GSD) of specific gravity-adjusted urinary concentrations of As, Cd, Pb, and Se (see Appendices 9 and 10 for unadjusted and creatinine-adjusted concentrations, arithmetic mean, range, and percentile values). Urinary As, Se, and Pb concentrations differed among villages.

Table 1-4 shows the results of multilevel analyses of the associations of urinary concentrations of As, Cd, Pb, and Se with the modernization-related variables (village, roof material, possessions index, and BMI) after adjusting for sex, age, and smoking status. The urinary concentrations of the four trace elements were significantly associated with village. Urinary Cd concentration was lower in the most modernized village (Na Lae) than in the least modernized village (Nam Nyon), while urinary Se concentration showed an increasing trend with the level of village modernization. Urinary As concentration did not show a linear trend with village modernization; it was lowest in Nam Nyon, but higher in Na Savang than in Na Lae. Urinary Pb concentration was higher in the most modernized village (Na Lae) than in

the least modernized village (Nam Nyon). In summary, urinary Se and Pb concentrations increased, and urinary Cd concentration decreased with the level of village modernization; urinary As concentration was not predicted by the degree of village modernization (see Figure 1-2 for a comparison of the least square means of urinary trace element concentrations among the villages).

Even after adjusting for the effect of village modernization, people living in a house with a concrete roof had higher urinary Se concentration. The possessions index and BMI were not associated with urinary concentration of As, Cd, Pb, or Se. Males had lower urinary Cd concentration and higher urinary Pb concentration than females; urinary concentrations of As, Cd, and Se increased with age, and urinary Cd and Pb concentrations were higher in smokers than in non-smokers.

1.4. Discussion

1.4.1. Urinary concentrations of As, Cd, Pb, and Se in marginal population of Northern Laos

First, I compared the urinary concentrations of As, Cd, Pb, and Se among the indigenous populations of Northern Laos to previously reported values in other populations (Table 1-5) (Aguilera et al., 2008; Batáriová et al., 2006; Becker et al., 2003; Caldwell et al., 2009; Combs et al., 2011; Ezaki et al., 2003; Fukui et al., 1999; Hasunuma et al., 1990; Hira et al., 2004; J. Huang et al., 1988; Ikeda et al., 2000; Inoue et al., 2014; Nakajima et al., 2005; Nordberg et al., 2005; Paschal et al., 1998; Ruiz et al., 2010; Tsuda et al., 1995; Wasowicz, 1989; Watanabe et al., 2001; Yang et al., 1983; Yao et al., 2021). The urinary concentrations of As and Cd in the present study were slightly higher than those reported in general populations, but lower than those in contaminated regions. The urinary Pb concentration of

the participants was equivalent to those reported in general populations, and much lower than those reported in contaminated regions. The urinary Se concentration in this study was lower than those in general populations. In summary, the study population had slightly higher urinary As and Cd concentrations, equivalent urinary Pb concentration, and lower urinary Se concentration in comparison to general populations in previous studies.

1.4.2. Potential health risks of heavy metal exposure and Se intake

The observed urinary concentrations of As and Cd implied potential health risks of exposure. The Agency for Toxic Substances and Disease Registry (2016) set the normal range of urinary total As concentration in the general population (no geographical contamination or occupational exposure) to < 100 µg/L. Urinary As concentration exceeded the normal range in 56 participants (16 %) in the present study. On the other hand, Gamo et al. (2006) reported that the risk of mild kidney disorder (evaluated based on β2-microglobulinuria) increased when urinary Cd concentration exceeded 2 µg/g creatinine, and 58 of the participants (17 %) in the present study exceeded this level. Since human population studies conducted in areas without known contamination of As or Cd have shown positive associations of As and Cd exposures with oxidative stress (Chung et al., 2008; M. Huang et al., 2009; Muzembo et al., 2012), which suggested that the As and Cd exposures observed in the present study might elevate oxidative stress among the participants. This aspect will be investigated in the below sections.

Urinary Se concentrations reported in populations thought to be Se deficient as follows: means of urinary Se concentrations were 7 µg/L in China (individuals with Keshan disease) (Yang et al., 1983), 11.4 µg/g creatinine in Poland (Wasowicz, 1989), and 9.15 and 9.05 µg/g creatinine (males and females, respectively) in India (Hira et al., 2004). In contrast,

urinary Se concentrations in populations living in regions where Se deficiency was not reported were 26 μg/L in China (Yang et al., 1983), 34.1–51.4 μg/g creatinine in Japan (Hasunuma et al., 1990), and 83.6 μg/g creatinine in the USA (Komaromy-Hiller et al., 2000). The urinary Se concentration of the participants in the present study fell in the range between the values reported in Se-deficient and Se-adequate populations. In fact, urinary Se concentrations of 85 participants (25 %) were < 8 μg/L, which is the sum of the mean and standard deviation of urinary Se concentration of patients with Keshan disease (Yang et al., 1983). Selenium deficiency is associated with increased risk of mortality, poor immune function, and cognitive decline (Rayman, 2012). In addition, Se is a cofactor of glutathione peroxidases (GPx), selenoproteins that act as antioxidative enzymes, so individuals with low Se nutritional status may have poor antioxidant capacity (Brigelius-Flohé & Maiorino, 2013). Therefore, Se deficiency might be a potential health problem in the study population.

As mentioned above, the urinary Pb concentration of the participants was equivalent to those reported in the general population, and it is unlikely that Pb had negative health effects on the present study population.

1.4.3. Comparison of urinary As, Cd, Pb, and Se concentrations among villages

In this study, I observed differences in urinary concentrations of trace elements among three villages with different levels of modernization. Urinary concentration of Cd was lower, while those of Se and Pb were higher, in the more modernized villages than in the least modernized village. Urinary As concentration was not predicted by the degree of modernization, although I observed differences among villages.

Before the survey, I assumed that exposure to As, Cd, and Pb, and intake of Se, would increase with modernization, as heavy metals are frequently present in industrially produced

commodities and Se is generally more abundant in modernized than in subsistence-oriented diets (Anyanwu et al., 2018; Miyazaki et al., 2004; York & Gossard, 2004). Urinary Pb and Se concentrations were consistent with these assumptions, while urinary As and Cd concentrations were not.

1.4.3.1. Modernization and urinary concentrations of Pb and Se

Urinary Pb concentration was higher in Na Lae than in Nam Nyon, which suggested that exposure to Pb increases with modernization in Northern Laos. It has been reported that, generally, more Pb is used and emitted in industrialized societies (IPCS, 1977). Potential sources of Pb exposure in Northern Laos are speculated to include industrial emissions (e.g., during smelting) and Pb-based paint. However, urinary Pb concentration in this study was in the range reported in general populations, suggesting that the increases in urinary Pb concentration in modernized villages may not be associated with health risks.

Higher urinary Se concentration in more modernized villages can be attributed to greater consumption of Se-rich foods by people who have adopted a modernized lifestyle. As urinary Se concentration was lower than previously reported values in general populations, a modernization-induced increase in urinary Se concentration would benefit the health of the residents of Northern Laos. The indigenous diet in this region consists of rice and wild edible plants, while people in modernized villages consume more meat and eggs (Khonje & Qaim, 2019; Miyazaki et al., 2004; NIH, 2021; York & Gossard, 2004; Yoshita et al., 1998). According to the food composition table in Japan, Se contents per 100 g of edible portion are 1 μg for wet rice and ≤ 3 μg for most leafy vegetables (e.g., cabbage, lettuce, and spinach); the values are far higher for meat (8–22 μg for beef, 13–27 μg for pork, and 14–22 μg for chicken) and eggs (24 μg) (Ministry of Education, Culture, Sports, Science and Technology,

2020). In the present study, meat was consumed more frequently in the more modernized villages, and urinary Se concentration was higher among participants who reported consuming meat more frequently (see Appendix 11-(A) and (B)). Therefore, a higher level of meat consumption in more modernized villages may have increased urinary Se concentration in the present study population.

1.4.3.2. Modernization and urinary concentrations of As and Cd

The reasons for the unexpected findings of a remarkable difference in urinary As concentration between Nam Nyon and Na Savang, and lower urinary Cd concentration in more modernized villages, were investigated in association with differences in the rice cultivation systems. The villagers in Nam Nyon grew mainly dry rice by swidden farming, while those in Na Savang cultivated rice in irrigated paddy fields. The villagers in Na Lae purchased rice produced mainly in irrigated paddy fields. Flooding during the growing season in paddy fields enables rice plants to incorporate As from the soil (Takahashi et al., 2004), while it can reduce rice-grain Cd concentrations (Arao et al., 2009). A field experiment revealed that As concentration in rice grains was 4.7–9.1 times higher when cropped under flooded conditions than when cropped under dry conditions, whereas Cd concentration in rice grain cropped under flooded conditions was only 1–3% of that of rice grain cropped under the dry conditions (L. Sun et al., 2014). Therefore, dry-grown rice is thought to have lower As and higher Cd concentrations than wet-grown rice, although the actual concentrations in rice grains depend on the As and Cd concentrations in the soil in each ecosystem. Nam Nyon residents consumed mainly dry-grown rice, while the people in the other villages usually consumed wet-grown rice; this could have led to the differences in urinary concentrations of As and Cd observed among villages. The lower urinary As concentration in Na Lae than in

Na Savang could be attributable to the lower rice consumption in the former than the latter village. The people in the more modernized villages were more likely to have been exposed to Cd from industrialized commodities such as fossil fuel, nickel-cadmium batteries (IPCS, 1992), and cement dust (Işıklı et al., 2006), but any such exposure may have been masked by the reduced Cd content of the rice consumed in the more modernized villages.

The findings suggested that the transition in rice cultivation systems from shifting farming to irrigated paddy farming, one of the important aspects of modernization in Northern Laos, had distinct impact on As and Cd exposures among the residents.

1.4.4. Urinary Se concentration and roofing materials

Even after controlling for village-level effects of modernization, urinary Se concentration was higher among the individuals living in more modernized houses with a concrete roof. As the roofing material was assumed to reflect household-level modernization, this finding was consistent with the above discussion, i.e., the modernized diet is rich in Se such that urinary Se concentration was higher in modernized individuals (York & Gossard, 2004; Yoshita et al., 1998). The participants living in a house with a concrete roof reported consuming meat more frequently than those living in a house constructed with other roofing materials (see Appendix 11-(C)).

1.4.5. Limitations

This study had four main limitations. First, although I assumed that village, roofing material, possessions index, and BMI well reflect the degree of modernization of participants, they were not statistically validated as modernization indices. The study villages were selected considering the modernization level (e.g., type of rice cultivation system and

accessibility to market), while the difference in modernization level among villages could not be shown quantitatively. Second, I measured trace element concentrations only in single spot urine samples, so the capacity for assessment of trace element exposure may have been limited. Urinary Pb concentration is not a sensitive biomarker of exposure, particularly in the low range of urinary Pb concentration observed in this study (Bergdahl et al., 1997; Fukui et al., 1999; Gulson et al., 1998; Shimbo et al., 2000; Sommar et al., 2014). Measuring concentrations of trace elements in whole blood may provide further information facilitating exposure assessments. Third, I did not measure concentrations of trace elements in environmental samples, such as soil and food, as I was not able to transport these samples. Rice was expected to be a major source of As and Cd exposures among the participants; thus, As and Cd concentrations in soil of farming fields would have significant effect on As and Cd concentrations in rice grains. Analyzing concentrations of trace elements in environmental samples would also be useful for clarifying the exposure/intake routes of each trace element. Finally, although seasonal changes in behavioral and dietary patterns among the participants, particularly among those in Nam Nyon and Na Savang, could affect their exposure to trace elements, I measured trace element concentrations in the participants' urine collected only in one season (dry season in Nam Nyon and Na Savang and rainy season in Na Lae). Investigation of seasonal changes in trace element exposure is the next study target.

1.5. Conclusions

In indigenous populations in mountainous areas of Northern Laos, urinary concentration of Cd was lower, while those of Se and Pb were higher, in more modernized villages. Urinary As concentration was lowest in the least modernized village, where the residents consumed mainly dry-grown rice. These findings could be explained by assuming a

Se-rich diet, greater exposure to industrial commodities in modernized villages, and lower Cd and higher As contents of wet- than dry-grown rice. Overall, modernization would probably have a positive impact on the health of local people due to increased Se consumption.

Chapter 2

2. Impact of modernization on oxidative stress

2.1. Introduction

Individual-level oxidative stress can be assessed by various biomarkers, each of which can reflect different aspects of oxidative stress. Urinary 8-hydroxy-2´-deoxyguanosine (8-OHdG: oxidized deoxyguanosine) and 8-isoprostane (a prostaglandin-like compound formed from arachidonic acid peroxidation) concentrations are the most common biomarkers of oxidative stress, which can reflect oxidative damage to DNA (Graille et al., 2020a; Kasai, 1997; Kasai et al., 1984, 2008; Loft et al., 1992) and lipid (Davì et al., 2004; Graille et al., 2020b; Roberts II & Morrow, 2002), respectively. Additionally, blood telomere length is a recognized biomarker of cumulative oxidative stress. Telomeres have a guanine-rich structure that is susceptible to reactive oxygen species (ROS) (Babizhayev et al., 2011; Blackburn, 1991; Houben et al., 2008; O'Donovan et al., 2011; J. Y. Y. Wong et al., 2014), and they shorten with every cell divisions, attrition of which can be accelerated under oxidative stress. Thus, telomere length can be regarded as a biomarker related to oxidative stress, especially that of cumulative oxidative DNA damage.

The objective of the present study was to examine the associations between modernization and three oxidative stress-related biomarkers (urinary 8-OHdG and 8-isoprostane concentrations and telomere length of blood DNA) in indigenous populations of Northern Laos. This case study will provide further insight into changes in oxidative stress in ethnic minorities undergoing the health transition process.

2.2. Methods

2.2.1. Study population and sampling

Characteristics of the study population and sampling has already been described in Chapter 1. Dried blood spot (DBS) samples had also been collected on Whatman 903 Protein Saver Cards (Cytiva, Marlborough, MA, USA) by finger prick in the survey and stored at −80℃ until the following biomarker analysis as well as urine samples.

2.2.2. Measurement of urinary 8-OHdG and 8-isoprostane concentrations and DBS telomere length

I measured urinary 8-OHdG and 8-isoprostane concentrations using liquid chromatography-tandem mass spectrometry (LC-MS/MS) following solid-phase extraction (Inaba et al., 2011; Oba et al., 2019). Urine was processed with a C18 solid-phase extraction cartridge (YMC Co., Ltd., Kyoto, Japan) for both 8-OHdG and 8-isoprostane. For 8-isoprostane only, further extraction followed using ENVI-Carb^{TM} cartridges (Merck KGaA, Darmstadt, Germany). For LC-MS/MS, I used a Xevo TQ-S spectrometer (Waters, Milford, MA, USA) with C18 columns in multiple-reaction-monitoring (MRM) mode. Electrospray ionization was positive for 8-OHdG and negative for 8-isoprostane. MassLynx software was used for data acquisition and analysis. The limits of quantitation were 0.05 and 0.1 μg/L for 8-OHdG and 8-isoprostane, respectively; the detection rate was 100% for both. To adjust for urinary dilution, urine specific gravity and urinary creatinine concentration were measured (Mizuno, Masuoka, et al., 2021).

I used urine specific gravity rather than urinary creatinine to adjust for urinary dilution, because it has been suggested that use of urine specific gravity is more appropriate than adjustment for creatinine in populations with large interindividual variations in muscle mass and meat intake (Suwazono et al., 2005), as was the case in the present study

population. I also calculated creatinine-adjusted concentrations to allow comparison of the results of the present study with those of previous studies. I compared the urinary 8-OHdG and 8-isoprostane concentrations with those reported in studies in Japan, China, Korea, Taiwan, and the USA, of apparently healthy adults with a mean BMI under 25. I excluded several studies from the analysis, including those conducted in small populations ($n < 50$), those targeting patients with diagnosed diseases, and those measuring concentrations using immunological assays (e.g., enzyme-linked immunosorbent assay [ELISA]), which are less accurate than chromatography (e.g., LC with MS or electrochemical detection).

I also measured telomere length in DNA. Chromosomal DNA was extracted from six DBS punches (3 mm in diameter) using a QIAamp DNA Investigator Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. Telomere length was measured by the quantitative polymerase chain reaction (qPCR) method (Cawthon, 2002; Mizuno, Hur, et al., 2021) using tel1-tel2 and 36B4u-36B4d primer sets on a LightCycler Nano instrument (Roche, Penzberg, Germany). This method provides the ratio of telomere repeat copy number (T) to single-copy gene number (S) (i.e., the T/S ratio).

2.2.3. Statistical analysis

Urinary 8-OHdG and 8-isoprostane concentrations were adjusted for specific gravity and logarithmically transformed to approximate a normal distribution. I used one-way ANOVA, followed by Tukey's multiple comparison test, to examine differences among villages in oxidative stress-related biomarkers (urinary 8-OHdG and 8-isoprostane concentrations and telomere length), and calculated Pearson's correlation coefficients for telomere length and urinary 8-OHdG and 8-isoprostane concentrations. Following these bivariate analyses, I performed multilevel analyses with household as a random effect to

examine the associations between modernization level of village and oxidative stress-related biomarkers after adjusting for covariates (sex, age, smoking status, drinking habits, and BMI). All analyses were performed using R software (version 4.1.0; R Development Core Team, Vienna, Austria).

2.3. Results

Table 2-1 shows the geometric means (GMs) and geometric standard deviations (GSDs) for the specific gravity-adjusted urinary 8-OHdG and 8-isoprostane concentrations, and the arithmetic means (AMs) and standard deviations (SDs) of telomere length. The unadjusted and creatinine-adjusted urinary 8-OHdG and 8-isoprostane concentrations are shown in Appendices 12 and 13. The participants in Nam Nyon showed lower urinary 8-OHdG concentration and longer telomeres compared to those in more modernized villages. In contrast, there were no differences in urinary 8-isoprostane concentration among the villages.

Table 2-2 shows the associations between the modernization level of villages and urinary 8-OHdG and 8-isoprostane concentrations and telomere length. In the multilevel analysis, the lowest urinary 8-OHdG concentration and the longest telomere were observed in the least modernized village (Nam Nyon). Urinary 8-isoprostane concentration did not differ by village. Males had lower urinary 8-isoprostane concentration than females, and smokers had higher urinary 8-isoprostane concentration than non-smokers. BMI was negatively associated with urinary 8-OHdG concentration and positively associated with urinary 8-isoprostane concentration.

Figure 2-1 shows scatterplots of telomere length and urinary 8-OHdG and 8-isoprostane concentrations. Telomere length was negatively correlated with urinary 8-OHdG concentration ($r = -0.15$, 95% confidence interval = -0.25, -0.04), but not significantly

correlated with urinary 8-isoprostane concentration (r = −0.01, 95% confidence interval = −0.12, 0.10).

2.4. Discussion

2.4.1. Urinary 8-OHdG and 8-isoprostane concentrations among indigenous populations in Northern Laos

Table 2-3 compares the urinary 8-OHdG and 8-isoprostane concentrations observed in the present study with those reported in previous studies of apparently healthy adult populations in Asia and the USA (El-Bayoumy et al., 2002; Il'yasova et al., 2018; Jacob et al., 2003; H. Kim et al., 2011; Nakano et al., 2003; Pan et al., 2008; Shimanoe et al., 2018; R.-H. Wong et al., 2008; Yuan et al., 2018). The mean urinary 8-OHdG concentration in the present study was lower than those reported in previous studies, whereas the mean urinary 8-isoprostane concentration was higher than those reported in previous studies. Therefore, the present study population may be characterized by lower oxidative DNA damage and higher oxidative lipid damage compared with the previously reported values among apparently healthy adult populations.

2.4.2. Associations of modernization with urinary 8-OHdG and 8-isoprostane concentrations and telomere length

The comparison of oxidative stress-related biomarkers among the villages found that higher urinary 8-OHdG concentrations and shorter telomeres were observed in the more modernized villages, whereas urinary 8-isoprostane concentrations did not differ by village.

The participants in this study had urinary 8-OHdG concentration lower than the range reported in previous studies, so I speculated that some of the lifestyle changes related to modernization exacerbated oxidative DNA damage (Aseervatham et al., 2013; Banerjee et al., 2001; Longo & Fontana, 2010; Macho-González et al., 2020). Conversely, urinary 8-isoprostane concentration in the participants of the present study was at the high end of the range of values reported in previous studies. Oxidative lipid damage has historically been high in the ethnic minorities of Northern Laos because of certain characteristics of their indigenous lifestyle (Aseervatham et al., 2013; Nasca et al., 2010; Sakano et al., 2009). This damage was not exacerbated by modernization-induced lifestyle changes. Previous studies have also used urinary 8-OHdG and 8-isoprostane concentrations as biomarkers of oxidative stress, but there has been little research into the difference between determinants of urinary 8-OHdG and 8-isoprostane concentrations (Gao et al., 2017; Kordas et al., 2018; Lai et al., 2016; Sakano et al., 2009; Xie et al., 2019). The cause of the differential responses of urinary 8-OHdG and 8-isoprostane concentrations to modernization should be explored further.

Since 8-OHdG is excreted into the urine during the course of base-excision repair of oxidative DNA damage (David et al., 2007), urinary 8-OHdG concentration could reflect both oxidative DNA damage and its repair (Cooke et al., 2005; Il'yasova et al., 2012; Wilson et al., 2011). Modernization-related lifestyle changes are expected to increase the capacity for DNA repair. For example, zinc intake is higher among people in more modernized villages; zinc is an essential component of enzymes involved in the repair of oxidative DNA damage (e.g., 8-oxoguanine glycosylase) (McAfee et al., 2010; York & Gossard, 2004). It follows that higher urinary 8-OHdG concentrations in more modernized villages are attributable to both modernization-induced oxidative stress and modernization-enhanced DNA repair capacity. Further studies are needed to precisely determine the modernization-related lifestyle changes associated with oxidative DNA damage and improvements in DNA repair capacity.

Telomere length is thought to be a biomarker of cumulative oxidative DNA damage (Babizhayev et al., 2011; Barnes et al., 2019), and differences among villages in telomere length were consistent with those in urinary 8-OHdG concentration. In fact, telomere length and urinary 8-OHdG concentration were negatively correlated. I note that recent studies have reported that differences in telomere length in adults were determined primarily by telomere length at birth (Benetos et al., 2013, 2019; Cui et al., 2016), and that telomere length can be inherited (Broer et al., 2013; J.-H. Kim et al., 2020). The differences in telomere length among the villages in this study may reflect the influence of both indigenous population-specific oxidative stress and modernization-induced oxidative stress.

2.4.3. Oxidative stress-related biomarkers and the risk of NCDs

Studies have reported close relationships between oxidative stress-related biomarkers and the risks for NCDs. Higher urinary 8-OHdG and 8-isoprostane concentrations and shorter telomeres have been observed among patients with NCDs, including CVDs, diabetes, and cancer (Davì et al., 2004; Patrignani & Tacconelli, 2005; Rizvi et al., 2015; Wu et al., 2004). Furthermore, some prospective studies have suggested that NCDs can be predicted by oxidative stress-related biomarkers. For example, the risk of death from CVDs was higher in those with higher urinary concentrations of 8-OHdG or 8-isoprostane, or shorter telomeres (Gohbara et al., 2021; Lin et al., 2015; Stefler et al., 2018). Therefore, although the causal relationship between oxidative damage and NCDs is still unclear, it is possible that modernization-induced oxidative DNA damage increases the risk for NCDs. High levels of oxidative lipid damage have already been shown to increase such risks in the indigenous populations of Northern Laos. Differences in the relationships of the three oxidative stress-

related biomarkers with NCD risk have not been explored. Additional prospective studies are needed to determine the sensitivity of each biomarker.

2.4.4. Strengths and limitations

To the best of my knowledge, this is the first study to investigate the association between modernization and oxidative stress in ethnic minorities under the momentous health transition process. I performed simultaneous evaluations of three oxidative stress-related biomarkers that would respectively reflect oxidative DNA and lipid damage and cumulative oxidative stress. Furthermore, urinary 8-OHdG and 8-isoprostane concentrations were measured by LC-MS/MS. This method provides far more reliable values than ELISA, particularly in low concentration ranges.

One important limitation of this study should be noted: I used only urinary 8-OHdG concentration as a proxy of oxidative DNA damage. Urinary 8-OHdG is an end product of oxidative DNA damage repair. Measurement of 8-OHdG in extracted DNA could assess oxidative DNA damage which has not been excised by the repair system and remains in DNA.

2.5. Conclusions

This comparative study of three villages in Northern Laos explored the impact of modernization on oxidative stress in ethnic minorities during the health transition process. Urinary 8-OHdG concentration was low, whereas urinary 8-isoprostane concentration was high, in comparison with values previously reported in apparently healthy adults. Oxidative DNA damage was higher in more modernized villages, but oxidative lipid damage did not differ by village. It is possible that population-specific experiences of modernization

influence the health transition process, and that oxidative stress is an important intermediate variable.

Chapter 3

3. Determinants and consequences of individual variation in oxidative stress

3.1. Introduction

Many studies have investigated factors that explain individual variations in oxidative stress among various populations, such as people in clinical settings, urban areas, or special environments (e.g., contamination, occupational exposure, and nutrient deficiency), who would be predisposed to NCDs (Aseervatham et al., 2013; Pilger & Rüdiger, 2006; Valavanidis et al., 2009). In contrast, few studies have targeted a marginal population with a low risk of NCDs, although studies regarding determinants of oxidative stress are essential considering the drastic health transition in such populations.

The study among indigenous populations in Northern Laos reported in Chapter 2 revealed that urinary 8-OHdG and 8-isoprostane concentrations and blood telomere length, all oxidative stress-related biomarkers that are widely used in epidemiologic studies, showed distinct patterns. Urinary 8-OHdG concentration, a marker of oxidative DNA damage (Valavanidis et al., 2009), exhibited an increasing trend with modernization, but remained lower than values reported in previous studies among apparently healthy adult populations. Urinary 8-isoprostane concentration, a marker of oxidative lipid damage (Montuschi et al., 2004), was higher than the values reported in previous studies among healthy adults; they were not affected by modernization. Blood telomere length reflects cumulative oxidative stress because its guanine-rich structure is susceptible to the effects of ROS; telomere shortening during each iteration of cell division is accelerated by oxidative stress (Houben et al., 2008; Reichert & Stier, 2017). People in more modernized villages exhibit shorter telomeres. However, it remains unclear which factors are associated with specific oxidative stress-related biomarkers in the study population.

Among potential determinants of oxidative stress in the people of Northern Laos, I suspect that trace element exposure (e.g., As, Cd, and Se) is of importance. In the current study population, As and Cd exposures were high in comparison to values reported in general populations without known contaminations, whereas Se intake was potentially deficient (Mizuno, Masuoka, et al., 2021). Furthermore, there were large interindividual variations in urinary concentrations of As, Cd, and Se in the study population; the determinants of these variations were presumed to reflect changes in exposure resulting from modernization, e.g., rice cultivation systems (the transition from dry-grown rice by swidden farming to wet-grown rice by paddy farming) and diet (greater consumption of animal-based foods). Experimental studies have shown that exposure to As and Cd elevates oxidative stress in organisms; specifically, As generates ROS during its methylation process (Yamanaka et al., 1990, 2001; Yamanaka & Okada, 1994) and Cd contributes to ROS production indirectly through the Fenton reaction involving the replacement of iron and copper in proteins by Cd (Casalino et al., 1997; Wätjen & Beyersmann, 2004). Human population studies have also shown positive associations of As and Cd exposures with oxidative stress (Chung et al., 2008; M. Huang et al., 2009; Muzembo et al., 2012). However, the associations were contradictory in areas without known serious contaminations by those toxicants (Büyükşekerci et al., 2018; Farzan et al., 2017; Hambach et al., 2013; He et al., 2020; M. Huang et al., 2009; S. S. Kim et al., 2019); furthermore, it has not been determined whether these exposures have distinct associations with individual oxidative stress-related biomarkers. In contrast, Se, an essential trace element, can suppress oxidative stress because it is a cofactor of GPx (Hatfield et al., 2016). Several compounds that contain Se can form complexes with As or Cd and suppress their toxicities (Zwolak, 2020).

Another potential determinant of oxidative stress among the people in Northern Laos is dietary patterns. Nutritionists have argued that dietary patterns strongly impact oxidative

stress. Considering that vegetables and fruits contain an abundance of antioxidative nutrients and phytochemicals (Kaur & Kapoor, 2001; Slavin & Lloyd, 2012), plant-based dietary patterns have generally been associated with lower oxidative stress (Aleksandrova et al., 2021). The previous field observations have revealed large interindividual variations in diet; some individuals depended heavily on dry-grown rice and wild edible plants, while others frequently consumed meats or energy-dense foods purchased from markets or shops.

The objective of Chapter 3 was to investigate the individual determinants of three oxidative stress-related biomarkers (urinary 8-OHdG and 8-isoprostane concentrations and telomere length in blood DNA) among indigenous populations in Northern Laos. Trace element exposures and dietary patterns were regarded as potential determinants. Furthermore, this study explored associations of oxidative stress-related biomarkers with blood pressure, a risk factor for NCDs, as a health consequence of oxidative stress. Thus, this case study in Northern Laos is expected to provide insights into oxidative stress in marginal populations who usually have close interactions with the surrounding environment, but who are currently experiencing the effects of drastic modernization.

3.2. Methods

3.2.1. Study population and sampling

Characteristics of the study population and sampling has already been described in Chapter 1. Consumption frequencies of 27 food items were also asked and included in this Chapter (see below for the details of the method).

3.2.2. Biomarker measurements

Biomarker measurements, urinary As, Cd, Se, 8-OHdG, and 8-isoprostane concentrations and blood telomere length, have been described in Chapters 1 and 2. As discussed in Chapter 1, urinary Pb concentration is judged to have limited information as an exposure biomarker, thus was not analyzed in Chapter 3.

3.2.3. Consumption frequencies of 27 food items

I surveyed the participants' dietary patterns by face-to-face interviews regarding weekly consumption frequencies of 27 food items. Of the 27 items, 4 were wild plant foods (bitter bamboo shoot, river weed/algae, other wild vegetables, and wild fruits), 8 were locally produced foods (aquatic animals [e.g., frog, crab, and shrimp], bush meats, insects/larvae, organs of animals, contents of animal intestines, blood of animals, organs of fish, and rice), and 15 were newly introduced foods (corn, tuber crops, meats, poultry/eggs, cultivated leafy vegetables, cultivated fruits, herbs, spices, fish, coffee/tea, noodles, processed meats, canned fishes, sweets/snacks, and oil/fat).

3.2.4. Statistical analysis

Principal component analysis (PCA) was applied to the consumption frequencies of 15 food items to extract the participants' dietary patterns. Of the 27 food items, 11 items were not used in the analysis because more than half of the participants reported no consumption; rice was also excluded because all participants reported regular daily consumption of rice. The first principal component score was defined as "wild plant food score," which will be explained later. Differences in score among the three villages were tested by ANOVA. Urinary As, Cd, Se, 8-OHdG, and 8-isoprostane concentrations were adjusted for specific

gravity, then logarithmically transformed to approximate a normal distribution. I examined associations of oxidative stress-related biomarkers (urinary 8-OHdG and 8-isoprostane concentrations and telomere length) with urinary trace element (As, Cd, and Se) concentrations, interaction terms between the concentrations (As × Se and Cd × Se), and/or wild plant food score by performing bivariate linear regression and multilevel analyses considering household as a random effect and adjusted for sex, age, BMI, smoking status, and drinking habits. The associations were also explored after stratifying the participants by villages. Additionally, associations of oxidative stress-related biomarkers with blood pressure were examined using bivariate linear regression and multilevel analyses adjusted for the use of antihypertensive medications and the above covariates. All analyses were performed using R software (version 4.1.0; R Development Core Team, Vienna, Austria).

3.3. Results

Table 3-1 shows the consumption frequencies of 15 food items together with the results of PCA. The scree plot (Appendix 14) revealed that only the first component provided a meaningful reflection of the participants' dietary pattern, because there was large difference in eigenvalues between the first and second components and the second and the subsequent components showed low percentage of variance explained (e.g., second = 11.6 %, third = 9.6 %). The second and the subsequent components also did not show interpretable dietary patterns of the participants according to their factor loadings. The factor loadings of food items in the first component were strongly positive for the consumption frequencies of bitter bamboo shoot, river weed/algae, and wild vegetables, while they were strongly negative for meats, cultivated fruits, and noodles. Therefore, the first component score was presumed to reflect interindividual variation in dietary reliance on wild plant foods (hereafter, "wild plant

food score"). Scores were highest among participants in Nam Nyon (the least modernized village) and lowest among participants in Na Lae (the most modernized village) (Appendix 15), supporting my hypothesis that modernization is associated with lower consumption of wild plants and higher consumption of animal meats.

Table 3-2 shows the associations of urinary trace element concentrations and wild plant food scores with three oxidative stress-related biomarkers. In the multilevel analysis (Model 2), urinary As concentration was positively associated with urinary 8-OHdG concentration, whereas urinary Cd concentration was positively associated with urinary 8-isoprostane concentration. Additionally, the interaction terms (As × Se and Cd × Se) were negatively associated with urinary 8-OHdG and 8-isoprostane concentrations, respectively (see Appendices 16 and 17 for the details of the interactions). Surprisingly, urinary Se concentration was positively associated with urinary 8-OHdG concentration. Wild plant food score was positively associated with telomere length but not with urinary 8-OHdG or 8-isoprostane concentration.

The results of the stratified analysis by villages are shown in Appendices 18–20. The positive association between urinary Cd and 8-isoprostane concentrations was observed in all villages, while the positive association between urinary As or Se and 8-OHdG concentrations was not observed in either of the villages, probably due to small variation in urinary As and Se concentrations in each village.

Table 3-3 the associations of urinary 8-OHdG and 8-isoprostane concentrations and telomere length with blood pressure in the participants. The three oxidative stress-related biomarkers were not associated with blood pressure in the multilevel analysis.

3.4. Discussion

The primary findings of this study were as follows. First, urinary As concentration was positively associated with urinary 8-OHdG concentration, while the interaction term between urinary As and Se concentrations was negatively associated with urinary 8-OHdG concentration. Second, urinary Cd concentration was positively associated with urinary 8-isoprostane concentration, whereas the interaction term between urinary Cd and Se concentrations showed a negative association with urinary 8-isoprostane concentration. Third, urinary Se concentration was positively associated with urinary 8-OHdG concentration. Fourth, wild plant food score was positively associated with telomere length. Fifth, no associations of oxidative stress-related biomarkers with blood pressure were found.

3.4.1. Determinants of oxidative stress

3.4.1.1. Associations between urinary As and 8-OHdG concentrations

I found a positive association of urinary As concentration with urinary 8-OHdG concentration, an indicator of oxidative DNA damage. It has been speculated that As generates ROS during its methylation process (Jomova & Valko, 2011; Valko et al., 2005); during the process, dimethylarsinic radical and dimethylarsinic peroxyl radical can be formed along with ROS production (Yamanaka et al., 1990, 2001; Yamanaka & Okada, 1994). Additionally, H_2O_2 is produced in the oxidation of As(III) to As(V). Indirect ROS production mechanisms are also possible: for example, iron released from ferritin by dimethylarsinous acid can lead to ROS production via the Fenton reaction (Ahmad et al., 2000; Lloyd et al., 1997). Most population studies thus far have reported a positive association between As exposure and urinary 8-OHdG concentration, even among people who inhabited areas where severe As contamination was not recognized (Chung et al., 2008; M. Huang et al., 2009); those findings are consistent with the present results.

3.4.1.2. Associations between urinary Cd and 8-isoprostane concentrations

Urinary Cd concentration was positively associated with urinary 8-isoprostane concentration. It has been proposed that Cd itself cannot generate ROS but can indirectly contribute to ROS production: Cd can replace iron and copper in cytoplasmic and membrane proteins (e.g., ferritin, apoferritin, and metallothionein), thereby increasing unbound free iron and copper ions that participate in oxidative stress via the Fenton reaction (Casalino et al., 1997; Henkler et al., 2010; Rani et al., 2014; Wätjen & Beyersmann, 2004). Only four human population studies have examined the associations between urinary Cd and 8-isoprostane concentrations. A study of older Japanese people with relatively high urinary Cd concentrations (arithmetic mean = 5.26 μg/g creatinine) found a positive association (Muzembo et al., 2012); studies among solderers in Belgium (Hambach et al., 2013), school children in Uruguay (Kordas et al., 2018), and pregnant women in the USA (S. S. Kim et al., 2019) found low urinary Cd concentrations of 0.8 and 0.2 μg/g creatinine (median; solderers and reference groups, respectively), 0.02 μg/L (median; specific gravity-adjusted), and 0.04 μg/L (geometric mean; unadjusted), respectively, indicating no associations. Cadmium exposure may need to exceed a specific threshold to elevate urinary 8-isoprostane concentration. The geometric mean urinary Cd concentration in the present study (1.38 μg/g creatinine) was slightly higher than the values in the Belgian study populations and far higher than the values in Uruguay (Kordas et al., 2018) or the USA (S. S. Kim et al., 2019); thus, this concentration is thought to be sufficient to increase urinary 8-isoprostane concentration through lipid peroxidation.

3.4.1.3. Associations between urinary Se and 8-OHdG or 8-isoprostane concentrations

The current findings imply that oxidative stress induced by As or Cd exposure was suppressed among individuals with high urinary Se concentration. Selenium is essential for the activation of GPx (Hatfield et al., 2016); thus, poor Se-related nutritional status increases oxidative stress. Evidence to support such a scenario was not obtained in the present study because urinary Se concentration was not negatively associated with urinary 8-OHdG or 8-isoprostane concentration. Instead, the negative associations of oxidative stress-related biomarkers with interaction terms between As and Se and between Cd and Se may indicate that Se protects against As- or Cd-induced oxidative stress. In animal studies, the formation of As-Se and Cd-Se compounds, which are less toxic than As alone and Cd alone, was proposed to be a possible mechanism by which Se protects against As or Cd toxicity (Gailer et al., 2002; George et al., 2016). The seleno-bis(S-glutathionyl) arsinium ion is a representative nontoxic As-Se compound (Gailer, 2012; H. J. Sun et al., 2014). The specific chemical structure of Cd-Se compounds has not been reported; previous studies have proposed that compounds containing an approximately 1:1 atomic ratio of Cd and Se can be complexed with several selenoproteins, resulting in the detoxification of Cd (Jamba et al., 1997; Sasakura & T. Suzuki, 1998).

The positive association between urinary Se and 8-OHdG concentrations in this study was unexpected. Excess Se intake may induce oxidative DNA damage (Wycherly et al., 2004); however, this may not have occurred in the present study because urinary Se concentrations were within the range of values reported in Se-deficient and Se-adequate populations (Mizuno, Masuoka, et al., 2021). In an earlier study, Se was observed to increase the excision of 8-OHdG from DNA (de Rosa et al., 2012). Considering that 8-OHdG in urine is a product of oxidative DNA damage and its repair, individuals with higher Se intake may

have had higher 8-OHdG concentrations in their urine when they had oxidative DNA damage because of exposure to prooxidants such as As.

3.4.1.4. Susceptibilities of DNA and lipids to oxidative stress induced by As and Cd exposures

The current findings imply that DNA and lipid are susceptible to oxidative stress induced by As and Cd, respectively. Biological mechanisms that explain the distinct susceptibilities of DNA and lipids to As-/Cd-induced oxidative stress are unknown. Nevertheless, the observations of the present study support the importance of using multiple oxidative stress-related biomarkers, each of which can reflect a specific aspect of oxidative stress. It may be possible to elucidate the determinants of oxidative stress specific to the targets of oxidative damage. Toxic heavy metal exposures, including As and Cd, are generally presumed to increase oxidative stress that causes oxidative damage to various biomolecules (e.g., DNA and lipids) in the body. However, the relationships between the exposures and the targets of oxidative damage may be more complex than expected.

3.4.1.5. Associations between the wild plant-based dietary pattern and oxidative stress

Wild plant food score was positively associated with telomere length, which agrees with previous findings that plant food-based diets were associated with longer telomeres (Crous-Bou et al., 2019; Tucker, 2021). Commonly consumed wild plants in Northern Laos are supposedly rich in antioxidative phytochemicals (e.g., carotenoids and phenolic compounds) (Afolayan & Jimoh, 2009; Glew et al., 2005; Maisuthisakul et al., 2007; Ng et al., 2012); thus, an indigenous diet involving these plants may provide protection against oxidative stress induced by exposure to prooxidants such as As and Cd.

3.4.2. Oxidative stress and blood pressure

Oxidative stress-related biomarkers were not associated with systolic or diastolic blood pressure in this study; in contrast, previous studies reported higher urinary 8-OHdG and 8-isoprostane concentrations and shorter telomeres among individuals with relatively high blood pressure (Bhupatiraju et al., 2012; Espinosa et al., 2007; Muzembo et al., 2012). Results have been inconsistent among studies targeting normotensive populations, in which significant associations were found in the USA (Anderson et al., 2018; Y.-Q. Huang et al., 2020) and Japan (Miyamoto et al., 2011), while no associations were found in Germany (Koriath et al., 2019) or Spain (Rangel-Huerta et al., 2015). Oxidative stress can impact blood pressure by damaging endothelial cells; the impact may become visible only when blood vessel hardness reaches a specific threshold. The lack of associations between oxidative stress-related biomarkers and blood pressure in the present study is presumably because the blood pressure of most study participants was within the normal range (16% of the participants exhibited hypertension).

3.4.3. Strengths and limitations

I simultaneously investigated three oxidative stress-related biomarkers and their determinants, which enabled me to clarify individual differences in determinants of oxidative DNA and lipid damage and cumulative oxidative stress. Furthermore, urinary 8-OHdG and 8-isoprostane concentrations were measured by LC-MS/MS, which provided more reliable values than ELISA, particularly in the low concentration range.

One important limitation should be noted. Although I assumed that a wild plant-based diet in less modernized villages would suppress oxidative stress, I did not evaluate actual

antioxidative capacity. Chemical composition in edible wild plants can vary among species and growing environments (Ghasemzadeh et al., 2015; Yahyaoui et al., 2018). As mentioned in Chapter 2, the assessment of oxidative DNA damage by measuring only urinary 8-OHdG concentration was another limitation. No investigation of physical activity and psychological stress, known predictors of oxidative stress (Møller et al., 1996) is another limitation. Impact of seasonality is the next study target as mentioned in Chapter 1.

3.5. Conclusion

Exposures to As and Cd may induce oxidative damage to DNA and lipids, respectively, although this oxidative damage may be attenuated by Se intake. Additionally, the participants' indigenous dietary pattern that depends on wild edible plants may provide some protection against telomere attrition. Oxidative stress-related biomarkers were not associated with blood pressure in this study population. Trace element exposure and a wild plant-based diet could be critical determinants of oxidative stress in indigenous populations of Northern Laos.

General conclusions

This case study in the indigenous populations inhabiting mountainous areas of Northern Laos showed:

1) Modernization was positively associated with urinary As and Se concentrations and negatively with urinary Cd concentrations.
2) Modernization was positively associated with urinary 8-OHdG concentration and negatively with telomere length, but not with urinary 8-isoprostane concentration.
3) Urinary As and Se concentrations were positively associated with urinary 8-OHdG concentration, while urinary Cd concentration was positively associated with urinary 8-isoprostane concentration. These associations were mitigated in the condition with higher urinary Se concentration. The wild plant-based dietary pattern was positively associated with telomere length.

Together, this study provided several significant insights into the relationship between modernization and oxidative stress in the indigenous populations of Northern Laos during the health transition process. Oxidative DNA damage was low in the least modernized village but has increased in more modernized villages probably by As exposure through consuming wet rice grown in paddy fields. On the other hand, oxidative lipid damage might have been historically prevalent because of Cd exposure from consumption of dry rice grown in swidden fields. The people in more modernized villages consumed more Se from animal-based foods, which probably protected oxidative stress induced by As and Cd exposures. Furthermore, the decrease in wild plant food consumption was expected to shorten telomeres. These findings proposed that relationships between the determinants and oxidative stress-related biomarkers among a people under modernization might be population specific and more complex than expected.

I have little evidence regarding the health consequences of oxidative stress in the study participants. It is speculated that the health transition began in recent decades and will drastically progress in the near future. Therefore, it would be worthwhile to continue to survey longitudinally in them, and to investigate the impact of oxidative stress during modernization on their health status, which would provide further insights into the unique health transition pattern in Northern Laos people.

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Tables

Table 1-1 Characteristics of study sites: three villages in Northern Laos

	Nam Nyon	Na Savang	Na Lae
Modernization	Least modernized	Intermediate	Most modernized
Location	Remote mountainous region	Flat basin	Provincial capital
Market accessibility	2.5-hour drive to the market in Namo district; accessible only in dry season	1-hour drive to the market in Namo district; accessible whole year	Easily accessible
Economy	Subsistence-oriented	In-between	Market-oriented
Rice	Dry rice (Swidden farming)	Wet rice (Irrigated paddy farming)	Wet rice (Purchased from market)
Diet	Dry rice, wild edible plants; low consumption of animal foods	Wet rice, cultivated plants; relatively high consumption of animal foods	Wet rice, vegetables, animal foods, store foods: most foods are purchased in market

Table 1-2 Characteristics of the study participants

Characteristics		All (n = 341)	Nam Nyon (n = 108)	Na Savang (n = 150)	Na Lae (n = 83)	*p* [e]
Household		210	43	97	70	
Sex [a]	Male	134 (39)	50 (46)	60 (40)	24 (29)	0.05 [f]
	Female	207 (61)	58 (54)	90 (60)	59 (71)	
Age (years) [b]		40 ± 11	35 ± 12	43 ± 11	43 ± 10	< 0.001 [f]
Smoking status [a]	Smoker	85 (25)	44 (41)	29 (19)	12 (14)	< 0.001 [f]
	Non-smoker	256 (75)	64 (59)	121 (81)	71 (86)	
Drinking habit [a]	Yes	169 (50)	50 (46)	71 (47)	48 (58)	0.22 [f]
	No	172 (50)	58 (54)	79 (53)	35 (42)	
Body mass index (kg/m^2) [b]		23.1 ± 3.7	22.3 ± 3.5	22.9 ± 3.4	24.6 ± 3.9	< 0.001 [h]
Underweight [a, c]		20 (6)	13 (12)	6 (4)	1 (1)	
Normal [a, c]		228 (67)	73 (68)	107 (71)	48 (58)	
Overweight [a, c]		72 (21)	18 (17)	30 (20)	24 (29)	
Obesity [a, c]		21 (6)	4 (4)	7 (5)	10 (12)	
Blood pressure (mmHg)						
Systolic blood pressure [b]		122 ± 19	117 ± 17	123 ± 20	125 ± 22	0.005 [f]
Diastolic blood pressure [b]		78 ± 12	76 ± 13	79 ± 12	81 ± 12	0.009 [f]
Hypertension [a, d]	Yes	55 (16)	11 (10)	27 (18)	17 (20)	0.11 [f]
	No	286 (84)	97 (90)	123 (82)	66 (80)	
Antihypertensive medication [a]	Yes	6 (2)	0 (0)	3 (2)	3 (4)	0.16 [f]
	No	335 (2)	108 (100)	147 (98)	80 (96)	
Roofing material	Concrete	154 (45)	12 (11)	73 (49)	69 (83)	< 0.001 [f]
	Others	187 (55)	96 (89)	77 (51)	14 (17)	
	Galvanized iron	182 (53)	93 (86)	75 (50)	14 (17)	
	Wooden boards	2 (1)	0 (0)	2 (1)	0 (0)	
	Cogon grass	3 (1)	3 (3)	0 (0)	0 (0)	
Possessions [a]						
Cell phone	Yes	284 (83)	59 (55)	145 (97)	80 (96)	< 0.001 [f]
	No	57 (17)	49 (45)	5 (3)	3 (4)	
Tractor	Yes	206 (60)	51 (47)	139 (93)	16 (19)	< 0.001 [f]
	No	135 (40)	57 (53)	11 (7)	67 (81)	
Motorcycle	Yes	282 (83)	58 (54)	145 (97)	79 (95)	< 0.001 [f]
	No	59 (17)	50 (46)	5 (3)	4 (5)	
Car	Yes	47 (14)	0 (0)	30 (20)	17 (20)	< 0.001 [f]
	No	294 (86)	108 (100)	120 (80)	66 (80)	
Truck	Yes	8 (2)	1 (1)	5 (3)	2 (2)	0.45 [f]
	No	333 (98)	107 (99)	145 (97)	81 (98)	
Television	Yes	303 (89)	76 (70)	144 (96)	83 (100)	< 0.001 [f]
	No	38 (11)	32 (30)	6 (4)	0 (0)	
Refrigerator	Yes	173 (51)	4 (4)	91 (61)	78 (94)	< 0.001 [f]
	No	168 (49)	104 (96)	59 (39)	5 (6)	

Possessions index [c, d]	5 (3–6)	2 (1–4)	5 (5–6)	5 (5–6)	< 0.001 [g]

[a] *n* (%), [b] Mean ± standard deviation, [c] Median (interquartile range), [d] Calculated by counting the number of possessions (Yes = 1/No = 0): range, 0–7, [e] *p* for difference between villages, [f] Chi-square test, [g] Kruskal-Wallis test, [h] Analysis of variance.

Table 1-3 Specific gravity-adjusted urinary trace element concentrations (μg/L)

Elements	All (n = 341)		Nam Nyon (n = 108)		Na Savang (n = 150)		Na Lae (n = 83)	
	GM	(GSD)	GM	(GSD)	GM	(GSD)	GM	(GSD)
Arsenic	40.1	(2.33)	18.5	(2.10)$^{\gamma}$	70.9	(1.81)$^{\alpha}$	39.2	(1.62)$^{\beta}$
Cadmium	1.38	(1.97)	1.46	(1.93)$^{\alpha}$	1.34	(2.05)$^{\alpha}$	1.35	(1.87)$^{\alpha}$
Lead	1.87	(1.91)	1.80	(2.00)$^{\beta}$	1.68	(1.87)$^{\beta}$	2.36	(1.74)$^{\alpha}$
Selenium	11.7	(1.50)	9.23	(1.44)$^{\gamma}$	11.6	(1.44)$^{\beta}$	16.0	(1.37)$^{\alpha}$

GM, geometric mean; GSD, geometric standard deviation.

[a] Results of Tukey's multiple comparison test. Different letters indicate significant differences for each element between communities ($\alpha > \beta > \gamma$).

Table 1-4 Associations between urinary trace element concentrations and modernization-related variables: the results of multiple linear regression analyses; sex, age, and smoking status were adjusted for (n = 341)

		Arsenic (μg/L) [a]		Cadmium (μg/L) [a]		Lead (μg/L) [a]		Selenium (μg/L) [a]	
Fixed effects		Coef.	(95% CI)	Coef.	(95% CI)	Coef.	(95% CI)	Coef.	(95% CI)
Village	Na Savang	1.19	(0.94, 1.44) ***	−0.21	(−0.44, 0.03)	−0.04	(−0.26, 0.17)	0.14	(0.01, 0.27) *
(ref. = Nam Nyon)	Na Lae	0.57	(0.31, 0.84) ***	−0.26	(−0.51, −0.01) *	0.38	(0.15, 0.62) **	0.45	(0.31, 0.59) ***
Roofing material = Concrete (ref. = Others)		1.19	(−0.10, 0.24)	0.17	(0.00, 0.34)	0.01	(−0.15, 0.17)	0.11	(0.01, 0.20) *
Possessions index [a]		0.57	(−0.03, 0.09)	−0.03	(−0.09, 0.04)	0.03	(−0.03, 0.09)	0.01	(−0.03, 0.04)
Body mass index (kg/m^2)		1.19	(0.00, 0.03)	−0.01	(−0.03, 0.01)	−0.02	(−0.04, 0.00)	0.00	(−0.01, 0.01)
Sex = Male (ref. = Female)		0.57	(−0.20, 0.10)	−0.40	(−0.58, −0.22) ***	0.26	(0.08, 0.44) **	0.07	(−0.03, 0.21)
Age (years)		1.19	(0.01, 0.02) ***	0.02	(0.02, 0.03) ***	0.00	(−0.01, 0.00)	0.00	(0.00, 0.01) ***
Smoking status = Smoker (ref. = Non-smoker)		0.57	(−0.07, 0.28)	0.44	(0.23, 0.66) ***	0.31	(0.10, 0.52) **	0.09	(−0.03, 0.21)
Random effect		Var.	ICC	Var.	ICC	Var.	ICC	Var.	ICC
Household-level		0.21	0.58	0.10	0.28	0.04	0.13	0.03	0.26
Individual-level		0.15		0.27		0.30		0.09	

CI, confidence interval; ICC, intra-class correlation.

[a] Specific gravity-adjusted and log-transformed, [b] Calculated by counting the number of possessions (Yes = 1/No = 0): range, 0–7.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Table 1-5 Comparison of urinary trace element concentrations between this study and data in the literature (geometric mean, μg/g creatinine)

Element	Location	Participants	Concentration	Reference
Arsenic	Northern Laos	n = 341	30.0	This study
	General populations			
	China	n = 123	56.23	(Nordberg et al., 2005)
	USA	n = 2,557	8.24	(Caldwell et al., 2009)
	USA		16.93	(Yao et al., 2021)
	German	n = 476 (no fish consumption)	2.98	(Becker et al., 2003)
	Contaminated areas			
	Bangladesh	n = 133 (male)	126–204 [a]	(Watanabe et al., 2001)
		n = 229 (female)	174–219 [a]	
	China	n = 122	288.40	(Nordberg et al., 2005)
Cadmium	Northern Laos	n = 341	1.07	This study
	General populations			
	China	n = 522	1.9	(Inoue et al., 2014)
	China	n = 123	0.86	(Nordberg et al., 2005)
	Japan	n = 10,753	1.26	(Ezaki et al., 2003)
	Japan	n = 133 (male)	2.1	(Nakajima et al., 2005)
		n = 160 (female)	3.1	
	East and Southeast Asia	n = 561 (female)	1.97	(Ikeda et al., 2000)
	USA	n = 2257	0.074–0.507 [a]	(Ruiz et al., 2010)
	USA	n = 9662	0.3	(Yao et al., 2021)
	Spain	n = 861	0.57	(Aguilera et al., 2008)
	Czech Republic	n = 657	0.29	(Batáriová et al., 2006)
	German	n = 4740	0.227	(Becker et al., 2003)
	Contaminated areas			
	China	n = 122	2.16	(Nordberg et al., 2005)
	Japan	n = 1420 (male)	4.6	(Nakajima et al., 2005)
		n = 1751 (female)	7.2	
Lead	Northern Laos	n = 341	1.40	This study
	General populations			
	China	n = 522	3.3	(Inoue et al., 2014)
	Japan	n = 470 (male)	1.04–3.17 [a]	(Jin et al., 2000)
		n = 430 (female)	1.15–3.35 [a]	
	East and Southeast Asia	n = 561	3.59	(Ikeda et al., 2000)
	USA	n = 496	1.88	(Paschal et al., 1998)
	USA	n = 9662	0.64	(Yao et al., 2021)
	Contaminated areas			
	Japan	n = 214 (male)	81	(Fukui et al., 1999)

	China	n = 40	47	(J. Huang et al., 1988)
Selenium	Northern Laos	n = 341	8.73 (9.31) [c]	This study
	General populations			
	China	n = 522	38.3	(Inoue et al., 2014)
	Japan	n = 431 (male)	37.5	(Hasunuma et al., 1990)
		n = 358 (female)	45.7	
	Japan	n = 193 (male)	30.8	(Tsuda et al., 1995)
	(20–29 years)	n = 187 (female)	37.8	
	USA	n = 106 (male)	51.8	(Combs et al., 2011)
		n = 155 (female)	57.7	
	Low selenium areas			
	China	n = 43 (Keshan disease patients)	7 (μg/L) [b, c]	(Yang et al., 1983)
	Poland	n = 62	11.4 [c]	(Wasowicz, 1989)
	India	n = 25 (male)	9.15 [c]	(Hira et al., 2004)
		n = 29 (female)	9.05 [c]	

[a] Range of geometric means in several subgroups (e.g., stratification by age) in the previous studies, [b] Unadjusted urinary selenium concentration, [c] Arithmetic mean.

Table 2-1 Urinary 8-OHdG and 8-isoprostane concentrations and telomere length of the study participants by villages

	All (n = 341)		Nam Nyon (n = 108)		Na Savang (n = 150)		Na Lae (n = 83)	
	GM	(GSD)	GM	(GSD)	GM	(GSD)	GM	(GSD)
8-OHdG [a, b]	3.89	(1.77)	3.27	(1.63) $^{\beta}$	4.23	(1.80) $^{\alpha}$	4.18	(1.80) $^{\alpha}$
8-isoprostane [a, b]	0.934	(1.58)	0.941	(1.47) $^{\alpha}$	0.908	(1.63) $^{\alpha}$	0.973	(1.64) $^{\alpha}$
	AM	± SD	AM	± SD	AM	± SD	AM	± SD
Telomere length [b, c]	0.80	± 0.11	0.84	± 0.12 $^{\alpha}$	0.79	± 0.11 $^{\beta}$	0.76	± 0.10 $^{\beta}$

GM, geometric mean; GSD, geometric standard deviation; AM, arithmetic mean; SD, standard deviation; 8-OHdG, 8-hydroxy-2'-deoxyguanosine.

[a] Specific gravity-adjusted urinary concentration (μg/L), [b] Results of analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Different letters indicate significant differences for each biomarker among villages ($\alpha > \beta > \gamma$), [c] T/S ratio determined by the qPCR method.

Table 2-2 Associations between villages and oxidative stress-related biomarkers: results of multiple linear regression analyses (n = 341)

		8-OHdG (μg/L) [a]		8-isoprostane (μg/L) [a]		Telomere length [b]	
Fixed effects		Coef.	(95% CI)	Coef.	(95% CI)	Coef.	(95% CI)
Village	Na Savang	0.26	(0.10, 0.41) **	−0.04	(−0.17, 0.08)	−0.052	(−0.085, −0.019) **
(ref. = Nam Nyon)	Na Lae	0.31	(0.13, 0.48) ***	−0.01	(−0.16, 0.14)	−0.085	(−0.122, −0.047) ***
Sex = Male (ref. = Female)		0.08	(−0.09, 0.26)	−0.15	(−0.29, 0.00) *	−0.018	(−0.052, 0.016)
Age (years)		0.00	(0.00,0.01)	0.00	(0.00, 0.01)	−0.001	(−0.002, 0.000)
Smoking status = Smoker (ref. = Non-smoker)		−0.05	(−0.25, 0.15)	0.20	(0.04, 0.37) *	−0.002	(−0.041, 0.036)
Drinking habit = Yes (ref. = No)		−0.03	(−0.16, 0.10)	0.02	(−0.08, 0.13)	−0.003	(−0.029, 0.022)
Body mass index (kg/m^2)		−0.03	(−0.05, -0.01) *	0.01	(0.00, 0.03) *	0.001	(-0.003, 0.004)
Random effect		Var.	ICC	Var.	ICC	Var.	ICC
Household-level		0.01	2.79	0.02	8.28	0.003	25.00
Individual-level		0.29		0.19		0.009	

8-OHdG, 8-hydroxy-2'-deoxyguanosine; CI, confidence interval; ICC, intra-class correlation.

[a] Specific gravity-adjusted and log-transformed, [b] T/S ratio determined by the qPCR method.

$p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Table 2-3 Comparison of urinary 8-OHdG and 8-isoprostane concentrations between the present and previous studies

	Location	n	Age (years) [a]	BMI (kg/m^2) [a]	Concentration (μg/g creatinine) GM	AM	Reference
8-OHdG	Laos	341	40	23.1	2.92	3.48	This study
	Japan	6517	60.3	22.7	3.9		(Shimanoe et al., 2018)
	Japan	2507	51 [b]	NA		4.52	(Nakano et al., 2003)
	China	185 (non-smokers)	40.4	24.4	5.5		(Pan et al., 2008)
	Taiwan	125 (males, non-smokers)	34.1	22.8	4.1		(Pan et al., 2018)
	Taiwan	129	51.7	24.6		4.3	(R.-H. Wong et al., 2008)
	Korea	102 (females, non-smokers)	55	24.1		6.5	(H. Kim et al., 2011)
8-isoprostane	Laos	341	40	23.1	0.700	0.780	This study
	China	610 (males)	57.1	22.1	0.402		(Yuan et al., 2018)
	USA	143	38.0	25.2		0.209	(Il'yasova et al., 2018)
	USA	75	37.9	25.1		0.211	(Il'yasova et al., 2018)

8-OHdG, 8-hydroxy-2'-deoxyguanosine; BMI, body mass index; GM, geometric mean; AM, arithmetic mean.

[a] AM of age/BMI in study populations, [b] Median, [c] Conversion from μmol/mol creatinine to μg/g creatinine was done based on molecular weights (8-OHdG = 299.24, 8-isoprostane = 354.48, creatinine = 113.12 (g/mol))

Table 3-1 Food consumption frequencies and factor loadings of principal component analysis of the study participants (n = 341)

Food items	Food consumption frequency [a] (days/week)	First component [b] Factor loading	Second component [b] Factor loading	Third component [b] Factor loading
Wild plant foods				
Bitter bamboo shoot	1 (0–2)	**0.56**	−0.22	0.12
River weed and algae	2 (0–3)	**0.47**	**−0.53**	0.19
Other wild edible vegetables	2 (0–3)	**0.60**	−0.19	−0.23
Locally produced foods				
Aquatic animals	1 (0–2)	**0.51**	−0.23	−0.03
Contents of intestine	1 (0–2)	0.26	**−0.42**	**0.56**
Blood of animals	2 (1–3)	0.22	−0.25	−0.37
Newly introduced foods				
Meats	2 (1–3)	**−0.74**	−0.05	−0.07
Poultry/eggs	2 (2–3)	**−0.41**	−0.36	0.15
Cultivated leafy vegetables	1 (0–3)	−0.15	−0.34	**−0.67**
Cultivated fruits	7 (5–7)	**−0.70**	0.02	0.11
Spice	2 (0–2)	−0.27	**−0.49**	−0.33
Fish	3 (2–7)	−0.37	−0.30	**0.52**
Noodles	2 (0–3)	**−0.45**	−0.14	0.07
Sweets and snacks	2 (0–2)	−0.39	−0.33	−0.15
Oil/fat	5 (2–7)	−0.20	**−0.61**	0.01
Eigenvalue	–	3.09	1.73	1.44
Variation explained	–	20.6	11.6	9.6

[a] Median (interquartile range), [b] The values are bolded if those are more than 0.40 or lower than −0.40.

Table 3-2 Associations of urinary trace element concentrations and wild plant food score with oxidative stress-related biomarkers in the study participants (n = 341)

	Model 1 [a]			Model 2 [a]		
	8-OHdG (μg/L) [b]	8-isoprostane (μg/L) [b]	Telomere length [b]	8-OHdG (μg/L) [b]	8-isoprostane (μg/L) [b]	Telomere length [c]
Fixed effect	Coefficients (95% CI)	Coefficients (95% CI)	Coefficients (95% CI)	Coefficients (95% CI)	Coefficients (95% CI)	Coefficients (95% CI)
Arsenic (μg/L) [b, d]	0.16 ***	0.08 **	−0.021 *	0.09 *	0.06	−0.008
	(0.09, 0.23)	(0.02, 0.14)	(−0.036, −0.007)	(0.01, 0.17)	(−0.01, 0.12)	(−0.025, 0.010)
Cadmium (μg/L) [b, d]	0.14 **	0.24 ***	−0.020 *	0.04	0.24 ***	−0.020
	(0.05, 0.22)	(0.17, 0.31)	(−0.38, −0.002)	(−0.06, 0.15)	(0.15, 0.32)	(−0.042, 0.002)
Selenium (μg/L) [b, d]	0.38 ***	0.18 **	−0.054 **	0.27 **	−0.06	−0.008
	(0.24, 0.53)	(0.06, 0.30)	(−0.083, −0.024)	(0.07, 0.46)	(−0.21, 0.09)	(−0.048, 0.031)
Arsenic×Selenium [b, d]				−0.20 *	0.03	0.016
				(−0.39, −0.02)	(−0.12, 0.18)	(−0.021, 0.054)
Cadmium×Selenium [b, d]				−0.03	−0.15 *	−0.014
				(−0.19, 0.13)	(−0.28, −0.02)	(−0.047, 0.020)
Wild plant food score [e]	−0.02	−0.01	0.011 **	0.00	−0.02	0.011 *
	(−0.06, 0.01)	(−0.04, 0.02)	(0.004, 0.018)	(−0.04, 0.04)	(−0.06, 0.01)	(0.002, 0.019)
Sex = Male				0.08	−0.06	−0.029
(ref. = Female)				(−0.10, 0.25)	(−0.20, 0.08)	(−0.064, 0.007)
Age (years)				0.00	0.00	0.000
				(−0.01, 0.00)	(−0.01, 0.00)	(−0.001, 0.001)
Body mass index (kg/m^2)				−0.03 **	0.02 **	0.000
				(−0.04, −0.01)	(0.00, 0.03)	(−0.003, 0.003)
Smoking status = Smoker				−0.10	0.14	0.015
(ref. = Non-smoker)				(−0.29, 0.10)	(−0.01, 0.30)	(−0.024, 0.054)
Drinking habit = Yes				−0.05	0.03	−0.006

(ref. = No)	(−0.18, 0.07)		(−0.07, 0.13)		(−0.032, 0.020)	
Random effect	Var.	ICC (%)	Var.	ICC (%)	Var.	ICC (%)
Household	0.03	10.90	0.02	9.69	0.004	28.81
Individual	0.25		0.16		0.009	

8-OHdG, 8-hydroxy-2'-deoxyguanosine; CI, confidence interval; ICC, intraclass correlation coefficient.

[a] Model 1: bivariate linear regression model; Model 2: multilevel model including all independent variables, [b] Specific gravity-adjusted and log-transformed urinary concentration (μg/L), [c] T/S ratio determined by the qPCR method, [d] Centering at the grand mean, [e] Principal component score calculated from food consumption frequencies.

$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Table 3-3 Associations of oxidative stress-related biomarkers with blood pressure in the study participants (n = 341)

	Model 1 [a]		Model 2 [a]	
	SBP (mmHg)	DBP (mmHg)	SBP (mmHg)	DBP (mmHg)
Fixed effect	Coefficients (95% CI)	Coefficients (95% CI)	Coefficients (95% CI)	Coefficients (95% CI)
8-OHdG (μg/L) [b]	−0.81 (−4.47, 2.84)	−0.28 (−2.61, 2.05)	−0.93 (−4.30, 2.44)	−0.33 (−2.50, 1.83)
8-isoprostane (μg/L) [b]	0.47 (−4.06, 5.00)	1.17 (−1.71, 4.05)	−2.02 (−6.16, 2.12)	−0.34 (−3.00, 2.31)
Telomere length [c]	−10.51 (−28.65, 7.62)	−5.74 (−17.28, 5.81)	4.74 (−11.85, 21.34)	2.71 (−7.91, 13.34)
Sex = Male (ref. = Female)			3.52 (−1.98, 9.01)	−0.47 (−4.00, 3.07)
Age (years)			0.64 *** (0.47, 0.80)	0.39 *** (0.28, 0.49)
Body mass index (kg/m^2)			1.26 (0.73, 1.80)	0.74 (0.40, 1.08)
Smoking status = Smoker (ref. = Non-smoker)			−1.87 *** (−7.89, 4.14)	−2.49 *** (−6.36, 1.37)
Drinking habit = Yes (ref. = No)			−0.81 (−4.79, 3.16)	0.35 (−2.21, 2.90)
Antihypertensive medications = Yes (ref. = No) [d]			18.24 * (3.85, 32.63)	10.78 * (1.53, 20.03)
Random effect			Var. ICC (%)	Var. ICC (%)
Household			22.85 7.83	6.22 5.17
Individual			269.06	114.10

SBP, systolic blood pressure; DBP, diastolic blood pressure; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; CI, confidence interval; ICC, intraclass correlation coefficient.

[a] Model 1: bivariate linear regression model; Model 2: multilevel model including all independent variables, [b] Specific gravity-adjusted and log-transformed urinary concentration (μg/L), [c] T/S ratio determined by the qPCR method, [d] Anti-hypertensive medication.

$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Figures

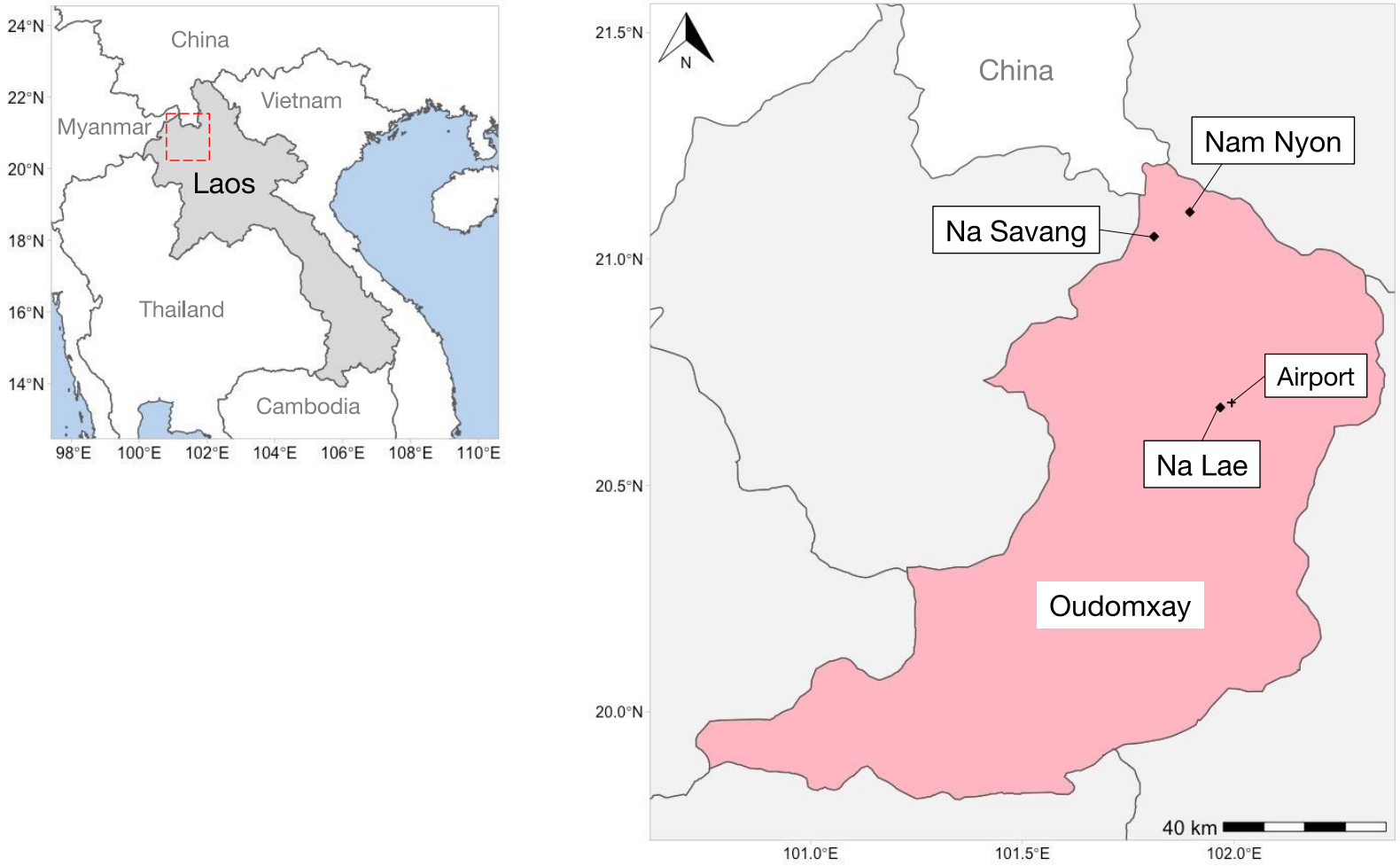

Figure 1-1 Study sites in Laos

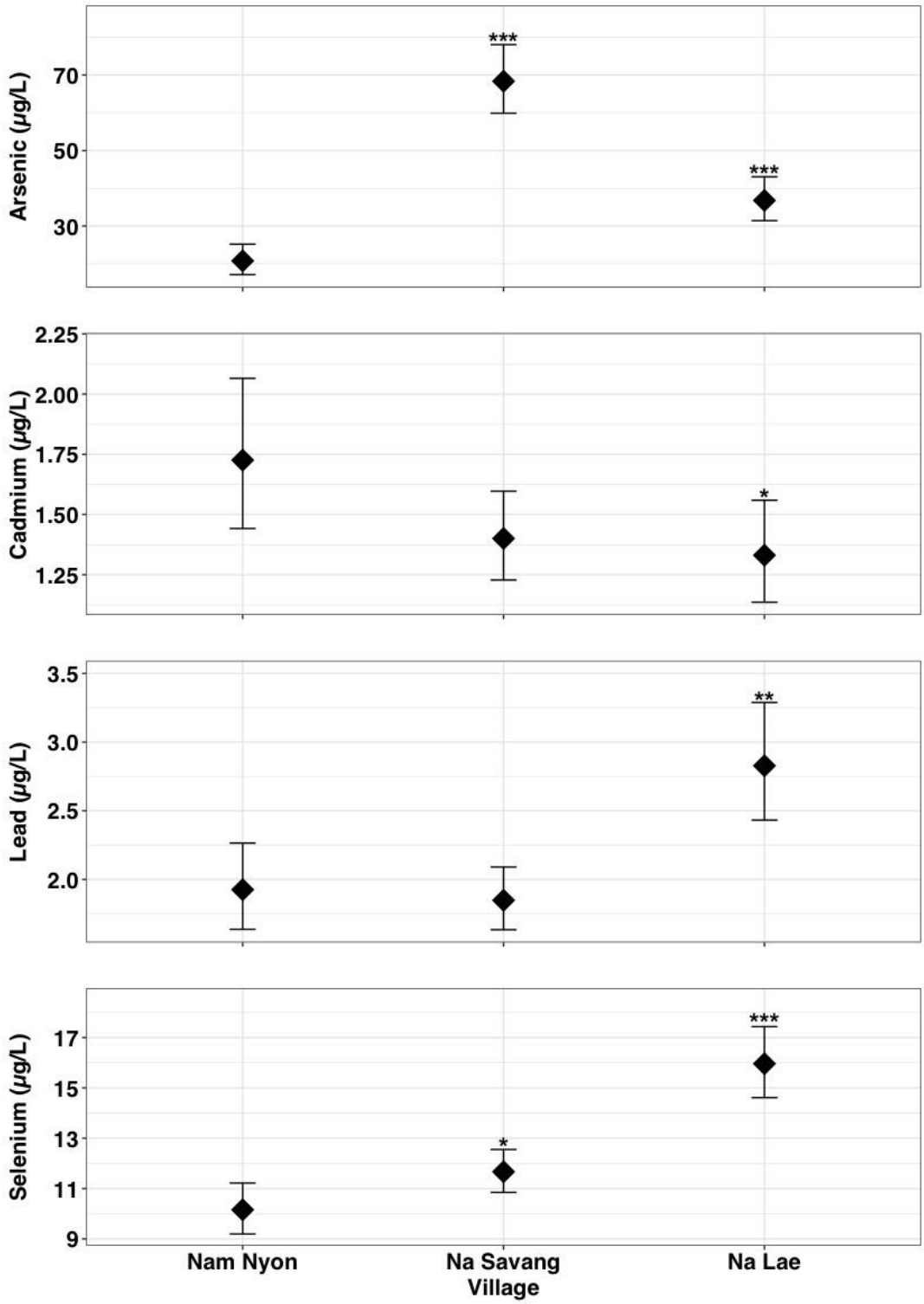

Figure 1-2 Least square means of urinary arsenic, cadmium, lead, and selenium concentrations (specific gravity-adjusted) in each village. The least square means were analyzed using multilevel models adjusted for roofing material, the possessions index, body mass index, sex, age, and smoking. Household ID was included as a random effect. Closed squares represent least square means, and error bars indicate the 95% confidence interval. Differences between villages (Nam Nyon vs. Na Savang/Na Lae) were examined by multilevel analyses: $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$.

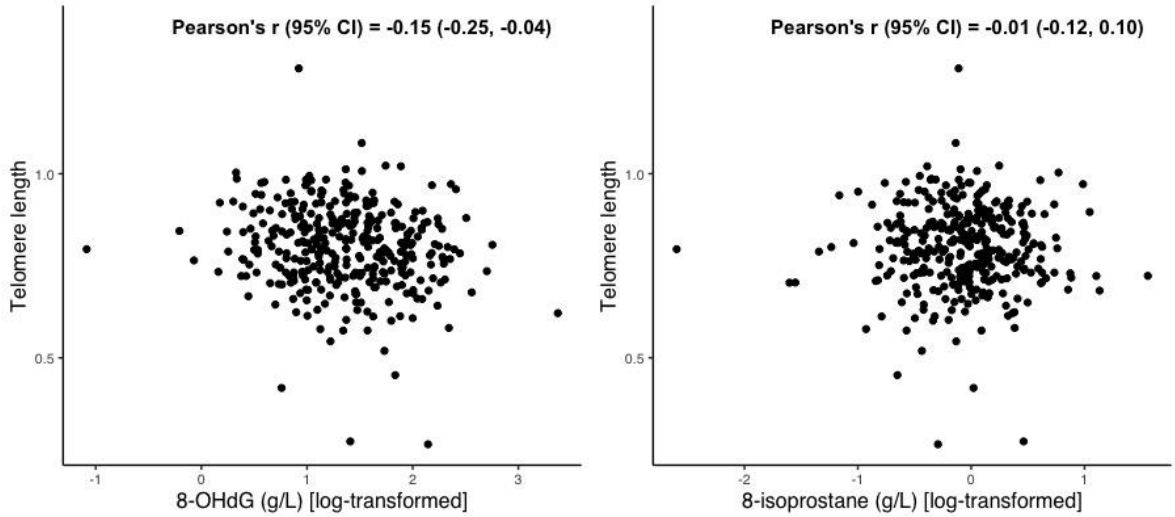

Figure 2-1 Correlations between telomere length and urinary 8-OHdG and 8-isoprostane concentrations (n = 341)

Telomere length is the T/S ratio determined by the qPCR method. Urinary 8-OHdG and 8-isoprostane concentrations were adjusted for specific gravity and logarithmically transformed.

8-OHdG, 8-hydroxy-2'-deoxyguanosine; CI, confidence interval.

Appendices

Appendix 1 Questionnaire (English version)

SURVEY QUESTIONNAIRE

1. ID number:	2. Village name:	4. Date: / /
3. Interviewer name:		5. Time:

6	What is your name?	
7	What is your sex?	Male / Female
8	In what date, month and year were you born?	Year: Month: Day:
9	Household #	
10	Ethnicity name	

11. Household members (the people who eat and work together with you)
Please give me the first name of each household member and write the sex and the relationship with you. *Relationship: Father, Mother, Grandfather, Older Sister, Younger Brother, Son, Daughter, Relative etc…***)**

Name	Sex	Age	Relationship

Personal data

12	Do you currently smoke? (If yes, how many cigarettes a day in average?)	Yes / No (cigarettes/day)
13	Do you drink alcohol? (If yes, how many days a week in average?)	Yes / No (days/week)
14	If you are woman, are you on your period?	Yes / No
15	If you are woman, are you pregnant?	Yes / No
16	If you are woman, are you lactating?	Yes / No
17	Which village were you born?	Village: District: Province:
18	If you were not born in this village, which year did you move to this village?	
19	Do you do farming?	Yes / No
20	What do you do except farming?	Trader, small shop, government officer, teacher, handicraft, construction or others ( )

Household Properties

21	What ha of land does your household own?	ha
22	Does your household own any of the following assets? Circle on the assets.	Cell phone/Smart phone, Tractor, Motorcycle, Car, Truck, TV, Refrigerator
23	What is roofing material?	Cogon grass, Wood, Galvanized iron, or Concrete

Do you (or your household) produce or sell following items?

No	Item	Produce	Sell
24	Wet rice	Yes/No	Yes/No
25	Dry rice	Yes/No	Yes/No
26	Corn (*Salii*)	Yes/No	Yes/No
27	Maize (*Khao phoot*)	Yes/No	Yes/No
28	Tobacco	Yes/No	Yes/No
29	Para rubber	Yes/No	Yes/No
30	Tuber crops (e.g. Taro, sweet potato, cassava etc)	Yes/No	Yes/No
31	Meats (water buffalo, cattle, pig, goat, dog etc.)	Yes/No	Yes/No
32	Poultry / Eggs	Yes/No	Yes/No
33	Cultivated vegetables (e.g. Chinese cabbage)	Yes/No	Yes/No
34	Cultivated Fruits	Yes/No	Yes/No
35	Herbs	Yes/No	Yes/No
36	Spices (e.g. Chili pepper, galangal, ginger, Chinese pepper)	Yes/No	Yes/No
37	Fish	Yes/No	Yes/No
38	Other aquatic animals *(frog, crab, shrimp etc.)*	Yes/No	Yes/No
39	River weed and Algae	Yes/No	Yes/No
40	Bamboo shoots, cardamom, mushrooms, tiger grass etc.	Yes/No	Yes/No
41	Wild vegetables		Yes/No
42	Bush meats		Yes/No

Do you eat following food items?

No	Food items	How many days did you eat in the last week?	Where did you get this? 1: Deep forest (*Pa ge*) 2: Shallow forest (*Pa lao*) 3: Paddy 4: Upland 5: Garden 6: River 7: Market or shop 8: Gift 9: Other (Specify)
43	Rice (dry/wet)		
44	Corn		
45	Tuber crops (e.g. Taro, sweet potato, cassava etc)		
46	Meats (water buffalo, cattle, pig, goat, dog etc.)		
47	Poultry / Eggs		
48	Cultivated leafy vegetables (e.g. Chinese cabbage)		
49	Cultivated fruits		
50	Herbs		
51	Spices (e.g. Chili pepper, galangal, ginger, Chinese pepper)		
52	Fish		
53	Other aquatic animals *(frog, crab, shrimp etc.)*		
54	River weed and Algae		
55	Bitter bamboo shoot		
56	Wild fruits		
57	Wild vegetables		
58	Bush meats		
59	Insects or larvae		
60	Organs of animals		
61	*Kii Pa, Phia*		
62	Blood of animals (Duck, pig, water buffalo, wild animals etc.)		
63	Organs of fishes		
64	Coffee/tea		
65	Noodles		
66	Sausages, other processed meat		
67	Canned fishes		
68	Sweets and snacks		
69	Oil/Fat		

70. Do you like to eat bitter foods? (Yes / No)
What bitter foods did you eat in the last week? ( )
How many days did you eat this? (1-2 / 3-5 / 6-7 days)

71. Do you like to eat astringent foods? (Yes / No)
What astringent foods did you eat in the last week? ( )
How many days did you eat this? (1-2 / 3-5 / 6-7 days)

72. Do you like to eat sour foods? (Yes / No)
What sour foods did you eat in the last week? ( )
How many days did you eat this? (1-2 / 3-5 / 6-7 days)

73. Do you like to eat hot/spicy foods? (Yes / No)
What hot foods did you eat in the last week? ( )
How many days did you eat this? (1-2 / 3-5 / 6-7 days)

Urine

Protein	Glucose	pH	Salt					
− +/- 1+ 2+ 3+ 4+	− +/- 1+ 2+ 3+ 4+	5 6 7 8 9						

Anthropometry

Blood Pressure (mmHg)	Blood Spot	Hb (g/dL)	Height (cm)	Weight (kg)	MUAC (cm)	Tri (mm)	Sub (mm)
/							
/							

This is the end of the questionnaire, thank you for participating.

Appendix 2 Information sheet for participants (English version)

資料 1

Information Sheet for Participants

Study Title: Does the diet in mountainous areas in Laos prevent non-communicable diseases?
Principal Investigator: Masahiro Umezaki

Thank you very much for your participation.

The objective of this study is to explore the favorable health effects of traditional Lao diets. In this study, we would like to conduct an interview with a questionnaire. Although We would ask names of yours and your family members, the identity of participants and their family members will not be disclosed. The questionnaire also includes question items about your household structure, physical activity and food intake. In addition, we would like to conduct anthropometric measurement and blood pressure measurement, as well as biological sample collection (urine, feces, saliva, scalp hair and blood). The biological samples will be used for the evaluation of the risk of non-communicable diseases (e.g., inflammation, oxidative stress, gut microbiota) and dietary characteristics (animal protein intake).

What we would learn from you will help us to understand the impact of dietary changes on health status in rural and also the mechanisms that could underlie health problems in other developing countries.

This study is approved by the Ethics Committee of the University of Tokyo. Your participation for this study is entirely voluntary and you may refuse to answer any question if you choose or may withdraw your consent to participate at any time without penalty. The interview, measurement and biological sample collection will take about 45 minutes. All the information we obtain will remain strictly confidential and your answer will never be identified.

You may ask any question about the study at this time and if you have further questions about this study, please do not hesitate to contact;

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Appendix 3 Informed consent form for participants (English version)

資料 2

Informed Consent Form for Participants

To: The Dean of Graduate School of Medicine and Faculty of Medicine,
The University of Tokyo
Study Title: Does the diet in mountainous areas in Laos prevent non-communicable diseases?
Principal Investigator: Masahiro Umezaki

I, after reading and having been explained to me the contents of this study, understand what is expected of me as a participant in the study.

I understand:

1. The purpose and procedure of the study
2. The consent of the questionnaire
3. That I will not be placed under any harm of discomfort
4. That I may refuse to answer any question if I don't want to answer
5. That I can withdraw from the study at any time without giving a reason
6. That I can withdraw from the study at any time (during or after study) without any harm or without in any way affecting the health service I receive
7. That any information I provide will be strictly treated in a confidential manner that I will not be identified in the reporting of the result

Date: / /

_____

Signature of the person who receive consent

Appendix 4 Ethical approval letter (The University of Tokyo)

(医)

審査番号	12033

西暦　2018年07月17日

審　査　結　果　通　知　書
実　施　許　可　通　知　書

倫理委員会の設置者、実施機関の長
東京大学大学院医学系研究科・医学部長　殿

倫理委員会委員長
東京大学大学院医学系研究科・医学部倫理委員会
非介入等研究倫理委員会

赤林　朗　㊞

審査依頼のあった件についての審査結果を下記のとおり通知いたします。

記

研究課題名	ラオス山岳部の「森林食」は非感染性疾患を抑制するか？
審査結果	■承認する　□条件付きで承認する　□変更を勧告する　□承認しない □該当しない　□既承認事項の取り消し
審査事項 （審査資料）	＜新規案件＞ ■研究の新規実施 ＜継続案件＞ □研究に関する変更 □その他（　　）
審査区分	■委員会審査（審査日：西暦2018年07月09日） □迅速審査（審査日：西暦　　年　月　日）
指摘事項および 理由・条件等	
備考	

研究責任者　　梅崎 昌裕　殿

依頼のあった研究に関する審査事項について上記のとおり決定しましたので通知いたします。
倫理委員会での審査結果が承認となりましたので、研究の実施を許可いたします。

西暦　2018年07月17日

倫理委員会の設置者、実施機関の長
東京大学大学院医学系研究科・医学部長
宮園　浩平（公印省略）

Appendix 5 Ethical approval letter (Ministry of Health, Lao PDR)

Lao People's Democratic Republic
Peace Independence Democracy Unity Prosperity

Ministry of Health
National Ethics Committee
for Health Research (NECHR)

No 074 /NECHR
Vientiane Capital 19 / 7 / 2018

Approval Notice

Dr. Satoshi YOKOYAMA
Email: s-yokoyama@nagoya-u.jp
Tel: +81-52-789-4742

RE: Ethical Approval for Health Research

Title: **"Population dynamics, reproduction and livelihood changes in small-scale communities of Laos"(Submission ID: 2018.22.MP)**

Dear Dr. Satoshi YOKOYAMA ,

The National Ethics Committee for Health Research of the Lao People's Democratic Republic have reviewed and approved your research.

Please note the following information about your approved research protocol:

Approval period: July 2018 – July 2019
Approved Subject Enrollment: 302
Study Site: Luangphrabang province and Oudomxay province
Sponsor: Japan Society for the Promotion of Science, **Budget:** 246,000,000LAK
Implementing Panel/Project Investigator: Dr. Satoshi YOKOYAMA

Please note that the Ethics Committee reserves the right to ask for further questions, seek additional or monitor the conduct of your research and consent process.

Principle Investigator is required to notify the Secretary of the National Ethic Committee for Health Research:

- **Any significant change to the project and the reason for that change, including an indication of ethical implications (if any);**
- **Serious adverse effects on participants and the action taken to address those effects;**
- **Any other unforeseen events or unexpected developments that merit notification;**
- **The inability of the Principal Investigator to continue in that role, or any other change in research personnel involved in the project;**
- **Any expiry of the insurance coverage provided with respect to sponsored clinical trials and proof of re-insurance;**
- **A delay of more than 12 months in the commencement of the project; and,**
- **Termination or closure of the project.**

Additionally, the Principal Investigator is required to submit a progress report on the anniversary of approval and on completion of the project.

President of National Ethics Committee for Health Research

Prof.Dr. Douangdao SOUKALOUN

Appendix 6 Material transfer agreement

Ministry of Health
Lao Tropical and Public Health
Ban Kaognoth, Samsenthai Road, Sisattanack District, Vientiane capital, Lao PDR
Tel: 856-21-214012; 250670

CUSTOMS INVOICE

Date: _____ August 2018

WAY BILL number: _ _ _ _ _ _ _ _ _

Shipper:
Lao Tropical and Public Health Institute
Samsenthai Road, Kaognot village, Sisattanak District, Vientiane, Laos
Attn: Sengchanh Kounnavong, MD, PhD
Tel: 856-20 55923232
E-mail: sengchanhkounnavong@hotmail.com

From:
Lao Tropical and Public Health Institute
Ban Kaognoth, Samsenthai Road, Sisattanack District
Vientiane, Lao PDR
Contact: Dr Masahiro Umezaki
Organization name: the University of Tokyo

SHIP TO

Dr Masahiro Umezaki
Organization name: the University of Tokyo
Address: 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan
City/Province: Tokyo
Postal Code Country: 113-0033
Phone: 81-3-5841-3487

Content: Non-infectious Human Stool (frozen/RNAlater), Urine (frozen), Hair, and Dried Blood Spots samples

Units	Description of Goods	Harmonized code		Unit Value	Total Value	Currency
300	Healthy human stool	0511.99	190	10	3000	JPN yen
300	Healthy human urine			10	3000	JPN yen
300	Healthy human hair			10	3000	JPN yen
300	Healthy human dried blood spot			10	3000	JPN yen

For research purpose
No Commercial Value – Value for Customs Purposes Only

Total number of packages: 1
Total weight: ____kg

Director General of Lao TPHI

ดร.แสงจัน ภูมมาวิ

Appendix 7 Detection limit and repeatability/reproducibility of measurements of urinary trace element concentrations

Elements	m/z	Detection limit (μg/L)	Repeatability [a] (%)	Reproducibility [a] (%)
Arsenic	75	0.0454	1.36, 1.93	3.06, 4.32
Selenium	78	0.220	1.81, 1.64	2.27, 4.33
Cadmium	111	0.00752	1.61, 2.88	8.05, 2.38
Lead	208	0.0103	2.68, 1.65	5.68, 1.99

[a] The values of repeatability and reproducibility were shown for each of Seronorm Trace Elements Urine L-1 and -2.

Appendix 8 Observed concentrations of trace elements in Seronorm Trace Elements Urine L-1 and -2 (μg/L)

Elements	L-1		L-2	
	Observed value [a]	Reference range [b]	Observed value [a]	Reference range [b]
Arsenic	159 ± 2	126–190	257 ± 5	209–314
Selenium	17.0 ± 0.3	12.6–19.0	70.8 ± 1.2	57.3–86.1
Cadmium	0.205 ± 0.003	0.12–0.27	4.34 ± 0.12	3.9–5.8
Lead	0.644 ± 0.017	0.36–1.08	79.1 ± 1.3	64.0–96.2

[a] Mean ± SD (n = 5); [b] Reference range represents the results of replicate analyses obtained through collaboration with several independent laboratories.

Appendix 9 Urinary trace element concentrations of the study participants in all or each of the villages

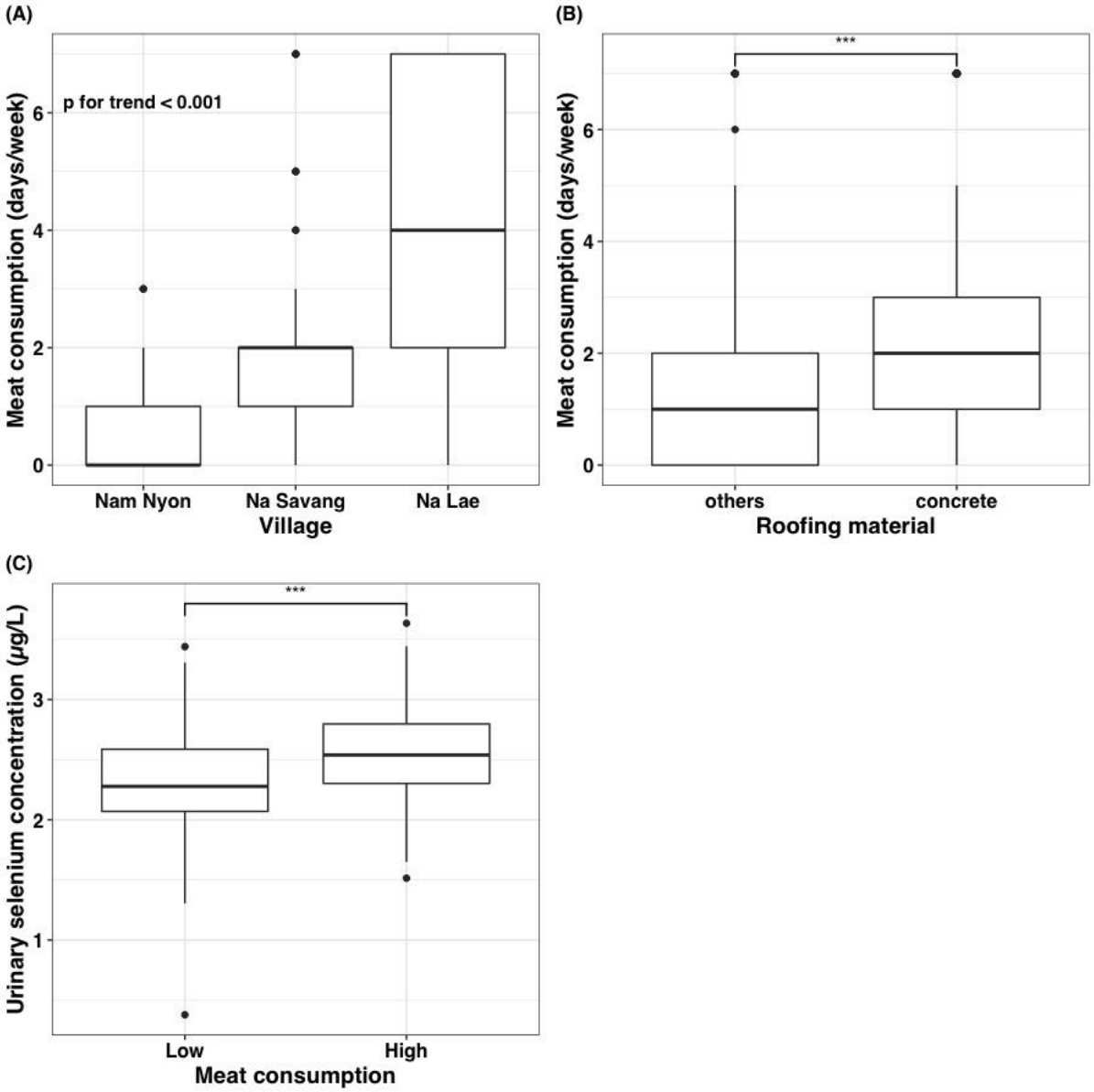
	All (n = 341)		Nam Nyon (n = 108)		Na Savang (n = 150)		Na Lae (n = 83)	
Unadjusted (µg/L)	GM	(GSD)	GM	(GSD)	GM	(GSD)	GM	(GSD)
Arsenic	40.3	(2.59)	21.6	(2.33)	72.5	(2.06)	31.6	(2.27)
Cadmium	1.39	(2.34)	1.70	(2.09)	1.36	(2.27)	1.09	(2.64)
Lead	1.88	(2.15)	2.10	(2.25)	1.72	(2.14)	1.91	(2.03)
Selenium	11.7	(1.84)	10.8	(1.72)	11.8	(1.77)	12.9	(2.08)
Creatinine-adjusted (µg/g creatinine)	GM	(GSD)	GM	(GSD)	GM	(GSD)	GM	(GSD)
Arsenic	30.0	(2.27)	13.1	(1.97)	55.3	(1.60)	29.5	(1.57)
Cadmium	1.03	(1.76)	1.03	(1.81)	1.04	(1.77)	1.01	(1.70)
Lead	1.40	(1.87)	1.27	(1.92)	1.31	(1.82)	1.78	(1.81)
Selenium	8.73	(1.43)	6.52	(1.33)	9.02	(1.32)	12.0	(1.30)

GM, geometric mean; GSD, geometric standard deviation.

Appendix 10 Arithmetic mean, standard deviation, and percentile values of urinary trace element concentrations in the study participants (n = 341)

Unadjusted (μg/L)	AM	± SD	Min.	5th	25th	50th	75th	95th	Max.
Arsenic	61.4	± 65.1	2.30	7.31	21.1	43.5	79.0	163	644
Cadmium	1.90	± 1.56	0.0966	0.281	0.837	1.43	2.54	4.52	11.4
Lead	2.52	± 2.68	0.149	0.525	1.20	1.93	3.12	5.53	34.9
Selenium	14.0	± 8.66	1.33	3.95	8.04	12.1	18.1	28.9	64.6
Specific gravity-adjusted (μg/L)	AM	± SD	Min.	5th	25th	50th	75th	95th	Max.
Arsenic	56.0	± 53.2	4.19	8.18	24.0	43.4	70.1	134	485
Cadmium	1.71	± 1.21	0.0618	0.458	0.901	1.40	2.16	3.99	7.38
Lead	2.35	± 2.26	0.0847	0.787	1.27	1.74	2.79	5.48	29.6
Selenium	12.6	± 5.21	1.46	6.37	8.72	11.7	15.3	22.9	37.9
Creatinine-adjusted (μg/g creatinine)	AM	± SD	Min.	5th	25th	50th	75th	95th	Max.
Arsenic	40.8	± 36.0	3.24	5.93	18.1	34.1	52.2	86.7	356
Cadmium	1.22	± 0.820	0.162	0.440	0.690	0.977	1.53	2.56	7.79
Lead	1.77	± 1.83	0.210	0.595	0.904	1.36	1.98	4.09	24.7
Selenium	9.31	± 3.42	3.49	4.86	6.66	8.81	11.1	15.8	24.1

AM, arithmetic mean; SD, standard deviation; 5th–95th, percentile values.



Appendix 11 Meat consumption, village, roofing material and urinary selenium concentrations (n = 341)

(A) Meat consumption frequency in the study participants of each village. The trend between meat consumption frequency and the level of village modernization (Nam Nyon < Na Savang < Na Lae) was examined using Jonckheere-Terpstra test. (B) Meat consumption in the two groups stratified according to roofing material. (C) Urinary selenium concentrations in the two groups of this study participants stratified according to meat consumption. Urinary selenium concentration was adjusted by urine specific gravity and logarithmically transformed. Meat consumption was stratified according to the median value (Low = "< 2 days/week", High = "≥ 2 days/week").

*** $p < 0.001$ (Mann–Whitney U test)

Appendix 12 Unadjusted and creatinine-adjusted urinary concentrations of oxidative stress markers

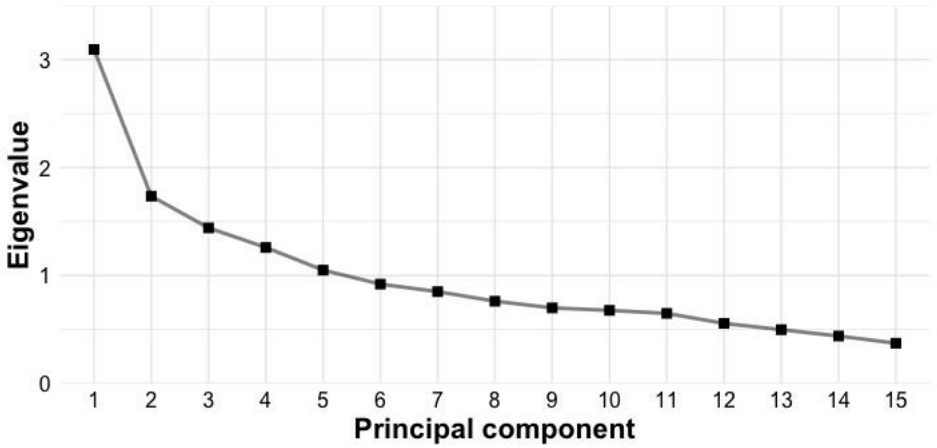
	All (n = 341)		Nam Nyon (n = 108)		Na Savang (n = 150)		Na Lae (n = 83)	
Unadjusted (µg/L)	GM	(GSD)	GM	(GSD)	GM	(GSD)	GM	(GSD)
8-OHdG	3.83	(1.99)	3.85	(2.03)	3.60	(2.02)	4.08	(1.91)
8-isoprostane	0.921	(1.92)	0.871	(1.89)	0.924	(2.09)	0.971	(1.760)
Creatinine-adjusted (µg/g creatinine)	GM	(GSD)	GM	(GSD)	GM	(GSD)	GM	(GSD)
8-OHdG	2.92	(1.79)	3.15	(1.80)	2.71	(1.66)	2.91	(1.88)
8-isoprostane	0.700	(1.58)	0.713	(1.50)	0.700	(1.60)	0.692	(1.65)

GM, geometric mean; GSD, geometric standard deviation.

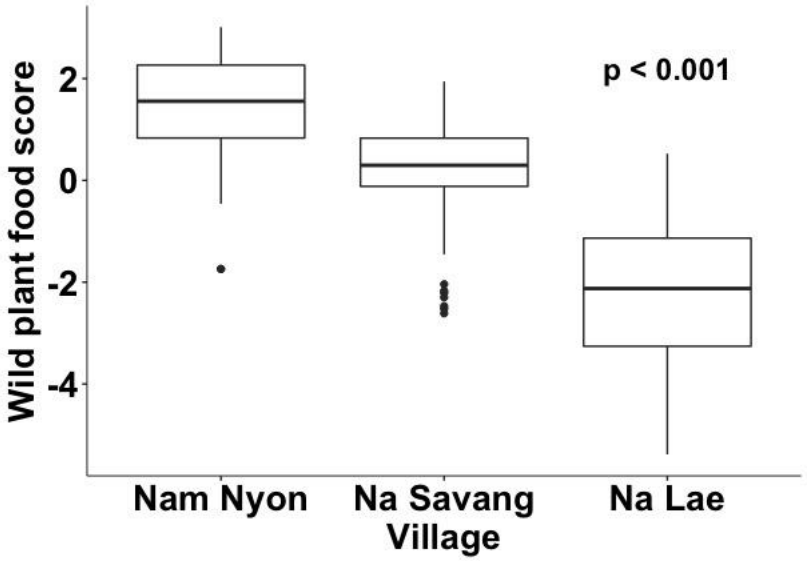
Appendix 13 Arithmetic means, standard deviation, and percentile values of urinary concentrations of oxidative stress markers (n = 341)

Unadjusted (μg/L)	AM	± SD	Min.	5th	25th	50th	75th	95th	Max.
8-OHdG	4.74	± 3.27	0.387	1.19	2.60	3.90	6.34	10.0	33.6
8-isoprostane	1.11	± 0.696	0.0780	0.274	0.665	0.998	1.43	2.27	7.13
Creatinine-adjusted (μg/g creatinine)	AM	± SD	Min.	5th	25th	50th	75th	95th	Max.
8-OHdG	3.48	± 2.51	0.661	1.21	1.94	2.82	4.26	7.68	29.2
8-isoprostane	0.780	± 0.441	0.140	0.338	0.535	0.696	0.940	1.45	5.93
Specific gravity-adjusted (μg/L)	AM	± SD	Min.	5th	25th	50th	75th	95th	Max.
8-OHdG	4.56	± 2.85	0.338	1.57	2.64	3.93	5.75	9.64	29.2
8-isoprostane	1.03	± 0.497	0.0740	0.477	0.697	0.947	1.21	1.87	4.75

AM, arithmetic mean; SD, standard deviation; 5^{th}–95^{th}, percentile values.



Appendix 14 Scree plot of principal component analysis with food consumption frequencies



Appendix 15 Wild plant food score among participants in the three villages
Wild plant food score was a principal component score calculated from the food consumption frequencies. Differences in the score among villages were compared by one-way analysis of variance.
Village: Nam Nyon (least modernized); Na Savang (somewhat modernized); Na Lae (most modernized).

Appendix 16 Associations between urinary arsenic and 8-OHdG concentrations in the participants (n = 341); simple slope analysis with conditions of low or high urinary selenium concentration

Outcome = 8-OHdG (μg/L) [a]	Low selenium (−1SD) [a, b]		High selenium (+1SD) [a, b]	
Fixed effect	Coef.	(95% CI)	Coef.	(95% CI)
Arsenic (μg/L) [a, c]	0.17	(0.07, 0.27) **	0.01	(−0.11, 0.13)
Selenium (μg/L) [a, c]	0.27	(0.07, 0.46) **	0.27	(0.07, 0.46) **
Arsenic×Selenium [a, c]	−0.20	(−0.39, −0.02) *	−0.20	(−0.39, −0.02) *
Cadmium (μg/L) [a, c]	0.05	(−0.06, 0.17)	0.03	(−0.10, 0.17)
Cadmium×Selenium [a, c]	−0.03	(−0.19, 0.13)	−0.03	(−0.19, 0.13)
Wild plant food score [d]	0.00	(−0.04, 0.04)	0.00	(−0.04, 0.04)
Sex = Male (ref. = Female)	0.08	(−0.10, 0.25)	0.08	(−0.10, 0.25)
Age (years)	0.00	(−0.01, 0.00)	0.00	(−0.01, 0.00)
Body mass index (kg/m^2)	−0.03	(−0.04, −0.01) **	−0.03	(−0.04, −0.01) **
Smoking status = Smoker (ref. = Non-smoker)	−0.10	(−0.29, 0.10)	−0.10	(−0.29, 0.10)
Drinking habit = Yes (ref. = No)	−0.05	(−0.18, 0.07)	−0.05	(−0.18, 0.07)
Random effect	Var.	ICC	Var.	ICC
Household	0.031	10.90	0.031	10.90
Individual	0.252		0.252	

8-OHdG, 8-hydroxy-2'-deoxyguanosine; SD, standard deviation; CI, confidence interval; ICC, intraclass correlation coefficient.

[a] Specific gravity-adjusted and log-transformed urinary concentration, [b] In the model of low Se: urinary Se concentration + 1×SD was included as the variable of that; In the model of high Se: urinary Se concentration − 1×SD was included as the variable of that, [c] Centering at the grand mean, [d] The principal component score calculated from the food consumption frequencies.

$^{*}p < 0.05$, $^{**}p < 0.01$

Appendix 17 Associations between urinary cadmium and 8-isoprostane concentrations in the participants (n = 341); simple slope analysis with conditions of low or high urinary selenium concentration

Outcome = 8-isoprostane (μg/L) [a]	Low selenium (−1SD) [a, b]		High selenium (+1SD) [a, b]	
Fixed effect	Coef.	(95% CI)	Coef.	(95% CI)
Cadmium (μg/L) [a, c]	0.30	(0.21, 0.39) ***	0.18	(0.07, 0.28) **
Selenium (μg/L) [a, c]	−0.06	(−0.21, 0.09)	−0.06	(−0.21, 0.09)
Cadmium×Selenium [a, c]	−0.15	(−0.28, −0.02) *	−0.15	(−0.28, −0.02) *
Arsenic (μg/L) [a, c]	0.04	(−0.04, 0.13)	0.07	(−0.02, 0.16)
Arsenic×Selenium [a, c]	0.03	(−0.12, 0.18)	0.03	(−0.12, 0.18)
Wild plant food score [d]	−0.02	(−0.06, 0.01)	−0.02	(−0.06, 0.01)
Sex = Male (ref. = female)	−0.06	(−0.20, 0.08)	−0.06	(−0.20, 0.08)
Age (years)	0.00	(−0.01, 0.00)	0.00	(−0.01, 0.00)
Body mass index (kg/m^2)	0.02	(0.00, 0.03) **	0.02	(0.00, 0.03) **
Smoking status = Smoker (ref. = Non-smoker)	0.14	(−0.01, 0.30)	0.14	(−0.01, 0.30)
Drinking habit = Yes (ref. = No)	0.03	(−0.07, 0.13)	0.03	(−0.07, 0.13)
Random effect	Var.	ICC (%)	Var.	ICC (%)
Household level	0.017	9.69	0.017	9.69
Individual level	0.161		0.161	

SD, standard deviation; CI, confidence interval; ICC, intraclass correlation coefficient.
[a] Specific gravity-adjusted and log-transformed urinary concentration (μg/L), [b] In the model of low selenium: urinary selenium concentration +1×SD was included as the variable of that; In the model of high selenium: urinary selenium concentration −1×SD was included as the variable of that, [c] Centering at the grand mean, [d] The principal component score calculated from the food consumption frequencies.
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Appendix 18 Associations of urinary trace element concentrations and wild plant food score with urinary 8-OHdG concentration [a] in the study participants by villages

	Nam Nyon (n = 108)		Na Savang (n = 153)		Na Lae (n = 83)	
Fixed effects	Coef.	(95% CI)	Coef.	(95% CI)	Coef.	(95% CI)
Arsenic (μg/L) [a, b]	0.03	(−0.15, 0.22)	0.14	(−0.03, 0.32)	0.05	(−0.30, 0.39)
Cadmium (μg/L) [a, b]	0.24	(0.01, 0.47) *	−0.05	(−0.22, 0.11)	0.07	(−0.24, 0.38)
Selenium (μg/L) [a, b]	0.07	(−0.45, 0.60)	0.17	(−0.22, 0.57)	0.48	(0.00, 0.96)
Arsenic×Selenium [a, b]	−0.15	(−0.57, 0.27)	−0.32	(−0.82, 0.17)	−0.69	(−1.49, 0.11)
Cadmium×Selenium [a, b]	0.06	(−0.41, 0.52)	−0.09	(−0.44, 0.27)	0.21	(−0.50, 0.92)
Wild plant food score [c]	0.01	(−0.10, 0.12)	−0.02	(−0.12, 0.09)	0.08	(−0.01, 0.17)
Sex = Male (ref. = Female)	0.05	(−0.36, 0.46)	0.20	(−0.05, 0.45)	−0.11	(−0.49, 0.27)
Age (years)	0.00	(−0.01, 0.00)	−0.01	(−0.02, 0.00)	0.01	(0.00, 0.03)
Body mass index (kg/m^2)	0.00	(−0.03, 0.03)	−0.04	(−0.06, −0.01) *	−0.03	(−0.06, 0.00)
Smoking status = Smoker (ref. = Non-smoker)	0.03	(−0.34, 0.40)	−0.18	(−0.48, 0.12)	0.18	(−0.25, 0.62)
Drinking habit = Yes (ref. = No)	−0.17	(−0.41, 0.07)	0.01	(−0.19, 0.21)	−0.16	(−0.44, 0.12)
Random effect	Var.	ICC	Var.	ICC	Var.	ICC
Household-level	0.031	14.21	0.000	0.00	0.000	0.000
Individual-level	0.190		0.298		0.273	

8-OHdG, 8-hydroxy-2'-deoxyguanosine; CI, confidence interval; ICC, intraclass correlation coefficient.

[a] Specific gravity-adjusted and log-transformed urinary concentration (μg/L), [b] Centering at the grand mean, [c] Principal component score calculated from food consumption frequencies.

* $p < 0.05$

Appendix 19 Associations of urinary trace element concentrations and wild plant food score with urinary 8-isoprostane concentration [a] in the study participants by villages

	Nam Nyon (n = 108)		Na Savang (n = 153)		Na Lae (n = 83)	
Fixed effects	Coef.	(95% CI)	Coef.	(95% CI)	Coef.	(95% CI)
Arsenic (μg/L) [a, b]	−0.01	(−0.15, 0.14)	0.19	(0.06, 0.32) **	0.00	(−0.30, 0.31)
Cadmium (μg/L) [a, b]	0.19	(0.01, 0.38) *	0.20	(0.08, 0.33) **	0.37	(0.10, 0.64) **
Selenium (μg/L) [a, b]	−0.24	(−0.67, 0.18)	−0.03	(−0.33, 0.27)	−0.10	(−0.51, 0.32)
Arsenic×Selenium [a, b]	−0.17	(−0.51, 0.18)	−0.21	(−0.59, 0.16)	0.23	(−0.46, 0.92)
Cadmium×Selenium [a, b]	0.11	(−0.28, 0.49)	−0.11	(−0.37, 0.16)	0.06	(−0.57, 0.68)
Wild plant food score [c]	−0.01	(−0.10, 0.08)	−0.12	(−0.20, −0.04) **	−0.04	(−0.11, 0.04)
Sex = Male (ref. = Female)	0.04	(−0.30, 0.38)	−0.01	(−0.20, 0.18)	−0.15	(−0.46, 0.16)
Age (years)	0.00	(−0.01, 0.01)	0.00	(−0.01, 0.00)	0.00	(−0.01, 0.01)
Body mass index (kg/m^2)	0.03	(0.00, 0.05) *	0.01	(−0.01, 0.03)	0.01	(−0.02, 0.04)
Smoking status = Smoker (ref. = Non-smoker)	0.08	(−0.22, 0.39)	0.10	(−0.12, 0.33)	0.19	(−0.18, 0.56)
Drinking habit = Yes (ref. = No)	−0.06	(−0.25, 0.14)	0.13	(−0.02, 0.27)	−0.06	(−0.30, 0.17)
Random effect	Var.	ICC	Var.	ICC	Var.	ICC
Household-level	0.010	6.45	0.007	3.81	0.086	40.47
Individual-level	0.139		0.164		0.127	

CI, confidence interval; ICC, intraclass correlation coefficient.

[a] Specific gravity-adjusted and log-transformed urinary concentration (μg/L), [b] Centering at the grand mean, [c] Principal component score calculated from food consumption frequencies.

*p < 0.05, **p < 0.01

Appendix 20 Associations of urinary trace element concentrations and wild plant food score with telomere length [a] in the study participants by villages

	Nam Nyon (n = 108)		Na Savang (n = 153)		Na Lae (n = 83)	
Fixed effects	Coef.	(95% CI)	Coef.	(95% CI)	Coef.	(95% CI)
Arsenic (µg/L) [b, c]	−0.013	(−0.062, 0.035)	0.026	(−0.010, 0.061)	0.029	(−0.035, 0.093)
Cadmium (µg/L) [b, c]	−0.032	(−0.090, 0.025)	−0.039	(−0.072, −0.007) *	0.002	(−0.055, 0.059)
Selenium (µg/L) [b, c]	0.037	(−0.101, 0.174)	−0.004	(−0.081, 0.073)	−0.046	(−0.136, 0.043)
Arsenic×Selenium [b, c]	0.039	(−0.066, 0.145)	−0.013	(−0.111, 0.085)	0.014	(−0.134, 0.162)
Cadmium×Selenium [b, c]	−0.027	(−0.143, 0.089)	−0.004	(−0.073, 0.065)	0.029	(−0.103, 0.161)
Wild plant food score [d]	0.025	(−0.003, 0.052)	−0.006	(−0.027, 0.015)	−0.009	(−0.025, 0.008)
Sex = Male (ref. = Female)	−0.014	(−0.115, 0.088)	−0.032	(−0.078, 0.015)	−0.049	(−0.119, 0.021)
Age (years)	0.000	(−0.002, 0.002)	0.001	(−0.001, 0.003)	−0.003	(−0.005, 0.000) *
Body mass index (kg/m^2)	0.002	(−0.005, 0.009)	0.000	(−0.005, 0.005)	−0.002	(−0.008, 0.004)
Smoking status = Smoker (ref. = Non-smoker)	0.002	(−0.090, 0.093)	0.016	(−0.041, 0.073)	0.011	(−0.070, 0.092)
Drinking habit = Yes (ref. = No)	−0.005	(−0.063, 0.054)	−0.017	(−0.054, 0.021)	0.022	(−0.030, 0.073)
Random effect	Var.	ICC	Var.	ICC	Var.	ICC
Household-level	0.006	36.30	0.004	32.98	0.000	0.000
Individual-level	0.010		0.008		0.009	

CI, confidence interval; ICC, intraclass correlation coefficient.

[a] T/S ratio determined by the qPCR method, [b] Specific gravity-adjusted and log-transformed urinary concentration (µg/L), [c] Centering at the grand mean, [d] Principal component score calculated from food consumption frequencies.

*p < 0.05