COMMUNAUTÉ FRANÇAISE DE BELGIQUE

UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

Contribution to the study of the mineral hypothesis in relation to the Kashin-Beck disease in Tibet Autonomous Region

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Dissertation originale présentée en vue de l'obtention du grade de

Docteur en sciences agronomiques et ingénierie biologique

Promoteur : Professeur Georges Lognay

Année civile 2016

"Je ne me demande pas où mènent les routes ; c'est pour le trajet que je pars."

Anne Hébert

Dermience Michaël (2016). Contribution to the study of the mineral hypothesis in relation for Kashin-Beck disease in Tibet Autonomous Region. PhD Thesis, Gembloux Agro-Bio Tech, University of Liege, Gembloux, Belgium, 207 pp., 24 tabl.

Abstract – A little known disease called Kashin-Beck disease (KBD) plagues the poor and rural populations in the Tibet Autonomous Region (T.A.R.) and in other provinces of the People's Republic of China. It is an endemic and chronic osteochondropathy affecting long bones and joints, sometimes as soon as of the early childhood. Although the etiology of this disease is not clearly established, little doubt remains as to the implication of multiple environmental factors. Intoxication by mycotoxins in cereals and by organic acids in water, deficiencies in selenium and iodine, are all factors having a place in the multifactorial etiology hypothesized. In T.A.R., diet, notably, differentiates the rural community, affected by KBD, from the other communities (nomads and city-dwellers), who remain unaffected. Because more than one chemical element is essential to a healthy bone metabolism, and because there is scarce data, if not any, on the topic, this thesis had to primary objective to investigate the mineral and trace element dietary status of young Tibetan children living in areas endemic for KBD. The first logical action step led us to determine which elements are involved in bone and joints metabolism through an exhaustive review of the scientific literature. Thirty elements were highlighted, and a dozen was deemed relevant in this context. An exploratory study on the Tibetan food composition concluded on a high risk of introducing important bias by using the existing food composition tables for nutritional assessment in T.A.R. Being inescapable tools, a specific food composition table was elaborated for our area of investigation with the close collaboration of the China National Center for Food Safety Risk Assessment (CFSA). During a scientific internship of 7 month in the CFSA, 19 chemical elements were analyzed in not less than 1119 samples of sixteen traditional foods and beverages of rural T.A.R. In order to assess the nutritional status of the children, a cross-sectional study was implemented. 250 preschool children aged 3 to 5 years old from three rural counties around Lhasa were enrolled. They were interviewed twice, at six month of interval, via the 24-hour recall method. The results suggest several imbalances in their dietary mineral intakes compared to the Chinese recommendations. Sodium and manganese intakes are too high, while they are too low for potassium, calcium, zinc, copper and selenium. The Tibetan diet is rich in fiber and in phytic acid, which are susceptible to decrease the bioavailability and to aggravate the deficiencies of the later elements. For this reason, we conducted an animal experimentation on a rat model to assess the apparent digestibility, the fecal excretion and the urinary excretion of minerals and trace elements in the traditional Tibetan dish called *tsampa pag*. This traditional dish consisting of roasted barley flour mixed with yak butter tea is the mainstay of the Tibetan diet. The results of this experiment suggest low bone mineral density, a possible secondary copper deficiency, and a possible manganese excess in rats that consumed *tsampa pag*. In view of the results presented, it would be interesting to compare the mineral intake between children living in endemic areas and in non-endemic areas. It would also be interesting to include more of elements known to affect bone metabolism in future studies.

Dermience Michaël (2016). Contribution à l'étude de l'hypothèse minérale en rapport avec la maladie de Kashin-Beck dans la Région autonome du Tibet. (Thèse de Doctorat) Gembloux, Belgique, Université de Liège, Gembloux Agro-Bio Tech, 207 pp., 24 tabl.

Résumé – Il sévit au sein des populations rurales de la Région Autonome du Tibet (R.A.T.), mais également dans d’autres provinces de la République Populaire de Chine, une maladie peu connue appelée maladie de Kashin-Beck (MKB). Il s’agit d’une ostéochondropathie endémique et chronique qui affecte principalement les os long et les articulations, parfois dès la petite enfance. Bien que l’étiologie de cette maladie ne soit pas clairement établie, peu de doutes subsistent quant à l’implication de multiples facteurs environnementaux. Les facteurs communément admis sont un empoisonnement par des mycotoxines présentent dans les céréales, une contamination de l’eau de consommation par des acides organiques, et des carences alimentaires, notamment en sélénium et en iode. Dans la R.A.T., l’alimentation est un facteur qui constitue une différence majeure entre les populations rurales, affectées par la maladie, et les habitants des villes et les nomades, qui ne sont guère affectés. Etant donné les nombreux éléments chimiques impliqués dans le métabolisme osseux, et le peu de données scientifiques en rapport avec les apports alimentaires de ces populations, cette thèse avait pour but principal l’étude du statut en minéraux et oligo-éléments des jeunes enfants tibétains vivant dans des régions endémique pour la MKB. La première étape logique fut de déterminer exhaustivement les minéraux impliqués, de façon directe ou indirecte, dans le métabolisme osseux. Trente minéraux furent répertoriés, et une dizaine d’entre eux furent jugé pertinents et considérés dans la suite de l’étude. Une évaluation des tables de composition alimentaires existantes conclut à un risque d’erreur important en cas d’utilisation de ces dernières en R.A.T. Etant un outil indispensable à toute étude nutritionnelle, une table de composition alimentaire spécifique a été élaborée en étroite collaboration avec le *China National Center for Food Safety Risk Assessment (CFSA)*. Durant un séjour scientifique de 7 mois au sein du CFSA, 19 éléments chimiques furent analysés dans seize catégories de produits alimentaires traditionnels collectés, en R.A.T. pour un total de 1119 échantillons. Enfin d’évaluer le statut minéral des enfants tibétains, une étude transversale impliquant 250 enfants âgés de 3 à 5 ans et habitant dans trois comtés ruraux de la préfecture de Lhassa a été mise en œuvre. Des données de consommation alimentaires quantitatives ont été récoltées deux fois pour chaque enfant à six mois d'intervalle par la méthode de rappel de 24 heures. Les résultats mettent en évidence plusieurs déséquilibres alimentaires par rapport aux recommandations chinoises (DRIs). Les apports en sodium et en manganèse sont trop élevés, alors qu'ils sont trop faibles pour le potassium, le calcium, le zinc, le cuivre et le sélénium. De plus, l'alimentation tibétaine étant riche en fibres et en acide phytique, un problème de biodisponibilité est susceptible d’aggraver les déficits d’ingestion mis en évidences pour certains éléments, et éventuellement de causer des carences secondaires. Dans cette optique, une expérimentation a été conduite dans le but d’étudier l’absorption et l’excrétion des minéraux chez des rats nourris avec de la *tsampa pag*. Ce plat traditionnel composé de farine d’orge grillée mélangée à du thé au beurre de yak étant le pilier de l'alimentation tibétaine. Les résultats de cette expérimentation suggèrent une teneur minérale osseuse faible, une possible carence secondaire en cuivre, et un possible excès en manganèse chez les rats ayant consommé la *tsampa pag*. Au vu des conclusions présentées, il serait intéressant de comparer les apports minéraux entre les enfants vivant en zones endémiques et ceux vivant en zones non endémiques. Il serait également intéressant d’intégrer d’avantages d'éléments connus pour avoir un impact sur le métabolisme osseux dans de futures études.

Acknowledgements

This thesis is not the result of a personal work, but the fruit of a convergence between several people who have crossed on the paths of Science, Love, Friendship, and Fortune. Because nobody is perfect, it would be presumptuous to claim I do not forget anyone, but I wish to express my sincere thanks:

To Professor Georges Lognay
Simply, without sycophancy, the best Teacher I ever had
He taught me more than Science, he instilled me Values

To Professor Jean-Paul Barthélemy
Who taught me the practical side of the word “Chemistry”
And who shared with me his love for Nature

To Françoise Mathieu and Williams Claus
With more people like them, the world would get better
Selflessness, is what they taught me

To Philippe Maesen
A velvet hand in an iron glove, and not the opposite
A man you can rely on

To the members of my thesis committee
For keeping me on the right scientific path

To Sophie, Lili, Fabienne, Stéphanie, Maryse, and Christophe
The lasts six years I may have spent more time with them than with everyone else,
and always in good mood

To Stéphanie, Carine, Tina, Michel, Laurent, Olivier, Nicolas, Francesco and Pierre
For sharing with me their passion for BBQ and good beers

To Yangzom lak, Tselha, Big Pema, Small Pema, Rinchen Lopsang, Lakpa Wangdu, Tashi Yangzom, Dikki Yangzom, Nima Kanden, Tsewang Rinzen, and Chungdak

To Xiao Wei Li, Lu Bing, Yue Bing, Xie Ke, Wang Dong Mei, Sun Zhong Qing, and all the friends I made in Beijing

They welcomed me like one of them

They opened my mind to another culture

To Viviane De Maertelaer

For guiding me in the meanders of statistics

To Philippe Goyens, Shancy Rooze, Camille Chasseur, and Maurice Hinsenkamp

For providing critical advice and experience

And for giving time to KBD Fund

To Ghislaine Dufourny, Fatma Yasar, Caroline Etienne, and Charlotte Nicolas

For providing essential support

To the Center for Disease Control and Prevention of Tibet Autonomous Region (P.R. China)

For their collaboration and support

To CLSA Hong Kong

Who financially support KBD Fund and part of this study

To Gelek, Tsering Namgye, Nyima Tashi, Chimy Namgye, Tashi, Dawa, Dorjee, and Tserin Cheuden,
For their essential contribution as interviewers

To the Tibetan families implicated in this work

For their welcome and their spontaneous participation

To Mathilde, Christopher, Alexandre, and Anthony

Who contributed to my work

And which I had the pleasure to supervise

To forty innocent rats

Sacrificed in the name of Science

To Randolph, Gaëlle, Fabien, Amaury, Simon, Karl, Max, Junior, Alexia, Bastien, Stéphane, Vincent, Laurent, Kevin, Max, Bruno, Pal, Axel, Stéphane, and many other

Because they are all facets of the precious gemstone that is Friendship

To my Grandparents, Marcel Dermience, Rosa Hanozet, Roy Tull, and Nicole Borry

All were sincerely beloved, all are gone too early

Traditions will be carried

To my Mother and my Father

Who love me unconditionally

I just do not would exist if they did not love each other

To my Two Brothers,

Because together we are never alone

Trismegistus

To my Love Oya,

She is my Yin, as far as I believe to be her Yang,

In anyway, “Ma cavalière pour l’éternité”

To me,

Not taking myself too seriously makes things much easier

Table of Contents

Chapter I.

"Understanding food to be like medicine, I will use it without desire or hate, not for pride's sake nor in arrogance, and not for beauty but just for sustenance"

A popular table prayer by the Buddhist teacher Nagarjuna (2nd - 3rd centuries AD)

Introduction to Kashin-Beck Disease

Nutrition is a major concern all-over the world. Few populations can boast of not having any health problems related to diet (Langley-Evans, 2015). Many countries fight with disease such as, diabetes or cardiovascular diseases, which are generally correlated to diets excessively rich in salt, sugar, fats, alcohol, etc. On the other hand, many populations suffer from deficiencies in one or several nutrients, mostly vitamins and minerals, causing moderate to severe impacts on their health and lifespan. Contrary to what one might think, nutritional excesses are not the preserve of rich countries, and nutrient deficiencies are not confined to poor countries (Popkin et al., 2012). Even worse, nutrition-related health problems are sometimes caused by both excesses in some nutrients and deficiencies in others, a problem which particularly affects low and middle income areas with easy access to foods of poor nutritional quality (Tzioumis and Adair, 2014).

Living at the top of the world since thousands of years, Tibetans have to adapt to a harsh environment, with annual precipitation around 400 mm, mean annual temperatures around 6°C, and a severely dry air most of the time (Malaisse et al., 2008). According to Haubruge et al. (2000), the Tibetan population of the Tibet Autonomous Region (T.A.R.) occupies four macro-ecosystems: the urban centers; the proximate suburban zones; the agricultural areas; and the pastoral areas, located at over 4500 m in altitude, where few people but nomads venture.

If suburban zones benefit from agricultural machineries and road networks, due to the continued growth of the urban centers, most agricultural areas are still relying on traditional subsistence farming and animal husbandry (Chasseur et al., 2008). Productivity is very low, but despite an inhospitable environment, rural Tibetans developed a way of life in subtle harmony with the wild nature surrounding them. If sheep and goats are bred in almost every village, yaks and their hybrid offspring are the hearts of the Tibetan traditional agricultural practices. Everything coming from this animal is used, from milk and meat to skin and hair, and even dung. But they are also highly appreciated for their active contribution to land ploughing. Few crop plants are adapted to the high altitude of the Tibetan plateau. Barley is the main cultivation, followed by potatoes and radishes. Wheat, beans, Chinese cabbages, and colza are cultivated when possible. Wild plants and mushrooms are also appreciable food sources. Considering these drastic conditions, in addition to the fact that most Tibetans generally observe Tibetan Buddhism, it is not surprising that the great majority of them consider food like a means of subsisting, far from the hedonic eating behavior of many other populations.

The rural population of T.A.R. is affected by a mysterious disease called Kashin-Beck disease (KBD), named after the physicians who first made the clinical descriptions, Dr. N.I. Kashin, and Dr.

E.V. Beck and his wife A.N. Beck (Mathieu and Hinsenkamp, 2008). Although its prevalence is decreasing in rural T.A.R., it is estimated that KBD affects between 0.74 million and 2.5 million people in China, Russia, and North Korea (Yamamuro, 2001; Mathieu and Hinsenkamp, 2008, Mathieu et al., 2015). This introduction does not pretend to provide an exhaustive review of the literature about Kashin-Beck disease (KBD), as a state-of-the-art review has been done quite recently (Malaisse and Mathieu, 2008). It aims at summarizing the current knowledge and introducing the background and the rationale of the present thesis.

KBD, also called “Big Bone Disease”, is a disabling disease defined as an endemic and chronic osteo-chondropathy. KBD people are of short stature, and present skeletal deformities and enlarged, stiff and painful joints. Consequently, severely affected people see their mobility of limbs decreasing and tend to lose muscle mass. Three to four stages in the evolution of the disease have been defined, according to the severity of the symptoms (Mathieu and Hinsenkamp, 2008). They are based on clinical and radiological criteria which can be observed from childhood and adolescence (Hinsenkamp et al., 2001; Mathieu et al., 1997). The pathophysiological features of KBD involve a focal chondro-necrosis of mature chondrocytes in the deep zone of the growth plate cartilage and the articular cartilage, as well as abnormal expressions of collagen types I, II, III, VI and X in the cartilage (Nesterov, 1964; Pasteels et al., 2001, Wang *et al.*, 2009). Peroxidation of the lipids of chondrocyte membrane is supposed to be responsible of their necrosis (Peng et al., 1992).

Despite broad and intensive research, the etiology of this disease keeps scientists in check for the last hundred years. Allander, (1994) realized an exhaustive review, covering a period from 1849 to 1992, of the scientific literature directly related to KBD. He highlighted not less than 499 publications. He noted that the first suspicions of Russian physicians were oriented towards toxic effects of mycotoxins. This hypothesis has been subject to many investigations since then (Chasseur et al., 2008, 2001, 1997, 1996; Chen et al., 2012; Haubruge et al., 2001; Shi et al., 2009). But no single study has yet been able to definitely demonstrate that one or several mycotoxins were capable of reproducing KBD lesions. Yet, this hypothesis should not be rejected so far because there is a definite relation between the presence of mycotoxin-producing fungi and KBD (Chasseur et al., 2008).

Then, investigations progressively drifted on the troubling correlation observed between selenium deficiency in soils and the prevalence pattern of KBD (Ge and Yang, 1993; Liquiang et al., 1991; Li et al., 2009; Tan et al., 2002; Tan and Huang, 1991; Zhang et al., 2011). The soil deficiency is susceptible to induce a nutritional deficiency, and since selenium is essential to bone health, through its antioxidant action via the glutathione peroxidase, there were good reasons to suspect it in the etiology of KBD. Many clinical trials were implemented to assess the therapeutic and preventive effect of selenium supplementation on KBD patient, but the conclusions of meta-analysis are again not definite (Jirong et al., 2012; Yu et al., 2015; Zou et al., 2009). Although sometime close, nobody has been able to reproduce the pathophysiological feature of the disease by inducing selenium deficiency, either *in vitro* or *in vivo* (Downey et al., 2009; Wei et al., 1986). Furthermore, several studies aiming at comparing the selenium status between KBD and non-KBD people were not able to highlight significant discrepancies (Chen et al., 2015; Ge and Yang, 1993; Moreno-Reyes et al., 1998).

The antagonistic action of mycotoxins and selenium deficiency was of course considered. Results were encouraging, and neither of the two factors can be discarded in the etiology of KBD, but once again, no definite conclusion was reached (Chen et al., 2012; Kang et al., 2013; Yao et al., 2010; Zhang et al., 2010).

Because iodine is essential to growth, and frequently deficient in high altitude, it has also been investigated in relation to KBD (Moreno-Reyes et al., 2006, 2003, 1998; Yao et al., 2011). Interestingly, these studies concluded that if selenium cannot be excluded in the etiology, iodine supplementation was more beneficial to people affected, or at risk. There is the rub; iodine deficiency is known to cause goiter and cretinism, but not KBD.

Another hypothesis was raised by few researchers, the presence of organic acids in water (La Grange et al., 2001; Peng et al., 1999; Ren et al., 1991). Indeed, humic and fulvic acids has been demonstrated to generate oxidative stress and disturb collagen metabolism, especially when selenium is deficient (Shaohua et al., 2004; Yang et al., 1993, 1991). But again, the lesions produced on animal models were not completely similar to those observed in KBD patients, and the exact mechanisms of action of these acids are not clear.

Deficiencies in other trace elements, namely boron, germanium and molybdenum were also anecdotally investigated and proposed as putative factor for the etiology of KBD, but there is little data and no further investigation were conducted (Fang et al., 2003; Peng et al., 2000; B. Zhang et al., 2010). Few authors also consider the implication of genetic factors that predispose to the disease. (Shi et al., 2011; Zhang et al., 2015). If it is true that children having a KBD-affected sibling seems more likely to contract the disease (Suetens et al., 2001; Shi et al., 2008), it cannot be excluded but again it remains to be proved.

Despite the numerous studies conducted, it must be said that the etiology of Kashin-Beck disease remains unclear. Today, most authors agree that KBD is more than likely of environmental origin, and its etiology is multifactorial (Mathieu and Hinsenkamp, 2008; Sudre and Mathieu, 2001; Suetens et al., 2001).

Apart from KBD considerations, several studies focusing on anthropometric measurements and nutritional status assessment conducted in T.A.R. revealed clinical signs of stunting and malnutrition (Chasseur et al., 2008b; Goyens et al., 2008; Harris et al., 2001; Kolsteren et al., 1995; Rooze et al., 2012). It is not necessary to look very far in order to find convincing evidence that this problem must take its roots in the poor nutritional status of the rural Tibetan diet. Food enquiries revealed that these populations are still relying on traditional subsistence farming and animal husbandry. Their diet is extremely monotonous, rich in cereals but with very low consumption of vegetables, fruits, meat, and dairy products (Dermience, 2010; de Voghel, 2008; Goyens et al., 2008).

This diet is obviously susceptible to lead to various nutrient imbalances, in particular as regards to micronutrients: vitamins, minerals, and trace elements. Yet, it is well established that micronutrients play a role in growth and bone metabolism. Surprisingly, to the best of our knowledge, only one study conducted by Wang et al. (2010) was implemented in order to assess the mineral intakes of rural Tibetans mothers. Another study of Liquiang et al., (1991) aimed at comparing the mineral intakes between KBD endemic areas and non-endemic areas. But it was conducted in the Shanxi Province and Inner Mongolia Autonomous Region, these areas are quite far from T.A.R., and the results date back twenty years ago.

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Chapter II.

"When it is obvious that the goals cannot be reached, don't adjust the goals,

adjust the action steps"

Confucius

Objectives

If we resume, KBD is an endemic osteo-chondropathy. Its etiology, although unclear, most probably finds its roots in environmental factors. T.A.R. soils present low, if not very low levels in several elements. More than one nutrient is essential to a healthy growth and bone metabolism. There is scarce data on the nutrition status of the rural Tibetan population in T.A.R.

It therefore made sense to implement a study aiming at assessing the nutrition status of the Tibetans living in rural areas endemic for Kashin-Beck disease. The focus was made on minerals and trace elements, because their concentrations in foods are intimately liked to soil levels, unlike macronutrients and vitamins. Furthermore, deficiencies of at least two elements, selenium and iodine, are frequently cited as possible causes of the disease. Young Tibetan children aged three to five were targeted because at this age the first symptoms of the disease may appear. So the original question of the thesis was formulated as following:

"What is the mineral dietary status of young Tibetan children living in rural areas endemic for Kashin-Beck disease?"

But this question immediately raises other questions, which must be previously addressed. Key sub-questions can be formulated in this way:

- What minerals or elements are involved in the bone and joints metabolism?
- Which ones are of relevancy in the present context?
- What is the most appropriate method to assess the mineral dietary status of these young Tibetan children?
- Do we have the appropriate tools to conduct such a study?
- Are there some additional factors that can influence the mineral dietary status and how to consider them?

Tentative answers to these questions are proposed all along the following chapters. The third chapter presents an exhaustive review of all the elements that have been found to impact bone and joint metabolism. The fourth chapter is dedicated to the evaluation of existing tools for nutritional assessment in rural Tibet, and the proposition of an original tool to address this issue. Chapter V is the core chapter of this thesis. It presents the results of a nutrition survey conducted in rural T.A.R. among 250 children. The sixth chapter investigates the concept of bioavailability of minerals, a major issue in nutritional assessment. Finally, the seventh chapter proposes a conclusion of the results and some perspectives in the context of KBD.

Chapter III.

"All things are poison and nothing is without poison; only the dose makes a thing not a poison."

Paracelsus

Introduction

Skeletal tissues form a complex system that serves several functions in mammals. It provides support for muscles, allowing movement. It protects vital organs, such as heart, or brain. It is the exclusive site of some vital mechanisms, such as hematopoiesis. And it is a strategic reserve for certain minerals.

Bone is a connective tissue resulting of subtle interlacing between organic and inorganic components (Marieb, 2005). As to the organic part, it contains fibrous and globular proteins that will confer its flexibility and resilience to bone. A gel-like substance, also called ground substance, made of proteoglycans and glycoproteins is also present, generating a favorable extracellular environment. This environment is essential to the actual bone architects, namely osteoblasts, osteoclasts, and osteocytes. These bone cells will generate, resorb, maintain and remodel the bone matrix throughout life. The inorganic matrix, the most important in weight, is predominantly made of hydroxyapatite crystals that confer hardness and rigidity to bones. Traces of many other elements are irremediably present in the mineralized matrix (Zaichick et al., 2010; Zaksas et al., 2008). These elements, as we will see in this chapter, may be absolutely essential, exclusively toxic, or sometimes both, depending on the concentration.

Bone metabolism seems influenced by numerous chemical equilibria, a fine gene regulation, and a complex communication between osteoblasts and osteoclasts (Asagiri and Takayanagi, 2007; Fuller et al., 2007; Ishimi et al., 1990; Kaji et al., 1996). For decades, the comprehension of this metabolism was the subject of many studies and investigations all around the world, and gave rise to a tremendous number of scientific publications. But, despite the considerable knowledge accumulated, we must admit that we are still far from a global understanding of all the mechanisms, chemical, molecular, and genetic, underlying bone and joints metabolism. This is evidenced by the global public health issue that constitutes osteoporosis, and our inability to find preventive or curative treatments of indisputable effectiveness (Body et al., 2011; Cheng et al., 2011; Deng and Recker, 2004; Gür et al., 2002; Heaney, 2000; Lelovas et al., 2008; Melton, 1993).

In the following review we aimed to bring our small contribution, providing an overview of the effects of chemical elements on bone metabolism and bone health. In the specific context of this thesis, it allowed us to select the elements of relevance that will be targeted in the nutrition survey.

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Effects of thirty elements on bone metabolism

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Adapted from the reference: Dermience, M., Lognay, G., Mathieu, F. & Goyens, Ph. 2015. Effects of thirty elements on bone metabolism. *J. Trace Elem. Med. Biol.* **32**, 86–106.

Abstract

The human skeleton, made of 206 bones, plays vital roles including supporting the body, protecting organs, enabling movement, and storing minerals. Bones are made of organic structures, intimately connected with an inorganic matrix produced by bone cells. Many elements are ubiquitous in our environment, and many impact bone metabolism. Most elements have antagonistic actions depending on concentration. Indeed, some elements are essential, others are deleterious, and many can be both. Several pathways mediate effects of element deficiencies or excesses on bone metabolism. This paper aims to identify all elements that impact bone health and explore the mechanisms by which they act. To date, this is the first time that the effects of thirty minerals on bone metabolism have been summarized.

Keywords

Bone metabolism, minerals; mineral deficiency; trace elements; heavy metals; osteoporosis

1. Introduction

The human skeleton is made of 206 bones. It plays several essential roles in the human body, including providing support, protecting vital organs, allowing movement, and storing minerals. Bones contain both organic and inorganic components. Organic components comprise osteoblasts, osteoclasts, osteocytes, the organic matrix (fibrous proteins), globular proteins (osteonectin and osteocalcin), and ground substances (proteoglycans and glycoproteins). The organic matrix confers flexibility and strength to bones. The inorganic fraction, 65% of total bone weight, is comprised of hydroxyapatite, which confers hardness and rigidity. Bones also contain small amounts of minerals and trace elements, which can be essential or toxic, depending on concentrations (Marieb, 2005).

The main objective of this review is to provide an overview of the effects of elements on bone metabolism and bone health. Thirty elements, known to impact bone metabolism, bone health, osteogenesis, and homeostasis and those used as anti-osteoporosis drugs, were reviewed. Because beneficial and adverse effects can depend on very narrow concentration ranges, elements are listed in alphabetical order and not grouped by mechanism of action. Majors elements (C, H, O, N), as well as sulfur as not been discussed in this review. It also does not include quantitative information, including it would require consideration of aspects as divergent as dietary intake, supplements, toxicological threshold, and pharmacological doses. Readers interested in quantitative values related to dietary intakes or supplements are referred to World Health Organization (WHO). The national recommendations from the Workshop Summary of the National Research Council on dietary reference intakes (West Suitor and Meyers, 2006a) provide exhaustive recommendations for many elements, depending on age and gender. For a reference on bone mineral trace element content, the reader is referred to (Zaichick et al., 2010), which lists bone composition of 59 elements, according to age and gender.

2. Impacts of thirty minerals on bone metabolism

2.1. Aluminium

Aluminium, an element widely distributed on earth, has a negative impact on bone health. Aluminium negatively affects collagen synthesis, inhibits bone formation, and impairs bone remodeling, which can lead to adynamic bone disease, a variety of renal osteodystrophy, and osteomalacia (Bronner, 2002; Malluche, 2002; Li et al., 2011b; Pilar Martínez et al., 2011). The deleterious impacts of aluminium are exacerbated when calcium and magnesium are deficient (Zafar et al., 2004; Krewski et al., 2007). Despite low bioavailability, 60% of aluminium stored in the body is in bones, which increases its half-life and makes toxicity cumulative (Malluche, 2002; Krewski et al., 2007). Despite strong affinity for bone, aluminium has not been correlated with occurrence or extent of osteoporosis and does not seem to play a role in etiology (Hellstrom et al., 2006; Hellström et al., 2008).

Adverse effects of aluminium are exerted through multiple pathways, affecting bone mineralization, cell number, and cell activities. At the intestinal level, (Mahieu et al., 2004) reported that chronic exposure to aluminium reduced absorption of inorganic phosphorus and promoted bone accretion. In contrast, Zafar *et al*. (Zafar et al., 2004) reported decreased calbindin (CaBP)-D9k expression, a gene coding for a protein that mediates transport of calcium through the enterocyte barrier, and lower calcium deposition in bone. How aluminium reduces synthesis of calcium-binding proteins is not known, but it may be involved in renal production of calcitriol (1α,25-Dihydroxycholecalciferol) (Mahieu et al., 2004; Zafar et al., 2004). However, there is no doubt that the deposition of aluminium on the mineralization front induces physiochemical changes that disturb calcium accretion and enhance calcium release.

Several studies revealed that toxicity of aluminium in osteoblasts and osteoclasts is caused by inhibition of proliferation and cellular activities (Malluche, 2002; Rousselle et al., 2002; Zafar et al., 2004). The mechanisms are not clear, although they seem to be closely related to parathyroid function. An experimental study suggested a direct toxic action of aluminium on osteoblasts (Rodriguez et al., 1990). In addition to this direct action, aluminium is known to decrease parathyroid function by accumulating in the gland and elevating serum calcium levels (Cannata Andía, 1996). Rats fed high levels of aluminium also presented with decreased calcitonin, osteocalcin, procollagen carboxy-terminal propeptide, and bone alkaline phosphatase levels (Li et al., 2011b).

At this stage of our knowledge, no doubt that aluminium negatively impacts bone metabolism. Human observations, animal testing, and in vitro experimentations, all conclude in deleterious effects of aluminium. However, the different pathways by which it acts are still to be cleared.

2.2. Arsenic

Arsenic is both essential and toxic. Essential roles are associated with methionine metabolism, and phosphorylation. However, deprivation of arsenic in rat and hamster models showed no pathological effects on bone metabolism (Uthus, 1990; Jacotot and Le Parco, 1992; Uthus, 1994) except depressed growth (Nielsen, 1991). However, other work in rats watered with a solution of arsenic (0.21mg/kg.b.w./day for 45 days) showed inhibition of endochondral ossification (Aybar Odstrcil et al., 2010), suggesting possible adverse effects on bone metabolism. Despite conflicting results, recommended intakes extrapolated from animal experiments are very low, ranging from 12 to 25 µg/day, and no human deficiency has ever been reported (Uthus and Nielsen, 1993).

To investigate the effects of arsenic trioxide, which is used in tumor treatment, Hu *et al*. (Hu et al., 2012) conducted an *in vivo* study on bone remodeling and osteoblast metabolism. Rats received intraperitoneal injections of saline or arsenic trioxide (5 or 10 mg/kg) for 4 weeks, and markers of bone turnover, osteocalcin, procollagen type I N-terminal propeptide, and C-terminal cross-linked telopeptide, were reduced. In addition, reduced bone mineral density and trabecular bone volume in the femur were observed (Hu et al., 2012). These effects may be mediated through decreased capacity of osteoclasts to adhere to the bone matrix. Consistent with this model, reduced expression of the vascular cell adhesion molecule-1 (VCAM-1) has been observed, which may impair resorption and remodeling of subchondral bone.

Another possibility is that arsenic disrupts osteoblast differentiation. This model is supported by decreased expression of transcription factors Runt-related transcription factor 2 (RUNX2) and osterix. Mineralization, due to depressed alkaline phosphatase and osteocalcin expression, also supports this possibility (Aybar Odstrcil et al., 2010; Hu et al., 2012). Expression of interleukin 6, which is produced by osteoblasts and known to stimulate osteoclasts and induce bone resorption, was also decreased (Ishimi et al., 1990; Hu et al., 2012). Finally, human studies have connected arsenic exposure to osteomalacia, higher prevalence of Paget's disease, and disrupted fetal development, but further studies are needed before conclusions can be reached (Lever, 2002; Kippler et al., 2012; Akbal et al., 2013). It is also worth noting that As under the form of arsenate substitutes for phosphate and decrease it transport (Ito et al., 2005).

The acute toxicity of arsenic is long known. However, quite few studies have interest for chronic low-level exposure to arsenic which obviously exerts deleterious effects on bone metabolism. Most of them are about animal testing and they are often far from being representative of the environmental exposure of humans to this element. Epidemiological studies on the exposure of human populations to As concomitant with a better comprehension of the physiological mechanism of its different chemical species would help to reach definite conclusion on the impact of this both essential and toxic element on bone metabolism.

2.3. Boron

Boron is not described as essential, because no biochemical function has been directly connected to it, although it is known to influence the activity of many enzymes (Hunt, 1996; Nielsen, 1997). Numerous studies have demonstrated beneficial effects of boron on bone health, including improved bone growth, strength, stiffness, hardness, mechanical properties, and trabecular bone microarchitecture (Newnham, 1994; Armstrong and Spears, 2001; Naghii et al., 2006; Nielsen, 2009). Boron may also treat arthritis by alleviating pain and discomfort and decreasing inflammation (Newnham, 1994). Interestingly, the boron content of healthy bones is higher than in arthritic bones, and the prevalence of arthritis is much lower in areas where daily intake of boron is 3 to 10 mg compared to areas where consumption is 1 mg or less (Newnham, 1994; Devirian and Volpe, 2003).

Deprivation of boron alone does not lead to adverse effects. However, it is associated with decreased chondrocyte density in proliferation zones within bone growth plates. Boron deficiency also exacerbates the symptoms of vitamin D and magnesium deficiencies, increasing urinary calcium. Accordingly, supplementation of boron improves bone calcification and decreases calciuria (Hunt, 1996; Devirian and Volpe, 2003; Nielsen et al., 2007; Nielsen, 2008). Boron deprivation also affects concentrations of several other minerals and trace elements involved in bone metabolism, including magnesium, copper, and zinc (Armstrong and Spears, 2001; Nielsen, 2008). These results are consistent with the suggestion that boron deficiency may impair bone healing by reducing osteogenesis, possibly by decreasing periodontal alveolar bone osteoblast surfaces (Gorustovich et al., 2008a, 2008b).

The biochemical functions of boron in humans are not yet elucidated. Data suggest a beneficial interaction occurs between boron and steroid hormones involved in bone metabolism (Sarazin et al., 2000; Sheng et al., 2001a; Naghii and Mofid, 2008). Indeed, 17β-estradiol appears to be a target, such that its concentration and efficacy are enhanced by boron, improving trabecular bone volume, bone growth plate density, and trabecular separation (Sheng et al., 2001a). Boron may also help prevent bone-related diseases such as osteoporosis, arthritis, and other steroid hormone dependent diseases, possibly by improving absorption of calcium, phosphorus, and magnesium and retention of calcium and magnesium (Sheng et al., 2001b). Although the role of boron in bone metabolism remains unclear, a current hypothesis implicates hydroxylation of estrogen (Naghii and Mofid, 2008). Similarly, another track considered by biochemists is that formation of boroesters with ribose-derived compounds can influence reactions involving S-adenosylmethionine and oxidized nicotinamide adenine dinucleotide (NAD^+) (Nielsen and Meacham, 2011).

It is not common that a chemical element presents so many beneficial effects on bone metabolism without being essential. Despite the fact that some researchers spend their career on the role of boron in the human metabolism, its molecular mechanisms are not completely elucidated. Performing speciation analysis, using for example IC-ICP-MS, in order to highlight the different species of boron in the human body would certainly help identify the exact roles and mechanisms of this particular element.

2.4. Cadmium

Toxic effects of cadmium in kidneys and bones are well established. Cadmium can cause renal tubular dysfunction, decrease bone mineral density, and cause hypercalciuria, all factors that can increase risk of fracture, osteomalacia, and osteoporosis (Wang et al., 2003; Kazantzis, 2004; Zhu et al., 2004; Brzóska and Moniuszko-Jakoniuk, 2005; Wu et al., 2010; Engström et al., 2011). Although cadmium toxicity affects both genders, elderly females are more susceptible than men, especially after menopauses (Alfvén et al., 2004; Åkesson et al., 2006; Brzóska et al., 2010; Chen et al., 2011).

Cadmium is so harmful, adverse effects are evident in young children with low chronic environmental exposure. Moreover, symptoms can appear soon after exposure, and may persistent even after exposure is reduced (Brzóska et al., 2004; Bhattacharyya, 2009; Chen et al., 2009b; Sughis et al., 2011). Itai-itai disease is an example of a disease caused by chronic cadmium exposure (Cai et al., 2001; Inaba et al., 2005). Postmenopausal women are the most commonly affected by itai-itai disease, which is characterized by osteoporosis with osteomalacia, renal tubular disorder, and renal anemia (Nogawa et al., 1987; Kazantzis, 2004; Uchida et al., 2010). Recent evidence indicates cadmium can also induce oxidative stress, revealing new putative factor in cadmium toxicity (Brzóska et al., 2011).

Although the toxicity of cadmium is well established, its mechanisms of action remain unclear. Growing evidence indicates the presence of direct and indirect pathways. Before any bone effects are observed, environmental exposure to cadmium affects the kidneys, causing renal tubular dysfunction (Trzcinka-Ochocka et al., 2010). It has been suggested that conversion of 25-hydroxycholecalciferol to its biologically active form in the kidney may reduce the efficiency of intestinal calcium absorption, but recent studies have challenged this model. Instead, recent work suggested that symptomatic cadmium exposure is adversely affected by hypovitaminosis D (Beattie and Avenell, 1992; Engström et al., 2009; Uchida et al., 2010).

The direct pathway implicated in cadmium toxicity is involved with calcium homeostasis, collagen matrix, and bone cell metabolism. Due to similar properties, cadmium can disturb calcium metabolism during osteogenesis and bone homeostasis, increasing calciuria and disturbing calciotropic hormones (Beattie and Avenell, 1992; Brzóska and Moniuszko-Jakoniuk, 2005; Schutte et al., 2008; Yokota and Tonami, 2008). Accordingly, calcium deficiency has been reported to enhance cadmium toxicity (Beattie and Avenell, 1992). Cadmium also affects homeostasis of other minerals involved in bone metabolism. Noël *et al.* (Noël et al., 2004) found that liver levels of iron, magnesium, and selenium decreased while copper, zinc, and manganese increased with increasing cadmium levels. The collagen matrix upon which bone mineral is deposited, is also altered by cadmium, possibly by stimulating proliferation and activity of osteoclasts. These effects have been connected to alteration of glycosaminoglycans and proteoglycans (Kazantzis, 2004; Sredzinska et al., 2004). Cadmium also acts on bone cells, reducing bone formation and promoting bone resorption. Increased number and activity of osteoclasts may be due to increased levels of serum parathyroid hormone and up-regulation of RANKL (receptor activator of nuclear factor kappa-B ligand) expression in osteoblasts (Beattie and Avenell, 1992; Chen et al., 2009a). Cadmium can also decrease osteoblast viability, mineralization capacity, and alkaline phosphatase activity (Bhattacharyya, 2009; Chen et al., 2009a).

2.5. Calcium

Calcium plays a critical structural role in bone. Indeed, 99% of calcium in the body is in the form of hydroxyapatite which is bone and tooth mineral. Deficient dietary consumption of calcium leads to lower bone mineral content and bone mineral density, and long-term deficiency can lead to rickets, osteomalacia, and osteoporosis (Marieb, 2005). Accordingly, high calcium intake, from food or supplementation, contributes to better calcium homeostasis, enhanced bone mineralization during growth, and decreased bone loss and reduced risk of osteoporotic fracture in the elderly (Heaney, 2000; Huncharek et al., 2008; Rizzoli et al., 2010). The beneficial effects of calcium supplementation on bone health are marked when children present low dietary intake, whereas effects are masked in healthy children with sufficient dietary consumption (Gibbons et al., 2004; Winzenberg et al., 2006).

Calcium also contributes to bone metabolism. Experiments in cell culture have shown that high concentrations of extracellular calcium stimulate osteoclast-like cell formation and bone resorption by mature osteoclasts (Kaji et al., 1996). This action may be mediated by osteoblasts. High extracellular calcium levels stimulate DNA synthesis in osteoblasts, evoking osteoclast proliferation activities (Sugimoto et al., 1994; Asagiri and Takayanagi, 2007). In contrast, high cytosolic calcium concentrations modify the osteoclast cytoskeleton. This may be mediated through calcium-signaling molecules expressed in podosomes, which modify cell adhesion and decrease bone resorption capacities (Miyauchi et al., 1990; Siddiqui et al., 2012).

Although the primary role of calcium in bone metabolism is the formation of hydroxyapatite, this ubiquitous element has multiple and complex regulatory functions. The complete understanding of the role of calcium on the bone metabolism requires a global vision of its different mechanisms of action, which is not yet possible in the present state of knowledge.

2.6. Chromium

Trivalent chromium is an essential trace element that plays numerous roles in carbohydrate and lipid metabolism (Martin, 2000). Yet, animal experiments and *in vitro* studies demonstrate that some forms, including hexavalent chromium, can induce oxidative stress and exert cytotoxic effects on bone cells. In several studies, rats that were given water enriched with dichromate during gestation produced fetuses with reduced ossification, especially in parietal, interparietal, and caudal bones, suggesting accelerated bone resorption activity and reduced bone formation (Junaid et al., 1996; Kanojia et al., 1998; Soudani et al., 2011).

Chromium intoxication from environmental exposure is not common, except in chromium-related industries and associated environments. However, metal implants used in surgery are made of chromium-containing alloys. These implants are subject to wear and corrosion, and release chromium into surrounding tissues. *In vitro* studies showed that chromium particles and ions induce osteolysis by enhancing bone resorption and reducing bone formation (Sansone et al., 2013). Reduced bone formation may be mediated via cytotoxic effects on osteoblasts, which reduce proliferation, inhibit osteocalcin release, and decrease alkaline phosphatase activity. These effects are associated with oxidative stress, which can be caused by imbalanced OPG/RANKL ratios, oxidation and nitration of proteins, and misregulation of antioxidant enzyme expression (Fleury et al., 2006; Zijlstra et al., 2012; Sansone et al., 2013). Chromium ions were also found to disturb the release of cytokines (TGF-β1, TNF-α, IL-β1, TNF-G) from osteoblasts, promoting proliferation of preosteoclasts and maturation into active osteoclasts, ultimately, enhancing bone resorption (Wang et al., 1996; Sansone et al., 2013).

2.7. Cobalt

Cobalt is an essential trace element since it is the core component of vitamin B12. There is no dietary recommendation related to cobalt itself because vitamin B12 cannot be synthetized in the human body and must be ingested as such (Martin, 2000). Apart from this role, cobalt is not known to play any other physiological roles. Yet, cobalt - like chromium - is a major component of some metal implants used in surgery, and its use raises the same concerns with regard to bone health. Both cobalt ions and cobalt-chromium particles can release from prosthetic implants due to wear and/or corrosion. This, in turn, causes loss of peri-implant bones (Sansone et al., 2013). Several *in vitro* studies investigated the link between cobalt and this form of osteolysis. Results revealed that cobalt influences bone resorption and bone formation by modulating bone cell metabolism. Indeed, cobalt ions affect osteoblast proliferation, size, and shape. Influences on osteoblastic activities decreases alkaline phosphatase levels and calcium accretion, which inhibits release of osteocalcin and collagen type 1 proteins (Anissian et al., 2002; Fleury et al., 2006; Queally et al., 2009; Sansone et al., 2013). *In vitro* experiments have also suggested that oxidative stress is an adverse effect of divalent cobalt ions. These effects may be mediated by the redox state in osteoblast-like cells, but the mechanism of action is not known, and contradictory results have been presented (Fleury et al., 2006; Tkaczyk et al., 2010a; Tkaczyk et al., 2010b; Zijlstra et al., 2012).

Cobalt ions also promote secretion of cytokines (TGF-β1, TNF-α, IL- β 1, IL-6, IL-8, and MCP-1) from osteoblasts, which leads to inflammation and osteoclast differentiation, maturation, and stimulation (Wang et al., 1996; Anissian et al., 2002; Queally et al., 2009; Devitt et al., 2010; Sansone et al., 2013). In contrast, some studies on cobalt-chromium wear particles indicate antagonistic actions, such as induction of apoptosis in osteoclasts and decreased secretion of some inflammatory factors such as prostaglandin E2 and interleukin-6 (Haynes et al., 1993; MacQuarrie et al., 2004).

2.8. Copper

Copper is considered an essential trace element, although copper deficiencies in humans have been reported only rarely. Several cases of children, under parenteral nutrition, and animals presenting symptoms associated with deficient copper consumption are documented in the literature. The symptoms of copper deficiency include decreased bone strength, impairment of bone formation and growth, reduced bone mineralization, reduced ossification of growth centers, and compromised cartilage integrity. Animals with copper deficiency have presented with deformed bones, hypoplasia, brittle bones, and frequent fractures (Dollwet and Sorenson, 1988; Keen et al., 1998; Sarazin et al., 2000). Effects of copper deficiency on bone metabolism are particularly evident in newborns affected by Menkes disease. A neurodegenerative disorder, Menkes disease impairs copper absorption, producing widespread effects, including bone change such as retarded growth, generalized osteoporosis, and flared metaphyses of the long bones (Kodama et al., 1999). Low to moderate copper deficiency is also associated with osteoporosis, indicating sufficient dietary intakes are important for maintaining bone and cartilage (Beattie and Avenell, 1992; Saltman and Strause, 1993; Aaseth et al., 2012).

The following physiopathological symptoms associated with copper have been reported: thinning of the cortex and trabeculae of long bones; epiphyseal separations; metaphyseal cupping and blurred margins; subperiosteal new bone growth; systemic porosis; rib fractures; enlarged costochondral junctions; flared, cupped, and irregular metaphyses; osteoporotic ulnar, tibial, radial, femoral, and fibular bones; periosteal elevation with superiosteal calcification, resembling scurvy; and higher soluble to insoluble collagen ratio (Dollwet and Sorenson, 1988; Beattie and Avenell, 1992; Keen et al., 1998). Various cuproenzymes (such as amine oxidase, ceruloplasmin, cytochrome-c oxidase, dopamine-β-monooxygenase, extracellular superoxide dismutase, lysyl oxidase, peptidylglycine-α-amidating monoxygenase, Cu/Zn-superoxide dismutase, and tyrosinase) mediate the effects of copper deficiency.

A main adverse effect attributed to copper deficiency is a lysyl oxidase disorder, which prevents crosslinking between collagen and elastin, reducing strength of the bone matrix (Dollwet and Sorenson, 1988; Beattie and Avenell, 1992; Sarazin et al., 2000). Reduction of superoxide dismutase may also contribute to inhibition of osteoblasts, as it is sensitive to free radicals generated by osteoclast activities (Keen et al., 1998; Sarazin et al., 2000). In contrast, excess copper can generate free radicals, which induce lipid peroxidation and interfere with bone metabolism. These effects are manifest as generalized loss of bone density, rickets, and anomalous osteophytes in Wilson's disease patients (Beattie and Avenell, 1992; Martin, 2000).

Copper, like iron and zinc, is also a ubiquitous element in the human body which plays many roles at different levels. It is an essential element but excess can have severe negative impacts. An overall comprehension of its homeostasis and physiology – which is not yet acquired – is mandatory to establish its exact role in the bone metabolism and to define an optimal interval of dietary intake.

2.9. Fluorine

The effects of fluorine are controversial. Beneficial actions on dental caries are proven, whereas influences on bone metabolism are poorly defined. Little doubt remains as to the dose-dependent effects of fluoride on bone cell and bone structural properties. However, its use as an anti-osteoporotic drug to prevent fractures has been questioned, even though recent studies revealed beneficial effects of low fluoride doses (Hillier et al., 1996; Lau and Baylink, 1998; Lehmann et al., 1998; Vestergaard et al., 2008). Fluoride supplementation combined with other anti-resorptive drugs such as estrogens (for postmenopausal women) and bisphosphonates seem to achieve better results than fluoride alone (Aaseth et al., 2012). Indeed, some authors suggested a positive correlation between fluoride intake and fracture incidence, but these conclusions were deemed inconclusive as they were reached through ecological studies that did not control for possible confounding variables (Cauley et al., 1995; Hillier et al., 1996; Allolio and Lehmann, 1999).

Toxicity of excess fluoride is not contested. Overconsumption leads to skeletal fluorosis, a disturbance of bone homeostasis characterized by a decreased bone mass in the peripheral skeleton concomitant with genu valgum and varum, sabre tibia, and secondary hyperparathyroidism (Beattie and Avenell, 1992; Everett, 2011; Aaseth et al., 2012).

Relatively high-dose fluoride also increases bone mineral density, bone volume, trabecular thickness, and cortical and trabecular new bone formation (Beattie and Avenell, 1992; Balena et al., 1998; Lehmann et al., 1998). However, bone formed in the presence of extra fluoride exhibits distinctly abnormal matrix characteristics, including osteocytic cellularity, irregular arrangement of osteocytes, and enlarged osteocyte lacunae, which weaken biomechanical properties. Moreover, fluoride-induced osteogenesis is appositional (i.e. without formation of new trabeculae), and long-term fluoride supplementation can cause impaired and delayed mineralization (Vigorita and Suda, 1983; Beattie and Avenell, 1992; Aaseth et al., 2012).

Fluoride has two main mechanisms of action in the human body. Fluorapatite has a strong affinity for calcium and hence for bone matrix, whereas fluoride acts to modulate osteoblast activity. Fluorapatite is less acid soluble, which may confer higher resistance to bone resorption by osteoclasts. However, it appears that its accretion is perpendicular to collagen fibers, in contrast to hydroxyapatite. It is also less conducive to bonding with proteins (Beattie and Avenell, 1992). Fluoride also enhances bone formation by stimulating osteoblast proliferation and activity, although no direct influences on osteoclasts have been described (Aaseth et al., 2012). Mitogenic activity may be exerted through the mitogen-activated protein kinase (MAPK) pathway (Lau and Baylink, 1998; Everett, 2011). Upregulation of IGF-1 production is another proposed mechanism of fluoride on osteoblasts (Turner et al., 1997; Lau et al., 2002).

Although fluoride has been subject to supplementation recommendations and used as anti-osteoporotic drug, its beneficial role is more and more controverted. Fluoride clearly impacts bone metabolism but deeper investigations are necessary to fully elucidate its molecular mechanisms, which is mandatory to reach a consensus on the beneficial use of fluoride as anti-osteoporotic drug.

2.10. Gallium

Gallium nitrate was investigated for use in cancer-related hypercalcemia. Hopes were based on results from use with other bone-related diseases, such as multiple myeloma, bone metastases, Paget's disease, osteopenia, and osteoporosis (Hall and Chambers, 1990; Lakatos et al., 1991; Stern et al., 1994; Bockman, 2003; Chitambar, 2003). Gallium nitrate inhibits bone resorption, even at low-doses, and leads to improved bone mineralization and biomechanical properties without any apparent cytotoxic effects on bone cells. However, the major concern about gallium nitrate was raised by its poor bioavailability. Ga^{3+}, ingested under the form of gallium nitrate, forms various unstable hydroxide species prone to precipitation. The formation of hydroxides is concomitant with H_3O^+, which makes it potentially harmful and unfit for human consumption (Verron et al., 2012a). Organic gallium compounds, such as gallium maltolate or yeast incorporated gallium, have better bioavailability and lower toxicity and are better candidates for an alternative antiosteoporotic treatment. (Ma and Fu, 2010a, 2010b).

Like many other elements, gallium is incorporated into metabolically active bone matrices, which modifies physical and chemical properties (Hall and Chambers, 1990; Donnelly et al., 1991; Lakatos et al., 1991; Bockman, 2003; Verron et al., 2012a). Gallium is reported to have anti-resorptive actions mediated by dose and time-dependent modulation of osteoclast activity through two main pathways. Although previously contested, gallium seems to decrease osteoclast differentiation rates by downregulating NFATc1 (Nuclear factor of activated T-cells, cytoplasmic 1) gene expression and playing a key role in RANKL-induced osteoclast differentiation. The second pathway involves blocking the TRPV5 (Transient receptor potential cation channel subfamily V member 5) Ca^{2+} channel, which is essential for osteoclast bone resorption (van der Eerden et al., 2005; Verron et al., 2012b).

Gallium organic compounds seem to have promising outcomes in the treatment of osteoporosis and show no acute toxicity. Yet, the use of gallium based drugs is quite recent, and the precautionary principle would recommend more investigation on possible side effects of chronic exposure to gallium before systematic use for the treatment of osteoporosis.

2.11. Germanium

Due to similarity with silicon, Seaborn and Nielsen (Seaborn and Nielsen, 1994) investigated germanium for beneficial roles in bone metabolism. They concluded that mineral salts of germanium may counteract some of the effects of silicon depletion, though the concentrations that produce positive and deleterious effects are very similar. An organic germanium compound (poly-trans-(2-carboxyethyl)germaniumsesquioxide or Ge-132) is less toxic and has been tested as an anti-osteoporosis drug in ovariectomized rats. The results, improvement of bone mineral density and bone mineral content, and maintenance or enhancement of bone strength, were encouraging (Matsumoto et al., 1991; Fujii et al., 1993; Jiang et al., 2004).

Few studies on the bone-related effects of germanium have been conducted. Although the use of germanium-based drugs in osteoporosis is low because more efficient medicines were discovered (Qin et al., 2013), the consequence of substitution of Ge to Si in the human body, and the possible Si-independent physiological roles of Ge deserve further considerations.

2.12. Gold

Gold salts have been used since the early 20th century as anti-rheumatic drugs. Although the molecular activities of gold are not known, few studies have investigated them. Gold salts, such as auranofin, aurothioglucose, and aurothiomalate, have inhibitory effects on bone resorption *in vitro* (Katz and Gray, 1986; Hall et al., 1996). The mechanism seems to be indirect, affecting the differentiation pathway of pre-osteoclastic cells and resulting in decreased proliferation of osteoclasts (Rousselle et al., 2002). A similar but weaker effect is observed on pre-osteoblastic cells (Rousselle et al., 2002; Chiellini et al., 2008). Vargas *et al*. (Vargas et al., 1987) suggested that gold acts by inhibiting prostaglandin E2 and interleukin-1. Another study revealed association of gold nanoparticles with RANKL, a major inducer of osteoclast formation (Sul et al., 2010).

2.13. Iron

Iron is an essential element for humans. *In vitro* and *in vivo* experiments have shown that iron deficiency disturbs bone homeostasis. Both bone formation and bone resorption are affected, resulting in decreased bone mineral density and mass, altered microarchitecture, and reduced strength (Medeiros et al., 2002; Harris et al., 2003; Medeiros et al., 2004; Parelman et al., 2006; Katsumata et al., 2009). However, the beneficial iron concentration window is narrow, and many recent studies have highlighted adverse effects of iron overload. Indeed, overconsumption may be involved in different types of osteoporosis, intensifying bone resorption and oxidative stress, dwindling bone biomechanical properties, and increasing risk of fracture (Mandalunis and Ubios, 2005; Jian et al., 2009; Yamasaki and Hagiwara, 2009; Tsay et al., 2010; Zarjou et al., 2010; Yang et al., 2011; Kim et al., 2012a; Kim et al., 2013). Iron overload has been documented in genetic diseases (e.g. hemochromatosis and thalassemia), iron over-supplementation (especially in the case of chronic renal failure), or increased iron serum level due to cessation of menstruation in postmenopausal women.

To our knowledge, only one study from Katsumata *et al*. (Katsumata et al., 2009) has described molecular mechanisms of iron deficiency in bone metabolism The authors reported decreased serum 1α,25-dihydroxycholecalciferol, insulin-like growth factor-I, and osteocalcin concentrations. Reduced regulatory factors and bone markers suggest iron deficiency reduces bone formation and bone resorption (Katsumata et al., 2009). Conversely, the effects of iron overload have been thoroughly investigated, revealing that it affects metabolism in both osteoblasts and osteoclasts. In osteoblasts, excess iron exerts antagonistic actions on pre-osteoblast cells, disrupting cell differentiation (Messer et al., 2009; Yamasaki and Hagiwara, 2009; Yang et al., 2011). Osteoblast activity is impacted through downregulation of ALP, HHIPL-2, osteocalcin, and CBF-α1 expression (Zarjou et al., 2010; Yang et al., 2011; Doyard et al., 2012; Zhao et al., 2012). It has been suggested that the ferroxidase activity of ferritin is responsible for these activities, but more studies are needed to confirm that model (Zarjou et al., 2010; Yang et al., 2011). Strong data does indicate that production of reactive oxygen species (ROS) occurs with iron overload, which induces oxidative stress in osteoblasts (Tsay et al., 2010; He et al., 2013). These effects are associated with high serum TNF-α, IL-6, TGF-1, and osteopontin, which stimulate osteoclasts and bone resorption through RANKL (Isomura et al., 2004; Tsay et al., 2010; Jia et al., 2012).

2.14. Lead

Lead accumulates in bones during fetal development, and adverse effects on bone metabolism present even after low-level of exposure (Beattie and Avenell, 1992; Berglund et al., 2000; Khalil et al., 2008). Lead causes growth retardation by inhibiting endochondral ossification. Increased bone turnover and reduced mineralization rates combine to decrease bone mineral density and mass, and, in the most severe cases, cause osteoporosis (Hamilton and O'flaherty, 1994; Hamilton and O'flaherty, 1995; González-Riola et al., 1997; Ronis et al., 1998; Ronis et al., 2001; Carmouche et al., 2005; Khalil et al., 2008; Jackson et al., 2010; Monir et al., 2010; Conti et al., 2012a; Conti et al., 2012b; Eric et al., 2012).

Lead is highly cytotoxic, affecting osteoblasts, osteoclasts, and chondrocytes. It affects hormonal secretion and hormonal-induced cell responses, particularly to 1α,25-dihydroxycholecalciferol and IGF-1; bone-related protein synthesis, including osteocalcin, collagen, osteopontin, and sclerostin; the calcium and second messenger, cAMP; and the Wnt/β-catenin pathway (Angle et al., 1990; Sauk et al., 1992; Eric et al., 2012). Surprisingly, lead stimulates chondrogenesis. However, stimulation is not beneficial due to delayed chondrocyte maturation, persistence of cartilage, and reduced bone formation (Zuscik et al., 2007). A study carried out by Dowd *et al.* raised a track for the molecular deleterious mechanism of lead (Dowd et al., 2001). It was already established that Pb(2+) can substitute to Ca(2+) in hydroxyapatite crystal. Besides, they demonstrated a higher affinity of lead compared to calcium for osteocalcin. Calcium-induced conformational changes in the protein increase its binding propensity to hydroxyapatite. The authors suggest that the substitution of calcium by lead, either in the complex with osteocalcin or in the hydroxyapatite would exacerbate the binding rate of the protein to the crystal. Although the role of osteocalcin is not clearly known, the consequence could be an alteration of bone remodeling.

2.15. Lithium

Lithium has been used for decades to treat bipolar disorders, and evidence of possible impacts on bone health has been revealed. Numerous case studies report lithium-associated hyperparathyroidism and hypercalcemia (Franks et al., 1982; Szalat et al., 2009). However, other studies of lithium on bone metabolism revealed conflicting results. Some studies suggest that lithium causes bone loss that can predispose or aggravate osteoporosis (Laroche et al., 1997; Lewicki et al., 2006), whereas other studies did not find any significant effects (Cohen et al., 1998). In contrast, several studies highlighted beneficial effects of lithium on bone metabolism, including inhibition of bone resorption and osteoclast formation, possibly via modulation of the canonical Wnt/β-catenin pathway (Pepersack et al., 1994; Clément-Lacroix et al., 2005; Zamani et al., 2009; Li et al., 2011a; Satija et al., 2013).

At the current state of knowledge, it is impossible to reach definite conclusion on the effects of lithium on bone metabolism. Some studies highlighted deleterious effects that have been later controverted, and some studies suggest beneficial effects. Lithium is probably an ambivalent element for which further studies are necessary to determine the likely narrow range of concentration with beneficial effects. A chronic toxicity consequent to lithium therapy is also possible and should be investigated, as well as the possible synergistic action with other factors affecting bone metabolism.

2.16. Magnesium

Magnesium is abundant in the human body. Approximately 50 to 60% of the 25 g present in the average human body is located in bones, and magnesium is essential for the function of 300 enzymes (Martin, 2000). Studies in animals and humans have revealed that magnesium deficiency causes impaired bone growth, osteopenia, skeletal fragility, and osteoporosis, even at low levels (Vormann, 2003; Rude et al., 2005; Rude et al., 2006; Rude et al., 2009). Deregulation of magnesium homeostasis may cause chronic chondrocalcinosis in myositis ossificans and in extra-skeletal ossification and calcification (Sctrick, 1991). Although mineralization defects have been observed after magnesium overload in postmenopausal women and patients with chronic renal failure, magnesium supplementation is thought to be beneficial for bone health (Martin, 2000; Vormann, 2003; Castiglioni et al., 2013).

The effects of magnesium deficiency are hard to define, given the wide diversity of magnesium requiring enzymes (e.g. alkaline phosphatases, ATP-ases, phosphokinases, the oxidative phosphorylation pathway). A comprehensive approach seems the best way to highlight the exact molecular effects of magnesium deficiency/supplementation. Decreased serum parathyroid hormone, calcitriol, and osteoprotegerin that occurs concomitant with increased substance P, TNF-α, IL1-β, IL6, and RANKL may partly account for reduced bone formation (Rude et al., 2009; Castiglioni et al., 2013).

2.17. Manganese

Manganese is a cofactor for numerous enzymes, playing many functional roles in living organisms. Manganese is essential for bone growth, and deficit has been associated with abnormal skeletal development in animals and humans, although deficiencies in humans are rare (Aschner and Aschner, 2005; EFSA Panel on Dietetic Products Nutrition and Allergies (NDA), 2009). Symptoms reported vary from defects in chondrogenesis (e.g. chondrodystrophy) to impaired osteogenesis and bone resorption (stunted bone growth, thickened bones, and epiphyseal dysplasia), and osteoporosis (Beattie and Avenell, 1992). Conversely, manganese overload can cause impaired bone development, in addition to neurotoxicity, its main effect (U.S. EPA Toxicity and Exposure Assessment for Children's Health, 2007).

Manganese superoxide dismutase protects osteoblasts against ROS emitted by osteoclasts (Matsumoto et al., 1991; Aschner and Aschner, 2005). However, it is also an essential cofactor for hydrolases and transferases (glycosyltranferases, xylosyltransferases, phosphohydrolases, and phosphotransferases) involved in the synthesis of cartilage proteoglycans (Matsumoto et al., 1991; Beattie and Avenell, 1992; EFSA Panel on Dietetic Products Nutrition and Allergies (NDA), 2009; Soetan et al., 2010). Clegg *et al*. (Clegg et al., 1998) also hypothesized that manganese deficiency alters IGF metabolism.

Like for magnesium, the implication of manganese deficiency on bone metabolism needs a comprehensive approach considering the high number of actions of this element as cofactor. However, unlike the magnesium, manganese has a relatively narrow window between recommended intake and tolerable upper intake (West Suitor and Meyers, 2006b). The bioavailability of manganese differs greatly depending of the source, and the chemical species. Acute toxicity consecutive to Mn ingestion is rare, but a potential chronic toxicity of Mn resulting from high daily intake should be more investigated, especially considering that no consensus has been reached on a Mn analysis (serum, plasma, etc.) that would be representative of the Mn status (Martin, 2000).

2.18. Mercury

Mercury is infamous for being a toxic heavy metal known to cause neurological symptoms (e.g. Minamata disease). Although no adverse effects of mercury on bone metabolism have been reported, few *in vitro* and *in vivo* studies on methylmercury revealed perturbation in calcium homeostasis, cytotoxicity in bone cells, and possible indirect action through modulation of estrogen secretion (Lundholm, 1995; Suzuki et al., 2004). In the light of these studies, it would certainly be interesting to further investigate the possible effects of mercury on bone metabolism.

2.19. Molybdenum

Molybdenum is an essential trace element, which serves as a co-factor for several redox enzymes. Due to active homeostasis mechanisms, including intestinal absorption that can vary from 25 to 90% and urinary excretion that can vary from 17 to 80%, molybdenum deficiency and overload are uncommon in human populations (Martin, 2000). Animal experiments have revealed that molybdenum deficiency inhibits growth, especially in early stages of development (Hathcock, 2004).

High molybdenum toxicity studies reported similar symptoms, including inhibition of fetal development, growth retardation, and skeletal deformities (Nadeenko et al., 1978; Vyskocil and Viau, 1999; Brem et al., 2009). The main mechanism involved is secondary copper deficiency due to increased urinary excretion. In addition, people with impaired copper metabolism are more susceptible to molybdenum toxicity (Khandare et al., 2013). Other direct actions molybdenum exerts on bone metabolism are presumed, but the mechanisms remain unknown and needs to be explored (Parry et al., 1993). The great advances made in the field of in vitro culture of human cells since the reported studies should allow to easier study the molecular mechanisms molybdenum on bone metabolism.

2.20. Phosphorus

Phosphorus, in the form of phosphate ions of hydroxyapatite, is a structural component of bones. Although human deficiency is rare, isolated cases of insufficient parenteral nutrition have revealed that deficiency can cause bone disorders, rickets, and osteomalacia (Martin, 2000). Several studies have also indicated adverse effects result from high-phosphate diets, including reduced bone formation with increased resorption and deteriorated biomechanical properties. Results suggest the mechanism involves increased parathyroid hormone secretion (Kemi et al., 2006; Huttunen et al., 2007; Kemi et al., 2008). However, adverse effects on bone health are disputed by *in vitro* experiments reporting inhibition of osteoclast differentiation and activity (Yates et al., 1991; Kanatani et al., 2003). Furthermore, a meta-analysis performed by Fenton *et al*. (Fenton et al., 2009) did not correlate high phosphate intake with bone demineralization. On the contrary, the study revealed that low urine calcium levels correlated with higher calcium retention. These results suggest that metabolisms of phosphorus and calcium are intimately linked and should not be considered separately. Moreover, the source of dietary phosphate can influence effects on parathyroid hormone secretion (Huttunen et al., 2006; Karp et al., 2007). Under physiological conditions, serum phosphate levels are regulated, at least in part, by fibroblast growth factor 23 (FGF23), a bone hormone secreted by osteocytes (Shimada et al., 2004; Lanske et al., 2014).

2.21. Platinum

Platinum is a rare element with unique properties that are valued in many applications. A recent study suggests that platinum cations that result from abrasion of catalytic converters may induce skeletal abnormalities (Stahler et al., 2013). However, these effects were not observed with platinum alone, but with a cocktail of platinum group metals including platinum, palladium, and rhodium. Further investigations are needed to confirm the harmful effects of platinum group metals on bone health, and to identify whether these effects may be attributed to one metal or a combination of several.

Platinum nanoparticles have recently been studied for ROS scavenging properties (Kajita et al., 2007; Watanabe et al., 2009). *In vitro* and *in vivo* experiments using platinum nanoparticles reported inhibition of osteoclast proliferation and activity, which are thought to down-regulate the ROS-induced RANKL pathway (Nomura et al., 2011; Kim et al., 2012b). These studies suggest platinum nanoparticles may have future application as new drugs for the treatment of bone diseases but not before exhaustive toxicological studies including platinum cation possibly resulting from the nanoparticles.

2.22. Potassium

Potassium is a ubiquitous, intracellular element essential to more than one metabolic pathway. Deficiency and excess have iatrogenic origins, and do not affect healthy individuals, whereas the elderly can be affected (Martin, 2000). Alteration of palatability and hyperkalemia are the main symptoms of potassium overload, while bone metabolism is relatively insensitive to potassium imbalances. A study performed by Macdonald *et al*. (Macdonald et al., 2008) did not find any beneficial effects of 2-years of potassium citrate supplementation on bone turnover and bone mineral density (BMD) in healthy post-menopausal women. Nonetheless, some human studies have reported enhanced calcium absorption, decreased urinary calcium and bone resorption markers (serum type I collagen C-telopeptide and urinary N-telopeptide), and lower serum parathyroïd hormone (PTH) due to supplementation of both potassium citrate and calcium citrate (Sakhaee et al., 2005; Karp et al., 2009). Potassium citrate supplementation is especially beneficial for postmenopausal women with high dietary sodium chloride intake (Sellmeyer et al., 2002; Harrington and Cashman, 2003).

2.23. Selenium

Selenium is an essential trace element. It is incorporated in selenoproteins in the form of selenocystein, which forms the active site of selenoenzymes. One major class of selenoenzymes is glutathione peroxidases, which are involved in ROS scavenging. Animal experiments showed selenium deficiency caused bone and joint abnormalities, including growth retardation, osteopenia, and chondronecrosis (Moreno-Reyes et al., 2001; Ren et al., 2007; Downey et al., 2009). Human studies have revealed correlations between soil selenium deficiency and the prevalence of Kashin-Beck disease, an endemic osteoarthropathy (Tan et al., 2002; Li et al., 2009). However, epidemiological studies have uncovered the multifactorial and environmental nature of the disease. According to these studies, selenium deficiency alone cannot explain the etiology (Chasseur et al., 1997; Moreno-Reyes et al., 1998; Moreno-Reyes et al., 2003; Chasseur et al., 2008a; Chasseur et al., 2008b). Nevertheless, several *in vitro* and *in vivo* studies confirmed harmful effects of selenium deficiency, including growth retardation, impaired bone and cartilage metabolism, and osteopenia (Yang et al., 1993; Moreno-Reyes et al., 2001; Ren et al., 2007; Downey et al., 2009). Selenium deficiency may also aggravate osteoporosis (Ebert and Jakob, 2007). Excessive selenium consumption also appears to be harmful for bone, inducing decreased bone mineral density and altering bone structure (Turan et al., 2000; Martiniakova et al., 2013).

The metabolism of osteoclasts, osteoblasts, and osteocytes is greatly influenced by ROS production and scavenging (Manolagas, 2010). The main activity of selenium was thought to be neutralization of ROS through glutathione peroxidases, but recent *in vitro* studies suggest selenite may have antioxidant activities that prevent osteoclast differentiation and induce osteoclast apoptosis (Chung et al., 2006; Moon et al., 2012). Recently, Sun *et al*. (Sun et al., 2011) hypothesized that selenoproteins regulate microRNAs that influence bone development, especially during endochondral ossification.

Since the first evidences of selenium essentiality and the highlighting of its anti-oxidant role, incorporated into glutathione peroxidases under the form of selenocystein, dozens of other selenoproteins have been discovered (Brown and Arthur, 2001; Burk et al., 2003). The roles of these proteins are not always exactly known, but it seems most of them have key roles in more than one metabolism pathway. Many researchers are focusing their studies on the possible correlations between selenoproteins levels and different diseases. No doubt the future holds surprises that could possibly lead to therapeutic applications.

2.24. Silicon

Silicon's essential role in bone and joint metabolism was revealed by studying animals on silicon-deficient diets (Schwarz and Milne, 1972; Carlisle, 1980). Although questioned by some researchers (Elliot and Edwards, 1991), the implication of silicon in bone health has been confirmed with additional, more recent studies, which report animals deprived of silicon present with skeletal disorders, reduced collagen synthesis, and reduced osteopontin-related proteins (Seaborn and Nielsen, 2002a, 2002c, 2002b; Nielsen and Poellot, 2004). Conversely, adequate silicon intake or supplementation has been shown to stimulate collagen secretion, enhance bone matrix mineralization and bone mineral density, and decrease bone resorption (Reffitt et al., 2003; Sripanyakorn et al., 2005; Maehira et al., 2008). Silicon is even thought to have beneficial roles in prevention of osteoporosis. Indeed, dietary silicon has been positively correlated with bone mineral density (Bronner, 2002; Jugdaohsingh et al., 2004; Price et al., 2013). Interestingly, silicon-containing implants and ceramics have superior biocompatibility compared to non-silicon-containing counterparts, because they enhance the formation apatite-like surface layers (Jugdaohsingh, 2007). Little doubt remains as to the essentiality of silicon for bone health. Although the biochemistry and mechanisms through which silicon acts are not yet elucidated, different effects of Si deprivation on other elements have been observed. Notably that Ca, Mg, Cu, Mn, and Mo concentrations in bone decrease when Si is deficient (Seaborn and Nielsen, 2002c). Silicon deprivation also appeared to decrease the liver concentrations of magnesium, calcium, phosphorus, zinc, and germanium (Seaborn and Nielsen, 1994). The biochemistry of silicon is probably complex and there is clearly an open research field that could benefit from the modern application of in vitro experimentation.

2.25. Sodium

Sodium is a ubiquitous element in the human body, and it is the extracellular counterpart of potassium. Although bone disease is not associated with sodium deficiency or excess, there is growing concern over the impacts of hyponatremia on osteoporosis in the elderly. Several epidemiologic studies have demonstrated increased risk of osteoporosis and increased rates of fall and fracture related to mild hyponatremia (Kinsella et al., 2010; Verbalis et al., 2010; Hoorn et al., 2011). Two likely mechanisms may be at play. First, unsteady gaits and falls can result from neurological complications of hyponatremia. Second, hyponatremia has a direct effect on bone metabolism (Ayus et al., 2012). Decreased bone mineral density is still controversial, but evidence indicates that hyponatremia may stimulate osteoclast proliferation and activity, possibly to mobilize sodium stored in bone (Teucher et al., 2008; Barsony et al., 2010). Although the exact mechanisms of hyponatremia are not yet elucidated, recent findings suggest that individuals prone to hyponatremia should be monitored for osteoporosis.

Since hyponatremia exacerbates osteoporosis, high salt diets are recommended. Indeed, it has been shown to increase bone resorption and cause calciuria, especially in people susceptible to osteoporosis, such as postmenopausal women (Teucher et al., 2008). Nevertheless, high sodium diet is also known to be deleterious, and since the window of optimal intake is quite narrow (West Suitor and Meyers, 2006b), people subject or at risk for osteoporosis should be carefully monitored for sodium intakes.

2.26. Strontium

It is not definite whether strontium is an essential element or whether overload of inorganic salts of strontium is harmful for bone health (Cabrera et al., 1999; Cohen-Solal, 2002). Several in vivo studies highlighted mineralization defects when rats are received high strontium intakes (Cohen-

Solal, 2002). There is also possible synergistic deleterious accumulation in bone with aluminium (Cohen-Solal, 2002). Nonetheless, organic salts of strontium, such as strontium ranelate (S12911-2) or distrontium (5-[bis(2-oxido-2-oxoethyl)amino]-4-cyano-3-(2-oxido-2-oxoethyl)thiophene-2-carboxylate)), are supposed good candidates for the treatment of osteoporosis. They have targeted anabolic effects on bone and prevent fracture. *In vitro* and *in vivo* studies have shown that strontium increases cartilage matrix secretion, stimulates osteoblast proliferation, enhances bone mineralization, and inhibits osteoclast differentiation and resorption activity (Buehler et al., 2001; Henrotin et al., 2001; Baron and Tsouderos, 2002; Takahashi et al., 2003; Marie, 2006; Reginster et al., 2009; Reginster et al., 2012; Cianferotti et al., 2013; Reginster et al., 2013). Several human studies on the use of strontium ranelate for preventing and treating postmenopausal osteoporosis demonstrated a reduced risk of fracture (Reginster et al., 2005; Meunier et al., 2009). These results indicated strontium was an ideal drug. However, the use of strontium ranelate induces severe risk of venous thromboembolic events, and DRESS syndromes (Drug Reaction with Eosinophilia and Systemic Symptoms) (Jonville-Bera and Autret-Leca, 2011). Furthermore, special attention should be paid to people with chronic renal failure. Indeed, chronic renal failure rats exhibited osteomalacic lesions when supplemented with Sr (Schrooten et al., 1998; Schrooten et al., 2003), although the effects seem reversible when stopping the supplementation (Oste et al., 2005). A recent *in vitro* study of Wornham *et al.*suggests that strontium may have inhibiting actions on osteoclasts and osteoblasts function with consequences on bone mineralization (Wornham et al., 2014).

The main mechanism underlying the action of strontium relies on chemical similarity to calcium, which is emphasized by preferential distribution of strontium in bone. The calcium-sensing receptor, which allows bone cells to react to small extracellular variations of Ca^{2+}, appears to be sensitive to Sr^{2+}. This receptor can induce cascade reactions (e.g. upregulation of OPG mRNA and downregulation RANKL mRNA) to normalize calcium homeostasis (Brown, 2003; Brennan et al., 2009). Strontium can also activate another cation-sensing receptor, which remains to be identified (Pi and Quarles, 2004; Marie, 2007). Furthermore, *in vitro* studies suggest the beneficial action of strontium is linked to PTH signaling and parathyroid hormone-related protein (PTHrP) production in osteoblasts (De Wolf et al., 2008; Bergmann et al., 2010).

2.27. Titanium

Metal alloy prostheses give rise to wear particles and ions that infiltrate the human body. These particles and ions may be responsible for periprosthetic osteolysis, which can induce loosening in cases of total joint arthroplasty. Titanium metal particles and ions were widely studied in the early

2000s, revealing that each species modulates bone cells through a different pathway. Indeed, wear particles are phagocytosed by osteoclasts, which can inhibit type 1 collagen synthesis through NF-κB signaling and down-regulation of procollagen α1[I] gene transcription (Yao et al., 1995; Kwon et al., 2000; Roebuck et al., 2001; Vermes et al., 2001; Kaabar et al., 2009). Secretion and release of chemokines (IL-6, IL-8, and MCP-1) has also been reported as a consequence of exposure to titanium wear particles in osteoblasts (Vermes et al., 2001; Fritz et al., 2002). However, there are conflicting results regarding the impact of titanium particles on osteoclast metabolism, some indicate they cause inhibition of osteoclast differentiation, and others indicate they enhance differentiation (Bi et al., 2001; Nakano et al., 2003). Titanium ions act differently since they are not phagocytosed, but their effect are also subject to controversy. Some data indicates they promote osteoclast-induced bone resorption, possibly through cytokine modulation. Other data indicates titanium ions induce osteoclast apoptosis (Wang et al., 1996; Matsunaga et al., 2001; Rousselle et al., 2002).

There is clearly no general consensus on the effects of Titanium on the bone metabolism, whether under the form of wear particles or under the form of ions. This lack of convergent results may find its roots in the heterogeneity between the methodologies implemented in the in vitro studies cited above. There is also heterogeneity between the markers targeted by the researchers. Furthermore, it is difficult to conclude on a stimulatory or inhibitory effect on bone due to the plethora of factors influenced in response to wear particles. Whereas in vitro studies could help to highlight the still unknown molecular mechanisms of some elements, animal studies seem mandatory to enable a comprehensive approach on titanium effect on bone health.

2.28. Uranium

Uranium is a ubiquitous trace element found on the surface of the earth. Typically underestimated, its abundance is 40 times higher than silver (Argonne National Laboratory), and workers in the uranium industry can experience toxic effects. All *in vivo* and human studies agree that non-lethal doses of uranium disturb bone growth (Ubios et al., 1991; Díaz Sylvester et al., 2002; Kurttio et al., 2005; Tasat et al., 2012). Impaired endochondral ossification, decreased bone formation, and inhibition of alveolar bone healing have been observed. The exact mechanisms of uranium toxicity are not known, but exposure may disrupt osteoblast differentiation, intensification of ROS production, and reduction of alkaline phosphatase activity (Tasat et al., 2007; Tasat et al., 2012).

2.29. Vanadium

Although no human vanadium deficiency has been reported, *in vivo* experiments revealed evidence of its essentiality. Indeed, animals deprived of vanadium exhibit bone deformities, disordered skeletal development, and retarded bone growth (Laizé et al., 2009). Furthermore, vanadium supplementation stimulates osteogenic cell proliferation and differentiation and type-I collagen production; increases bone mineral density, mineralization, and formation; stimulates alkaline phosphatase activity; and improves biomechanical properties and bone formation rates (Johnson and Henderson, 1997; Cortizo et al., 2006; Facchini et al., 2006; Laizé et al., 2009). Due to these properties, vanadium is being investigated as a drug for the treatment of osteoporosis, but the concentration range for beneficial effects is narrow and adverse effects are associated with relatively low levels (Beattie and Avenell, 1992; Laizé et al., 2009).

Vanadium may act through two mechanisms, one structural and one physiological. Structurally, vanadate can substitute for phosphate in hydroxyapatite crystals (Laizé et al., 2009). Physiologically, the role of vanadium is not completely understood, but it is closely related to its ability to inhibit phosphotyrosyl protein phosphatase and the sodium-potassium-ATPase and its ability to produce insulin-like activities (Krieger and Tashjian, 1983; Johnson and Henderson, 1997; Barrio and Etcheverry, 2006; Laizé et al., 2009). In contrast, adverse effects of vanadium are mainly associated with the ability to induce apoptosis (Cortizo et al., 2006). Toxicity differs according to species; vanadium (V) is more toxic than vanadium (IV) compounds (Barrio and Etcheverry, 2006).

Vanadium has long been studied for its toxicity, and more recently for its essentiality. The numerous possible oxidation states (-1, 0, +2, +3, +4, and +5) result in a very complex chemistry. Furthermore, vanadium is categorized as an ultra-trace element, with very low concentrations in foods and biological tissues, making its determination difficult and sensible. If vanadium may have future therapeutic applications, a complete characterization of its biochemistry will be mandatory considering the low-level toxicity of this element. This should be facilitated by the increasingly efficient elemental analysis.

2.30. Zinc

Similar to other transition metals, Zinc is always present in the human body. Involved in more than 200 enzyme activities, it is mainly active in synthesis of protein and activation of DNA and RNA through zinc finger proteins. It is also needed in prostaglandin synthesis and for antioxidant defenses (Martin, 2000; Ma and Yamaguchi, 2001). Numerous *in vivo* studies and human observations have confirmed that zinc deficiency leads to bone growth retardation, various skeletal abnormalities, and osteopenia (Ma and Yamaguchi, 2000; Cho et al., 2007; Kim et al., 2009; Seo et al., 2010). A variety of additional symptoms have also been described, such as abnormal development of ribs and vertebrae, agenesis of long bones, club foot, cleft palate, micrognathia, impaired ossification, defective mineralization, and bowing of the long bones (Beattie and Avenell, 1992). Acrodermatitis enteropathica, a rare disease characterized by impaired absorption of zinc, reveals the essential role of zinc in bone metabolism. Zinc deficiency, induced by the disease, gives rise to retarded and impaired bone growth (Gupta et al., 2014).

Zinc supplementation has also been shown to favorably modulate bone turnover by stimulating osteoblast bone formation while inhibiting osteoclast differentiation. These effects combine to increase bone strength (Yamaguchi and Kishi, 1996; Ovesen et al., 2001; Peretz et al., 2001; Hadley et al., 2010; Nagata and Lönnerdal, 2011). Zinc supplementation also alleviates toxic effects of other metals, particularly cadmium (Beattie and Avenell, 1992; Brzóska et al., 2008), and it may prevent or treat several types of pathological bone loss (Yamaguchi, 2010).

As mentioned previously, experimental evidence suggests that zinc may promote osteoblast and chondrocyte differentiation and activity while, at the same time, inhibiting osteoclast metabolism (Moonga and Dempster, 1995; Kim et al., 2009; Seo et al., 2010). Many proteins, enzymes, zinc finger proteins, and bone markers are closely related and sensitive to zinc homeostasis, but distinguishing what is a cause and what is a consequence is difficult. A study by Ovesen *et al*. (Ovesen et al., 2001) supports the idea that the beneficial effects of zinc on bone metabolism are similar to the effects of growth hormone and insulin-like growth factor 1. Zinc storage proteins, like metallothionein, and zinc transporters are particularly important in zinc homeostasis and are implicated in osteoblast-induced mineralization (Fong et al., 2009; Nagata and Lönnerdal, 2011). Alkaline phosphatase, collagenase, and aminoacyl-tRNA synthetase are some of the zinc-dependent enzymes, which can impact bone metabolism and are likely to be affected by imbalanced zinc levels (Beattie and Avenell, 1992; Yousef et al., 2002; Yamaguchi, 2010). Gli-similar 3 (Glis3) and Odd-skipped related 2 (Osr2) are zinc finger transcription factors that promote osteoblast differentiation (Beak et al., 2007; Kawai et al., 2007). Zinc may also affect several aspects of osteoblast differentiation, presumably by modulating gene expression of runt-related transcription factor 2 (Runx2) (Yamaguchi et al., 2008; Kwun et al., 2010). In osteoclasts, zinc inhibits the RANKL and TNFα differentiation pathways (Fong et al., 2009; Yamaguchi, 2010). The consequences of zinc accretion in the skeleton are also notable (Beattie and Avenell, 1992).

Although extensively studied, zinc is a transition metal that plays so many roles in the organism that it is extremely difficult to have a global overview of its biochemistry. Mapping its numerous chemical species, including its protein forms, and their flows in the organism would certainly be of great help in the progression of the knowledge of exact biological roles of this multipotent element.

3. Concluding remarks

The skeleton is more than just a frame designed to enable movement and provide protection. The biology and biochemistry of the skeleton are very complex and rely on subtle equilibria between cells, organic molecules, and inorganic constituents. As reviewed in this paper, bones are often affected by minerals found in the environment, and they can be essential, deleterious, or both, depending on intake levels. In many cases deficiency and excess affect bone metabolism. Despite recent advances in knowledge in this area, much remains to be discovered to fully elucidate the impact of minerals on bone metabolism. Osteoporosis, for example, is a multi-factorial bone disease that affects more than 200 million people worldwide. To date, no drugs treat osteoporosis satisfactorily, indicating non-pharmacological prevention may be attained through nutrition and lifestyle (Body et al., 2011). The modern tools of science and ever growing technology should help advance knowledge of the effects of minerals on bones to benefit medicine, nutrition science, and toxicology. The ultimate objective is to precisely understand and characterize all the molecular mechanisms of all the elements – essential or not – susceptible to enter our body. Then, an integrative view could be achieved by mapping all the chemical species, including organic forms, and their flows in the organism, just like a proteome for elements.

Table 1. Summary table of the effects thirty elements on bone metabolism, including the mechanisms and the interactions between elements

Element	**Essential / Toxic**	**Beneficial / Deleterious effects**	**Main mechanisms**	**Particular interaction(s) with other elements**
Aluminium	Toxic	Disrupted collagen synthesis[a]; Adynamic bone disease[a,b]; Renal osteodystrophy[a,b]; Osteomalacia[a,b]	Bone accretion[a]; Disruption of gene expression[a]; Inhibition of bone cells proliferation and activities[a,b,c]	Exacerbation of deleterious effects when Ca and Mg are deficient; Decrease absorption of inorganic phosphorus; Alteration of iron metabolism; Possible synergistic deleterious accumulation in bone with strontium
Arsenic	Essential; Toxic	<u>Beneficial</u> Methionine metabolism[a,b]; Phosphorylation[a,b] <u>Deleterious</u> Deficiency: Depressed growth[a]; Excess: Inhibition of endochondral ossification[a]; Reduced bone mineral density and trabecular bone volume[a]; Osteomalacia[a]	Disruption of osteoblast differentiation[a]; Decreased capacity of osteoclasts to adhere to the bone matrix[a]; Reduced expression of the vascular cell adhesion molecule-1 (VCAM-1)[a]; Depressed alkaline phosphatase and osteocalcin expression[a]	Arsenate substitutes for phosphate and decrease its transport

Table 1. (Continued)

Element	**Essential / Toxic**	**Beneficial / Deleterious effects**	**Main mechanisms**	**Particular interaction(s) with other elements**
Boron	Not essential but beneficial; Not toxic	Supplementation: Improved bone growth, strength, stiffness, hardness, mechanical properties, and trabecular bone microarchitecture[a,b]; Lower arthritis prevalence[b] Deprivation: Impaired bone healing, decreased chondrocyte density in proliferation[a] zones	Possible beneficial interaction with steroid hormones[a,b]	Boron deficiency exacerbates the symptoms of vitamin D and magnesium deficiency, increase urinary calcium, and decrease copper and zinc concentration in bones
Cadmium	Toxic	Decrease bone mineral density[a,b]; Hypercalciuria[a,b]; Increase risk of fracture, osteomalacia, and osteoporosis[a,b]; Itai-itai disease[b]	Cadmium disturb calcium metabolism and calciotropic hormones[a,b]; Alteration of collagen matrix[a] Stimulation of osteoclasts proliferation and activity[a,c]; Decrease osteoblast viability, mineralization capacity, and alkaline phosphatase activity[a,c]; Increased serum levels of parathyroid hormone and up-regulation of RANKL[a,c]	Cadmium interact with calcium metabolism and causes hypercalciuria; Cadmium decrease liver concentration of iron, magnesium, and selenium but increase levels of copper, zinc, and manganese

Table 1. (Continued)

Element	**Essential / Toxic**	**Beneficial / Deleterious effects**	**Main mechanisms**	**Particular interaction(s) with other elements**
Calcium	Essential	Component of hydroxyapatite[a,b]; Deficiency: Lower bone mineral content and bone mineral density[a,b]; Rickets[b]; Osteomalacia[b]; Osteoporosis[b]	Stimulation of osteoclast-like cell formation and bone resorption by mature osteoclasts[c]; Increased osteoblasts proliferation and activity[c]	Calcium homeostasis is complex and influenced by numerous other elements (boron, cadmium, cobalt, germanium, lead, lithium, mercury, silicon, strontium); Intimately linked with phosphorus, sodium, and potassium metabolism
Chromium	Essential (Cr^{3+}); Toxic (Cr^{6+})	Beneficial Cr^{3+}: Carbohydrate and lipid metabolism Deleterious Cr^{6+}: Reduced ossification[a,c]; Accelerated bone resorption and reduced bone formation[a,c]	Cr^{6+}: Oxidative stress and cytotoxic effects on bone cells which can be caused by imbalanced OPG/RANKL ratios, oxidation and nitration of proteins, and misregulation of antioxidant enzyme expression [a,c]; Disturb the release of cytokines (TGF-β1, TNF-α, IL-β1, TNF-G) from osteoblasts[c]	Cobalt-chromium wear particles have possible synergistic deleterious actions, such as induction of apoptosis in osteoclasts and decreased secretion of some inflammatory factors such as prostaglandin E2 and interleukin-6

Table 1. (Continued)

Element	**Essential / Toxic**	**Beneficial / Deleterious effects**	**Main mechanisms**	**Particular interaction(s) with other elements**
Cobalt	Essential (under the form of cobalamin); Toxic (corrosion/wear of prosthetic implants)	Deleterious: Loss of peri-implant bones[b,c]; Influence bone resorption and formation[a,c]	Affect osteoblast proliferation, size, and shape[c]; Decreases alkaline phosphatase levels and calcium accretion, which inhibits release of osteocalcin and collagen type 1 proteins[c]; Induce oxidative stress[c]; Promote secretion of cytokines (TGF-β1, TNF-α, IL- β 1, IL-6, IL-8, and MCP-1)[C]	See Chromium
Copper	Essential; Toxic	Deficiency: Decreased bone strength[a,b], impairment of bone formation and growth[a,b]; Reduced bone mineralization[a,b]; Reduced ossification of growth centers[a]; Compromised cartilage integrity[a,b]; Deformed bones[a,b]; Hypoplasia, brittle bones, and frequent fractures[a,b]; Osteoporosis[b]	Lysyl oxidase disorder preventing crosslinking between collagen and elastin, reducing strength of the bone matrix[a]; Reduction of superoxide dismutase[c]; Excess copper can generate free radicals, which induce lipid peroxidation and interfere with bone metabolism	See boron, cadmium, molybdenum, and silicon

Table 1. (Continued)

Element	**Essential / Toxic**	**Beneficial / Deleterious effects**	**Main mechanisms**	**Particular interaction(s) with other elements**
Fluorine	Not essential but possibly beneficial; Toxic	Beneficial Controverted anti-osteoporotic drug[b,c] Deleterious Excess: Skeletal fluorosis[b]; Decreased bone mass with genu valgum and varum, sabre tibia, and secondary hyperparathyroidism[b] Impaired and delayed mineralization[a,b]	Possible accretion to bone matrix under the form of fluorapatite; Mitogenic activity through the mitogen-activated protein kinase (MAPK) pathway[a,c]; Upregulation of IGF-1[a,c]	
Gallium	Not essential but beneficial; Not proven to be toxic at pharmacological doses	Supplementation: Inhibit bone resorption[a,b]; Improve bone mineralization[a,b]	Incorporation into bone matrix[a,c]; Modulation of osteoclast activity: by downregulating NFATc1 gene expression[c]; by playing a key role in RANKL-induced osteoclast differentiation[c]; by blocking the TRPV5 Ca^{2+} channel[c]	
Germanium	Not essential but possibly beneficial; Toxic	Organic form (Ge-132): Anti-osteoporosis effect: Improvement of bone mineral density and bone mineral content[a]; Maintenance or enhancement of bone strength[a]	Possibly same role than Si when substituted[a]; Undetermined other possible Si-independent effects[a]	Substitute to silicon; Ge supplementation decrease Si and Mo bone concentrations but overcome calcium, zinc, sodium, iron, manganese, and potassium low concentrations in bones due to Si deprivation

Table 1. (Continued)

Element	Essential / Toxic	Beneficial / Deleterious effects	Main mechanisms	Particular interaction(s) with other elements
Gold	Not essential but beneficial; Not toxic	Inhibitory effect on bone resorption[c]	Indirect mechanism affecting the differentiation pathway of pre-osteoclastic cells and resulting in decreased proliferation of osteoclasts[a]; Inhibition of prostaglandin E2 and interleukin-1[a]; Inhibition by gold nanoparticles of osteoclast formation induced by RANKL[c]	
Iron	Essential; Toxic	Deficiency: Decreased bone mineral density and mass[a,b,c]; Altered microarchitecture[a]; Reduced bone strength[a,b] Excess: Osteoporisis[b]; Oxidative stress[a]; Dwindling bone biomechanical properties[a]; Increasing risk of fracture[b]	Deficiency: Decreased serum 1α,25-dihydroxycholecalciferol, insulin-like growth factor-I, and osteocalcin concentrations[a] Excess: Disruption of pre-osteoblast cells differentiation through downregulation of ALP, HHIPL-2, osteocalcin, and CBF-α1 expression[a,c]; Production of reactive oxygen species (ROS) which induces oxidative stress in osteoblasts[a]; Increase of serum TNF-α, IL-6, TGF-1, and osteopontin which stimulate osteoclasts and bone resorption through RANKL[a,c]	See aluminium, cadmium and germanium

Table 1. (Continued)

Element	**Essential / Toxic**	**Beneficial / Deleterious effects**	**Main mechanisms**	**Particular interaction(s) with other elements**
Lead	Toxic	Growth retardation by inhibiting endochondral ossification[a]; Increased bone turnover and reduced mineralization[a,b]; Decrease bone mineral density and mass[a,b]; Cause osteoporosis in the most severe cases[a,b]	Highly cytotoxic; Affects hormonal secretion and hormonal-induced cell responses (1α,25-dihydroxycholecalciferol and IGF-1)[a]; Affects bone-related protein synthesis (osteocalcin, collagen, osteopontin, and sclerostin)[a]; Affects cAMP and the Wnt/β-catenin pathway[a]; Negatively stimulates chondrogenesis[a]	Pb(2+) can substitute to Ca(2+) in hydroxyapatite crystal; Higher affinity of lead compared to calcium for osteocalcin
Lithium	Not essential; Possibly beneficial; Possibly deleterious	Lithium causes bone loss that can predispose or aggravate osteoporosis (controverted)[b]; Inhibition of bone resorption and osteoclast formation[a,c]	Possible modulation of the canonical Wnt/β-catenin pathway[a,c]	Complex effect on calcium homeostasis

Table 1. (Continued)

Element	**Essential / Toxic**	**Beneficial / Deleterious effects**	**Main mechanisms**	**Particular interaction(s) with other elements**
Magnesium	Essential	Deficiency: Impaired bone growth[a,b]; Skeletal fragility[a,b]; Osteopenia[a,b]; Osteoporosis[a,b]; Chronic chondrocalcinosis[a,b]; Myositis ossificans[a,b] Excess: None, except occasional mineralization defects after magnesium overload in postmenopausal women and patients with chronic renal failure	Hard to define, given the wide diversity of magnesium requiring enzymes; Decreased serum parathyroid hormone, calcitriol, and osteoprotegerin[a,b]; Increased substance P, TNF-α, IL1-β, IL6, and RANKL[a,b]	See boron, cadmium, and silicon; Mn can substitute to Mg in different enzymes; Mg concentration in bone decrease when Si is deficient
Manganese	Essential; Toxic	Deficiency: Abnormal skeletal development; Defects in chondrogenesis (e.g. chondrodystrophy); Impaired osteogenesis and bone resorption; Excess: Impaired bone development[a] (and neurotoxicity)	Cofactor for numerous enzymes (glycosyltranferases, xylosyltransferases, phosphohydrolases, and phosphotransferases)[a,b]; Manganese superoxide dismutase protects osteoblasts against reactive oxygen species (ROS) emitted by osteoclasts [a,b]; Deficiency alters IGF metabolism[a]	See cadmium, germanium, magnesium, and silicon

Table 1. (Continued)

Element	**Essential / Toxic**	**Beneficial / Deleterious effects**	**Main mechanisms**	**Particular interaction(s) with other elements**
Mercury	Toxic	Unknown effect on bone to date	Cytotoxicity in bone cells and possible indirect action through modulation of estrogen secretion[a,c]	Perturbation in calcium homeostasis
Molybdenum	Essential; Toxic	Deficiency: Inhibits growth, especially in early stages of development[a] Excess: Inhibition of fetal development[a]; Growth retardation[a]; Skeletal deformities[a]	Co-factor for several redox enzymes[a,b]; Excess: Induces secondary copper deficiency due to increased urinary excretion[a]	Interaction with copper metabolism; See silicon
Phosphorus	Essential; Potentially deleterious at high levels (controverted)	Component of hydroxyapatite[a,b]; Deficiency (rare): Bone disorders; Rickets; Osteomalacia Excess: Reduced bone formation with increased resorption and deteriorated biomechanical properties[a,b], Controversy: Inhibition of osteoclast differentiation and activity[c]; Low urine calcium levels correlated with higher calcium retention[b]	Excess: Increased parathyroid hormone secretion[a,b]	See aluminium, arsenic, and vanadium; Intimately linked with calcium metabolism; Influenced by Si deficiency

Table 1. (Continued)

Element	**Essential / Toxic**	**Beneficial / Deleterious effects**	**Main mechanisms**	**Particular interaction(s) with other elements**
Platinum	Not essential; Possibly beneficial (Pt nanoparticles); Possibly deleterious (wear particles)	Beneficial: Inhibition of osteoclast proliferation and activity[c] Deleterious: Skeletal abnormalities (in combination with palladium and rhodium)[a]	Beneficial: Down-regulation of the ROS-induced RANKL pathway[c]	Synergistic toxic effect with palladium and rhodium
Potassium	Essential	Enhanced calcium absorption and decreased urinary calcium due to supplementation of both potassium citrate and calcium citrate[b]	Decrease in bone resorption markers (serum type I collagen C-telopeptide and urinary N-telopeptide), and lower serum parathyroïd hormone (PTH) [b]	Intimately linked to sodium and calcium metabolism
Selenium	Essential; Toxic	Deficiency: Bone and joint abnormalities, including growth retardation, impaired bone and cartilage metabolism, osteopenia, and chondronecrosis[a,c]; Aggravation of osteoporosis Possible implication in the etiology of Kashin-Beck disease (endemic osteoarthropathy) Excess: Decreased bone mineral density and altered bone structure[a]	Neutralization of ROS through glutathione peroxidases (selenoproteins)[a,b]; Selenite may also have antioxidant activities[c]; Selenoproteins regulate microRNAs that influence bone development (especially during endochondral ossification)	

Table 1. (Continued)

Element	**Essential / Toxic**	**Beneficial / Deleterious effects**	**Main mechanisms**	**Particular interaction(s) with other elements**
Silicon	Essential	Deficiency: Skeletal disorders[a]; Reduced collagen synthesis[a]; Reduced osteopontin-related proteins[a];	Not yet elucidated	Ca, Mg, Cu, Mn, and Mo concentrations in bone decrease when Si is deficient Silicon deprivation decreased the liver concentrations of magnesium, calcium, phosphorus, zinc, and germanium.
Sodium	Essential; Deleterious at high levels	Increased risk of osteoporosis[b]; Increased rates of fall and fracture related to mild hyponatremia[b]	Hyponatremia may stimulate osteoclast proliferation and activity, possibly to mobilize sodium stored in bone[b]	Intimately linked to potassium and calcium metabolism
Strontium	Not proven to be essential but potentially beneficial; Toxic	Beneficial: Organic salts of strontium increase cartilage matrix secretion, stimulates osteoblast proliferation, enhances bone mineralization, and inhibits osteoclast differentiation and resorption activity[a,b,c]; Deleterious: Severe risk of venous thromboembolic events, and DRESS syndromes (Drug Reaction with Eosinophilia and Systemic Symptoms)[b]; Chronic renal failure rats exhibited osteomalacic lesions	Inhibiting actions on osteoclasts and osteoblasts function[c]; Influence on calcium-sensing receptor which induces cascade reactions (e.g. upregulation of OPG mRNA and downregulation RANKL mRNA)[c];	Chemical similarity to calcium

Table 1. (Continued)

Element	**Essential / Toxic**	**Beneficial / Deleterious effects**	**Main mechanisms**	**Particular interaction(s) with other elements**
Titanium	Not essential; Possibly toxic (wear particles and ions)	Wear particles: Conflicting results, some indicate they cause inhibition of osteoclast differentiation, and others indicate they enhance differentiation[c]; Titanium ions: Conflicting results, some say they promote osteoclast-induced bone resorption, other say they induce osteoclast apoptosis[c]	Wear particles: Phagocytosed by osteoclasts, which can inhibit type 1 collagen synthesis through NF-κB signaling and down-regulation of procollagen α1[I] gene transcription[c]; Induces secretion and release of chemokines (IL-6, IL-8, and MCP-1) from osteoblasts[c];	
Uranium	Toxic	Impaired endochondral ossification[a]; Decreased bone formation[a]; Inhibition of alveolar bone healing[a]	Disruption of osteoblast differentiation[a,b,c]; Intensification of ROS production[a,b,c]; Reduction of alkaline phosphatase activity[a,b,c]	

Table 1. (Continued)

Element	Essential / Toxic	Beneficial / Deleterious effects	Main mechanisms	Particular interaction(s) with other elements
Vanadium	Essential; Toxic	Deficiency: Bone deformities, disordered skeletal development, and retarded bone growth[a]; Supplementation: Stimulates osteogenic cell proliferation and differentiation and type-I collagen production[a,c]; Increases bone mineral density, mineralization, and formation; Stimulates alkaline phosphatase activity[a,c]; Improves biomechanical properties and bone formation rates[a,c]	Ability to inhibit phosphotyrosyl protein phosphatase and the sodium-potassium-ATPase and to produce insulin-like activities	Vanadate can substitute for phosphate in hydroxyapatite crystals
Zinc	Essential	Deficiency: Retarded and impaired bone growth[a,b]; Osteopenia[a,b]; Various skeletal abnormalities: abnormal development of ribs and vertebrae, agenesis of long bones, club foot, cleft palate, micrognathia, impaired ossification, defective mineralization, and bowing of the long bones[a,b]; Supplementation: Modulate bone turnover by stimulating osteoblast bone formation while inhibiting osteoclast differentiation[a,c]; Increased bone strength	Promotion of osteoblast and chondrocyte differentiation and activity[a,c]; Inhibition of osteoclast metabolism[a,c]; Many proteins, enzymes, zinc finger proteins, and bone markers are closely related and sensitive to zinc homeostasis (metallothionein, zinc transporters, alkaline phosphatase, collagenase, and aminoacyl-tRNA synthetase, Gli-similar 3 (Glis3) and Odd-skipped related 2 (Osr2), etc.) Modulation of gene expression of runt-related transcription factor 2 (Runx2);	Zinc supplementation alleviates toxic effects of other metals, particularly cadmium

[a] Effect or mechanisms highlighted in vivo on laboratory animals; [b] Effect or mechanisms highlighted in vivo on human; [c] Effect or mechanisms highlighted in vitro

Acknowledgments

We gratefully acknowledge Professor Maurice Hinsenkamp (M.D. PhD) and Professor Pierre Bergmann (M.D. PhD) for providing proofreading and corrections.

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Chapter IV.

"A knowledge of the chemical composition of foods is the first essential in dietary treatment of disease or in any quantitative study of human nutrition"

McCance and Widdowson

Introduction

In chapter three we concluded that at least thirty chemical elements were known to impact bone metabolism. Investigating all of these elements in the context of the Kashin-Beck disease would be of course not relevant. We can discard *prima facie* all elements which are not essential, as well as those exclusively experimented in the treatment of bone disorders: Ga, Ge, Li, Au, Pt, Sr, and Ti. Thus, we start with a basis of twenty –three elements to investigate.

Back to our main objective, nutritional assessment intends to describe the nutritional status, but also to identify both the risks of deficiency or excess in individuals or group of individuals (Gibson, 1990). Regardless of the design of the study, which will be discussed in the introduction of the next chapter, the basic concept is to collect information on food consumption, and to link this information with food composition database, usually called food composition tables (FCTs). The outcome is an estimated value of the intake, most of the time the daily intake, in one or several nutrients for individuals, group of individuals, or an entire population. There are several methods to collect food consumption data. As it is quantitative, not so expensive, and implementation is achievable considering field constraints, the 24-hour food recalls interview method has been selected for this study. It is not the simplest and the fastest method, but semi-quantitative questionnaire, such as food frequency questionnaire, are not suitable for quantifying minerals because they occur in a wide range of food items in variable concentrations (Gibson and Ferguson, 2008).

It is the same for food composition tables, which can be considered as a tool. When the objectives of the study are clearly defined, the FCTs that will be used must be carefully chosen, as it is critical to achieve a good quality of results, especially when dealing with mineral and trace elements (Gibson and Ferguson, 2008). Ideally, the values listed in the FCTs should be as close as possible to the true composition of the food consumed by the individuals. In practice, many potential issues must be considered. The FCT could be of questionable quality. It may not be appropriated to the study for geographical reasons. More simply, food items could be missing, which is probable to occur when conducting a survey in a population with specific traditional foods, such as Tibetans. When dealing with minerals composition, one might also remember that multiple factors influence the mineral composition of foods. Agricultural practices, climate, altitude, geochemistry, the variety of cultivars, physiological state and maturity, and post-harvest treatments are all factors known to affect the mineral composition of cereals and vegetables (Greenfield and Southgate, 2003; Rodríguez et al., 2011; Yada et al., 2013).

In rural T.A.R. where foods originate mainly from local agriculture and artisanal production, one must further consider the processing methods, handling, and food preparation, which are also susceptible to impact the final mineral concentrations (Greenfield and Southgate, 2003; Lisiewska et al., 2009; Campo et al., 2013). Many food composition tables or database are indexed by the FAO (FAO, 2014). The China Food Composition Table (China CDC, 2009) logically prevailed to be the most appropriate for this study as it is the national FCTs. Furthermore, the quality of this table appears to be honorable according to the criteria of Gibson & Ferguson (2008). However, several food items consumed in T.A.R. are missing, and the USDA National Nutrient Database (US Departement of Agriculture, 2010), was selected in addition, as it is the most comprehensive FCTs.

In the first part of this chapter we looked to evaluate the relevance of the two tables for a nutrition survey in T.A.R. In this purpose, we analyzed the mineral composition of various foodstuff originated from T.A.R., and we performed a statistical comparison with the two FCTs. Without surprise, significant discrepancies were highlighted, preventing their use in a nutritional survey in rural Tibet without introducing considerable bias. It led us to establish a FCT specific to the area of investigation. To generate this FCT, not less than 1119 samples of various foodstuffs, all locally grown in T.A.R., were analyzed by ICP-MS for 19 minerals at the China National Center for Food Safety Risk Assessment (CFSA). The results are presented in the second part of this chapter.

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The relevance of food composition data for nutrition surveys in rural Tibet: pilot study in the context of Kashin-Beck Disease

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Adapted from the reference: Dermience, M., Mathieu, F., Barthelemy, J.-P., Maesen, P., Romnee, J.-M., De Maertelaer, V., Denchen, Y., Tsewang, P., & Lognay, G. 2013. The relevance of food composition data for nutrition surveys in rural Tibet: pilot study in the context of Kashin-Beck Disease. *Biotechnology, Agronomy, Society and Environment [=BASE]*, **17**(1), 32-42.

Abstract

Kashin-Beck disease (KBD) is an endemic and chronic osteo-chondropathy. This disease principally occurs in the Tibet Autonomous Region and in several provinces of the People's Republic of China. The etiology of the disease remains obscure although environmental factors are assumed to be involved. Diet, in particular, differentiates the rural community, affected by KBD, from the other communities (nomads and city-dwellers), who remain unaffected. In anticipation of a nutrition survey, this study aimed to measure the mineral content (Ca, P, Mg, Fe, Zn, Mn, Cu, Ni, Se, Al, Sr, Mo, Cd, As, Pb, Hg, Cr, and Co) of eight Tibetan staple foods and to compare the results against two food composition tables (FCTs). Foods were sampled in twenty households selected from both an endemic and a non-endemic area of rural Tibet. Ten minerals involved in bone metabolism were measured using atomic and molecular spectrometric methods. Results revealed that a very limited number of food /constituent pairs showed a variation in mineral composition during a single year of testing for a given region. In addition, results showed significant differences in mineral content between the endemic and the non-endemic area, especially for wheat flour. Following our analysis of the mineral content of the Tibetan food samples, results were statistically compared with similar foods listed in two food composition tables: the USDA National Nutrient Database (USDA Food Search for Windows, Version 1.0, database version SR23), and the China Food Composition Table (book 1, 2nd edition). More than 50 to 60% of p-values < 0.05 were highlighted, suggesting the inappropriateness of using FCTs as a reference for nutrition surveys in rural Tibet, and emphasizing the need for analysis of traditional foods. Differences were found to be more or less marked depending on the element considered, and calcium content seemed to show the greatest difference. Although it is obviously too early for definite conclusions to be reached (insufficient number of samples by food and insufficient number of foods analyzed), it seems that the present pilot-study indicates significant discrepancies between measured and tabulated values of the mineral content of certain foods. A more complete survey would therefore seem mandatory.

Résumé

La maladie de Kashin-Beck se traduit par une ostéochondropathie endémique et chronique. Cette maladie se rencontre principalement dans la Région Autonome du Tibet et dans plusieurs provinces de la République Populaire de Chine. Bien que toujours incertaine, l’étiologie de cette maladie est vraisemblablement environnementale et multifactorielle, et l’apport en minéraux est supposé y jouer un rôle prépondérant. L’alimentation en particulier différencie les populations rurales touchées par la maladie des autres groupes (nomades et citadins). L’objectif de la présente étude, préliminaire à une enquête nutritionnelle épidémiologique, est l’analyse de la composition minérale (Ca, P, Mg, Fe, Zn, Mn, Cu, Ni, Se, Al, Sr, Mo, Cd, As, Pb, Hg, Cr et Co) de huit aliments de base au Tibet et la comparaison des résultats avec deux tables de composition alimentaires. Vingt familles furent recrutées dans des régions rurales endémiques et non-endémiques aux alentours de Lhassa, et les aliments y ont été échantillonnés. Une dizaine de minéraux impliqués dans le métabolisme osseux a été analysés par diverses méthodes spectrométriques atomiques et moléculaires. Les résultats semblent indiquer qu’un nombre très limité de couples aliments/constituants présente une variation de la composition minérale au cours d’une même année pour une région donnée. Par contre, il y a des différences significatives entre les régions endémiques et non-endémiques, surtout pour la farine de blé. Les contenus en minéraux des aliments Tibétains ont ensuite été comparés à la composition d’aliments similaires provenant de tables de composition alimentaire : USDA National Nutrient

Database (USDA Food Search for Windows, Version 1.0, database version SR23), et China Food Composition (livre 1, 2ème édition). Plus de 50 à 60% des p-valeurs sont inférieures à 0.05 suggérant l'inadéquation des tables de composition alimentaire dans le cas d'étude nutritionnelle en région rurale tibétaine, et l'importance de l'analyse des aliments traditionnels. Les différences sont plus marquées en fonction des éléments considérés, et les teneurs en calcium semblent les plus éloignées des tables. Le nombre d'échantillons par aliments et surtout le nombre d'aliments échantillonnés sont trop faible pour tirer des conclusions définitives. Cependant, il semble que la présente étude pilote indique des divergences significatives entre les valeurs mesurées et les valeurs des tables. Une étude plus complète est donc indispensable.

Keywords

Human diseases, bone diseases, disease control, food composition, foods, diet, flour, calcium, minerals, Tibet.

Mots-clés

Maladies humaines, maladies des os, métabolisme osseux, lutte contre les maladies, composition des aliments, régime alimentaire, farines, calcium, minéraux, Tibet.

1. Introduction

Today, Kashin-Beck disease (KBD) principally occurs in the Tibet Autonomous Region and in several western provinces of the People's Republic of China. It is estimated that KBD affects between 0.74 million and 2.5 million people in China and other parts of Asia; between 10 and 30 million people are estimated to be at risk (Yamamuro, 2001; Mathieu et al., 2008a; Wang et al., 2008; Wang et al., 2009). KBD is an endemic and chronic osteochondropathy characterized by short stature and skeletal deformities, especially of the long bones and joints. Joints become enlarged, stiff and painful. Mobility of limbs becomes limited and consequently muscles can become atrophied. Symptoms appear during childhood and may worsen progressively. The pathophysiological feature of KBD is a focal chondronecrosis of mature chondrocytes in the deep zone of the growth plate cartilage and the articular cartilage (Mathieu et al., 2008a; Wang et al., 2008; Wang et al., 2009).

Although many studies have been conducted and many others are still underway, the etiology of the disease remains unknown. Nevertheless, a multifactorial hypothesis has been proposed: a combination of selenium and iodine deficiency, high concentrations of organic matter in drinking water (fulvic acids), and mycotoxin poisoning via fungal infection of cereals (Yang et al., 1993; Moreno-Reyes et al., 1998; Haubruge et al., 2001; Suetens et al., 2001; Mathieu et al., 2008a; Mathieu et al., 2008b; Li et al., 2009; Shi et al., 2009).

According to several authors (Haubruge et al., 2000; Malaisse et al., 2008), Tibetan populations share 4 macro-ecosystems: the urban zone; the suburban zone, which is mechanized and has access to communication media; the agricultural zone, relying on subsistence farming; the pastoral zone located at over 4500 m in altitude and featuring nomads and yaks. KBD is only encountered in the agricultural zone and endemic areas are limited to poor, isolated and rural communities. Diet is a major factor differentiating these four groups. In addition, an unpublished study by de Voghel et al. (2008) highlighted the poorly diversified diet of those living in endemic areas and the probable deficiencies of several nutrients involved in bone metabolism.

In anticipation of a nutrition survey in the region, one of the objectives of this study aimed to measure the mineral content of the main staple foods of Tibetan peasants living in an endemic area (EA). Generally, nutrition studies are based on food composition tables (FCTs) (Gibson, 1990; Church, 2006; Egan et al., 2009). The USDA National Nutrient Database for Standard Reference (US Department of Agriculture, 2010) and The China Food Composition Table (National Institute of Nutrition and Food Safety China CDC, 2009) were selected for this study. Nevertheless, the composition of foods as listed in national FTCs may be quite different from the composition of foods in a given local area. Another of our objectives was to ensure that the results of the food analyses carried out in the present study matched with the reference values of FCTs. We wanted to be certain that a Tibetan nutrition survey based on FCTs would not introduce a major bias into the estimation of daily intake, as sometimes occurs (Moon et al., 1996). With this in mind, the mean mineral content of Tibetan food samples was statistically compared to the reference values of similar foods listed in the two selected FCTs. The aim was to determine the most appropriate FCT to use as a reference. Given the symptoms of KBD, this study focused on several minerals involved directly or indirectly in bone metabolism: Ca, P, Mg, Fe, Zn, Mn, Cu, Ni, Se, Al, Sr, Mo, Cd and As (Matsumoto et al., 1991; Sctrick, 1991; Jacotot et al., 1992; Martin, 2000; Sarazin et al., 2000; Moreno-Reyes et al., 2001; Baron et al., 2002; Pi et al., 2004; Marieb, 2005). A third objective of our study was to make a comparison of mean food mineral content between an endemic and a non-endemic rural area in Tibet. Screening for heavy metals was also undertaken (Pb, Hg, Cr, Co).

2. Materials and methods

2.1 Study area and sampling

Ten households from Nimo and Lhundrop counties (high KBD prevalence areas near Lhasa) were enrolled into the study. They were selected from the Kashin-Beck Disease Fund database according to two criteria: presence in the family of a 3 to 5 year old child (at this age, children are weaned and eat home cooking) and of an older KBD-affected child. Children with an affected sibling are more likely to develop the disease (Suetens et al., 2001). The application of these criteria is especially important for the ongoing nutrition survey. Ten additional households were enrolled in Rinpung, a non-endemic area. These also included a young child.

Food sampling was carried out twice in the endemic area, in winter (February 2010 = EAw) and in spring (May 2010 = EAs), in order to assess variance caused by a possible seasonal effect. One sampling campaign was also carried out in the non-endemic area (July 2011= NEA). Staple foods, which were selected based on the findings of a previous nutrition survey (Goyens et al., 2008), were collected from each family. The foods were: roasted barley flour, wheat flour, black tea, rice, potatoes, Chinese radish and yak butter. Samples of raw foods were taken from the top of their respective containers in order to get closer to the conditions of consumption. The container itself was not suspected of being the cause of heterogeneity in the mineral content of the samples taken. Samples were collected in single-use “food quality” plastic bags and stored in a place safe from humidity. Sometimes, one or several foods were unavailable in some households during the sampling period. The eighth staple food to be analyzed, Chinese cabbage, was bought in Lhasa. This food, frequently consumed, is not grown at home and families buy it in Lhasa. The day after sampling, in order to avoid contamination and deterioration, potatoes and Chinese cabbages were cut and dried at 105 °C in an air oven until an unchanging dry weight was obtained, enabling determination of dry matter (DM) content and long term storage.

2.2 Reagents and glassware

The laboratory glassware used was cleaned by soaking overnight in a 6N nitric acid bath and rinsed at least four times with distilled water. All reagents were of analytical grade. Ultrapure water was used throughout the analyses.

2.3 Sample digestion

The sampling portion was about one gram, weighed with an analytical balance (AE 200 Mettler) in a Teflon container and placed in an HPR 1000/6 rotor. Six milliliters of 65% nitric acid (AnalR Normapur, VWR Prolabo) and 1 ml of 35% hydrogen peroxide (analytical reagent, Merck) were added. Samples were then submitted to microwave assisted mineralization in a high performance microwave digestion unit, MLS 1200 mega (Milestone, Brøndby, Denmark) with an MLS Mega 240 terminal and EM 45 A exhaust module. The following optimized microwave program was implemented: 250 W: 2 min; 0 W: 2 min; 250 W: 6 min; 400 W: 5 min; 600 W: 5 min; ventilation: 10 min. The program used was based on the manufacturer's recommendations for use (Milestone Cookbook of Microwave Application Notes for MDR technology, January 1995). The rotor was then cooled down for a period of one hour. Mineralized solutions were quantitatively transferred into 50 ml volumetric flasks and diluted to volume with ultrapure water. Teflon containers were rinsed at least 3 times with ultrapure water. Black tea was brewed in the traditional Tibetan way (R. Wangla, personal communication): approximately 10 grams of tea were weighed precisely and then boiled in 100 ml of ultrapure water for 1h 30. Infusions were then filtered through folded filter papers (MN 616 1/4 18.5 cm diameter; Macherey-Nagel, Düren, Germany) and diluted to 250 ml with ultrapure water. 20 ml samples of this solution were transferred with a volumetric pipette into a Teflon container and were directly mineralized under the aforementioned conditions.

2.4 Atomic absorption spectroscopy procedures

Most of the mineral elements were measured using atomic absorption spectroscopy (Ca, Mg, Fe, Zn, Mn, Cu, Ni, Se, Al, Sr, Mo, Cd, As, Pb, Hg, Cr et Co, by flame atomic absorption spectrometry (FAAS), electrothermal atomic absorption spectrometry (ETAAS), hydride generation and cold vapor generation) in Belgium (Bureau Environnement et Analyses de Gembloux, BEAGx). Phosphorus was measured using UV-Vis spectrophotometry at 700 nm on a UV-1205 Shimadzu (Shimadzu, Kyoto, Japan) with an Epson LX-300 printer, using the Scheele method (heptamolybdate reagent). Most selenium levels were found to be below the limit of quantification (LOQ). Results were therefore confirmed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) on several samples at the Walloon Agricultural Research Centre (CRA-W), Agricultural Product Technology Unit.

A Perkin-Elmer 1100 B flame atomic absorption spectrometer (Perkin-Elmer, Waltham, USA) was used for Ca, Mg, Zn, Fe, Mn, Cu, Ni, Al, Sr, and Mo analyses. Dilutions with spectral buffer $LaCl_3$ 2 g/l in HNO_3 2% were performed for Ca, Mg, Al and Mo. When dilutions were required for other elements, a solution of HNO_3 2% in ultrapure water was used. Dilutions were performed with a Microlab® 500 series diluter (Hamilton Company, Bonaduz, GR, Switzerland). A background correction method using a deuterium lamp was applied to all the minerals tested except for calcium. Cu, Ni, Cd, Pb, Cr and Co analyses were performed with a Perkin-Elmer AAnalyst 800 electrothermal atomic absorption spectrometer (Perkin-Elmer, Waltham, USA) with P-E AS 800 autosampler. A matrix modifier (Bernd Kraft GmbH, Duisburg Germany) ($Pd+Mg/HNO_3$ 2 M) was used. Background correction based on the Zeeman Effect was also applied. In both devices, the light source was provided by hollow cathode P-E lumina lamps. Arsenic and selenium were measured using the hydride generation method (HG). Two milliliters of a solution of 5% KI (Fisher) and 5% ascorbic acid (Acros Organics) and 1 ml of 37% HCl (analytical reagent, Merck) were added to 2 ml of mineralized solutions. No KI was added for Se analysis. The whole solution was homogenized and allowed to reduce for a period of 45 min before dilution to 10 ml with ultrapure water. Samples were analyzed using a Perkin-Elmer AAnalyst 800 equipped with a quartz cell and with a P-E As 90plus autosampler. The reagent solutions used for hydride generation were HCl 10% and $NaBH_4$ 0.5% (for As) and 0.02% (for Se) and NaOH 0.005%. Mercury was analyzed using cold vapor generation (Co.Vap.) on a P-E FIMS-400 equipped with a S10 autosampler. A 5 ml sample portion was taken from each solution. Calibration curves were obtained with multi-element standard solutions (Bernd Kraft GmbH), except in the case of phosphorus and strontium, for which KH_2PO_4 and $Sr(NO_3)_2$ solutions (Merck) were used.

2.5 Statistical methodology

Statistical analyses were performed with the Minitab 15.1 software. Parametric tests were performed after checking the conditions of application (homoscedasticity and normality of the populations). Otherwise, transformations of variables were tested using the Box-Cox method. As a last resort, nonparametric tests were applied. In cases where no significant difference was found between the mineral composition of the analyzed food samples, the *Tukey Significant Difference* method was used for the classification of statistical means.

The mineral composition of the analyzed foods was then compared against two FCTs. The China Food Composition Table (book 1, 2nd edition) was chosen because it is probably the most appropriate for nutritional survey for the Tibet Autonomous Region. A comparison with the USDA National Nutrient Database was also made because it represents one of the most comprehensive FCTs in terms of number of food types listed and number of samples per food tested.

2.6 Quality control

The methods used (mineralization and measurements) were validated via certified reference materials (CRMs). Two CRMs: White cabbage BCR®-679 and Wheat flour ERM®-BC382, were obtained from the European Institute for Reference Materials and Measurements (Geel, Belgium). These CRMs were selected because they include almost all the elements of interest and because of their similarity with our sample matrices. However, these CRMs do not include a certified value for selenium. The CRMs were processed and analyzed here following the same procedures as for the samples. Measurement of each element was performed as described above in at least four replicates. Dry matter content was determined according to the provider's instructions. Statistical comparison between measurement results and certified values was also made according to the provider's instructions (Linsinger, 2005). Besides the validation by CRMs (through demonstration of recovery and repeatability), supplementary parameters were also assessed, such as limits of quantification (LOQs), linearity ranges, calibration curves and repeatability on samples.

3. Results and discussion

3.1 Validation of methods

Data were expressed in mg·kg^{-1} of dry matter. Results of measurements were compared with the certified values following an appropriate procedure (Linsinger, 2005). **Table 1** shows the mineral content values from the CRMs for white cabbage and wheat flour in comparison with data obtained in the laboratory. As Δm, the difference between the certified value (C_{CRM}) and the mean measured value (C_m), must be lower than U_Δ, the expanded uncertainty, the analytical method was validated for the following elements: Ca, P, Mg, Fe, Zn, Mn, Cu, Ni, Mo and Cd but not for Sr. Se, As, Pb, Hg, Cr, Co and Al content was not certified in the reference materials.

Relative standard deviations (RSDs) were calculated on five replicates of CRM, and five replicates of one barley flour sample. No RSD exceeded 10% and most were below 5%. Linearity ranges were defined by the interval between the LOQs and the upper limit of the calibration curves. All determination coefficients (R^2) of the calibration curves were higher than 0.998. LOQs were calculated for each element on eight blanks based on the average blank value plus ten times the standard deviation of blanks. Outliers were highlighted and set aside via the Grubbs' test (Miller et al., 2005). They are expressed in mg.kg^{-1} of dry weight: Ca: 30, P: 50, Mg: 10, Fe: 10, Zn: 3, Cu: 0.65, Mn: 1, Se: 0.05, Na: 35, K: 60, Ni: 0.4, As: 0.02, Sr: 9, Mo: 11, Cd: 0.05, Pb: 0.5, Hg: 0.03, Al: 30, Cr: 0.5, Co: 0.5.

Table 1. Comparison of mineral content values from CRMs for white cabbage and wheat flour in comparison with data obtained in the laboratory

	White cabbage BCR®-679 mg / kg (*µg / kg)			*Wheat flour ERM®-BC382* mg / g				
	$C_{CRM} \pm s_{CRM}$	C_m	s_m	$C_{CRM} \pm s_{CRM}$	C_m	s_m	Δ_m	U_Δ
Ca[2]	-	-	-	0.21 ± 0.018	0.203	0.017	0.007	0.019
P[1]	-	-	-	1.19 ± 0.07	1.26	0.01	0.071	0.071
Mg[2]	-	-	-	0.247 ± 0.010	0.253	0.011	0.006	0.013
Fe[2]	55 ± 2.5	55.3	3.1	-	-	-	0.3	3.4
Zn[2]	79.7 ± 2.7	81.6	2.8	-	-	-	1.9	3.4
Cu[3]	2.89 ± 0.12	2.96	0.22	-	-	-	0.07	0.23
Mn[3]	13.3 ± 0.5	13.7	0.8	-	-	-	0.4	0.8
Ni[4]	27 ± 0.8	27.5	2	-	-	-	0.5	1.9
Sr[3]	11.8 ± 0.4	7.6	0.5	-	-	-	4.3	0.6
Mo[3]	14.8 ± 0.5	13.9	1.5	-	-	-	0.9	1.4
Cd*[4]	1.66 ± 0.07	1.95	0.36	-	-	-	0.29	0.37

[1] n=8; [2] n=7; [3] n=5; [4] n=4; Δm, the difference between the certified value (C_{CRM}) and the mean measured value (C_m), must be lower than U_Δ, the expanded uncertainty. U_Δ is obtained by multiplication of u_Δ (the combined uncertainty of result and certified value) by a coverage factor k, equal to 2. There is no significant difference between the data obtained in the laboratory and the certified value if $\Delta m \leq U_\Delta$.

Analysis of food samples and comparison with food composition tables In the Tibetan food samples analyzed, some of the studied elements were shown to be present at a level below the limit of quantification (Ni, Se, Mo, Cd, Pb, Hg, Cr and Co). The levels of aluminum in the foods analyzed were higher than the limit of quantification but no reference values were found in the FCTs. Therefore, none of the aforementioned elements will be discussed further in this study. As we have seen, one of the food samples analyzed here was black tea. In rural Tibet, this is quite an expensive commodity. It is brewed to a highly concentrated level, and then drunk in very diluted form. Consequently, its mineral composition is extremely poor and this food will therefore also not be discussed here. Furthermore, another of our foods originally chosen for analysis, yak butter, could not be analyzed in this study for reasons of stability and conservation during the transport of samples.

Analyses of variance between food samples taken from the endemic area and the non-endemic area were conducted for the following elements: Ca, P, Mg, Fe, Zn, Cu, and Mn. Three staple food items, which were available in each area, were considered: roasted barley flour, wheat flour and rice (no potato was collected in the non-endemic area, and no radish was collected in the endemic area). Mean mineral content and p-values for these three staple foods are listed in **Table 2**. The results show no significant variation in the mineral composition of barley flour and wheat flour during the year of sampling. They also reveal a very limited variation in the mineral composition – iron and manganese – of rice between the two seasons of sampling in the endemic area. On the other hand, the three staple food items presented some significant differences in mineral composition between the non-endemic area and the endemic area. Wheat flour showed the greatest difference in composition, with five significantly different minerals out of seven. Manganese was found to be present at significantly higher levels (p-value < 0.01) in the endemic area both in roasted barley flour and in wheat. Conversely, calcium content was found to be significantly lower in barley flour and in rice (p-value < 0.5 and < 0.001 respectively). Within the context of a poorly diversified diet, as is the case in rural Tibet (Liquiang et al., 1991; Suetens et al., 2001; Dang et al., 2004), these differences in mineral composition might lead to differences in daily mineral intake.

Mineral composition (Ca, P, Mg, Fe, Zn, Mn and Cu) of the sample Tibetan foods was analyzed and means were calculated by area (Rinpung: non-endemic area, NEA; Nimo and Lhundrop: considered as one endemic area, for two seasons, spring and winter = EAs and EAw). Means were compared using one-sample Student t-tests against the mineral composition of foods listed in the two chosen FCTs (USDA and China). Certain foods listed in the FCTs were chosen for comparison with the samples based on their similarity. Results are summarized in **Table 3**. The table lists the Tibetan food samples and the food items from the FCTs. For the sake of readability, all p-values of one comparison (Tibetan sample/food item) are shown together for all minerals, and expressed as a percentage by category of p-value (p>0.05; 0.001<p<0.05; p<0.001). As can be seen in the first category (p>0.05), most food items from the FCTs present a different composition to the Tibetan foods analyzed. Except for *potato flesh and skin, raw* and *potato, white flesh and skin, raw* from the USDA FCTs and *bok choi, white* from the China FCTs, significant differences of more than 50% can be seen between the mineral composition of the Tibetan samples and that of the food items listed in the FCTs. Many even show a 60-70% difference. It is clearly sensible to determine a cut-off point when considering including or rejecting a similarity between food samples and FCTs, for example, concerning mineral content. the Canadian Food Inspection Agency tolerates a 20% difference between a labeled and a measured value for commercial products (Canadian Food Inspection Agency, 2003), whereas the French Association Nationale des Industries Alimentaires recommends a 40% level of accepted tolerance (Association Nationale des Industries Alimentaires, 2009). However, no mention is made by these agencies of the accumulation of differences, and our results showed that foods studied can be very different in term of mineral composition when compared against similar foods listed in the two national FCTs. Nevertheless, the distribution of p-values seemed to be different according to the mineral element, as can be seen in **Table 4**. Overall p-values for all foods investigated are shown for each FCT and for each mineral element, and are expressed as percentages. In both FCTs, and for most minerals, less than 50% of p-values are above 0.05, meaning that most of the mineral content levels in the sampled Tibetan foods were significantly different from those of the food items listed in the FCTs. The calcium content in the China FCTs was found to differ considerably from the calcium content in the Tibetan samples (only 5% of p-values > 0.05). Concerning the other elements, percentages of p-values > 0.05 ranged from 15 to 53%. Despite the small number of samples analyzed in the present study, this preliminary insight suggests that a nutritional survey in rural Tibet, based on food composition tables, could introduce a bias in the calculation of daily mineral intake, especially for calcium.

Dry matter (DM) content was measured in all the food samples. The means of dry matter content of barley flour, wheat flour and rice are listed in **Table 2**. The means of DM content for these three staple foods were also calculated by area by food item and compared using one-sample Student t-tests to similar foods listed in the two FCTs (USDA and China). The mean DM content of Tibetan barley flour was found to be significantly higher (p-values < 0.001) than the values found in both FCTs. One probable explanation for this comes from the fact that, in Tibet, barley is first roasted before grinding into flour. Overall, the DM content of our wheat flour and rice samples was quite close to the values shown in the FCTs (73% of p-values > 0.05). The DM content of Tibetan potato samples matched with only one food item from the USDA table (*potato, boiled in skin, flesh without salt*). Significant differences were found compared to other food items listed in the USDA and China tables (p-values < 0.05 and less). The DM content of the Tibetan dried radish samples was also found to be higher than that of its USDA homologue (*radish, oriental, dried*; p-value < 0.05). By contrast, the DM content of the Tibetan cabbage sample was found to be lower than the corresponding DM value for cabbage in both the USDA and the China FCTs (p-values < 0.01).

Table 2 Mean values of mineral elements (mg·kg^{-1} DM) by area and variance-values

	Average (mg·kg^{-1} dry matter)			p-values (one-way ANOVA)		
	NEA	EAw	EAs	NEA/EAw	NEA/EAs	EAw/EAs
Barley flour (n=30)						
Ca	290	396	384	*	*	/
P	3953	3978	4083	/	/	/
Mg	1227	1156	1158	/	/	/
Fe	188	115	144	*	/	/
Zn	25	23	23	/	/	/
Mn	21	16	18	**	**	/
Cu	6.1	4.1	3.7	*	*	/
Dry matter (%)	91	93	93			
Wheat flour (n=29)						
Ca	258	330	287	/	/	/
P	2832	1588	1748	*	*	/
Mg	889	409	435	*	*	/
Fe	127	40	48	***	***	/
Zn	20	11	13	*	/	/
Mn	22	10	11	**	**	/
Cu	3.0	3.0	2.6	/	/	/
Dry matter (%)	87	88	87			
Rice (n=24)						
Ca	30	80	73	***	***	/
P	1063	1156	1123	/	/	/
Mg	262	261	208	/	/	/
Fe	13	4.2	22	***	***	***
Zn	15	15	14	/	/	/
Mn	12	9.4	11	*	/	*
Cu	1.7	2.5	1.9	*	/	/
Dry matter (%)	87	87	86			

/: $p \geq 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; NEA: non-endemic area; EAw: endemic area in winter; EAs: endemic area in spring. Mean mineral composition (mg·kg^{-1} of dry matter) is listed by area (endemic and non-endemic) for the following Tibetan sample foods: roasted barley flour, wheat flour and rice. Analysis of variance was performed on these means. P-values are listed in the three next columns according to this code: / = p≥0.05; *= p<0.05; ** = p<0.01; *** = p<0.001.

Table 3 Comparison of mineral composition between Tibetan foods analyzed and foods listed in two national Food Composition Tables (FCTs) expressed as relative proportions of p-values obtained from applying one-sample t-tests

Tibetan sample	**Table**	**Food item**	**% p-value ≥ 0.05**	**% 0.001<p-value<0.05**	**% p-value < 0.001**
Roasted barley flour	China	Barley grain	14.3	23.8	61.9
(n=30)		Naked barley grain	23.8	14.3	61.9
	USDA	Barley flour or meal	38.1	19	42.9
Wheat flour	China	Wheat flour, standard grade	42.9	33.4	23.8
(n=29)		Wheat flour, refined, special grade 1	42.9	33.4	23.8
		Wheat flour, refined, special grade 2	42.9	33.4	23.8
	USDA	Wheat flour, white, all-purpose, unenriched	19	76.2	4.8
		Wheat flour, white, all-purpose, enriched, bleached	28.6	66.6	4.8
		Wheat flour, white, all-purpose, enriched, unbleached	28.6	66.6	4.8
Rice	China	Rice, grain	9.5	23.8	66.7
(n=24)	USDA	Rice, white, short-grain, raw	42.9	33.3	23.8
		Rice, white, short-grain, raw, unenriched	42.9	42.9	14.3
		Rice, white, medium-grain, raw, enriched	14.3	42.9	42.9
		Rice, white, medium-grain, raw, unenriched	14.3	52.3	33.3
Potato	China	Potato, white	21.4	71.4	7.1
(n=16)	USDA	Potato, boiled in skin, flesh without salt	35.7	28.5	35.7
		Potato, boiled in skin, skin without salt	21.4	21.4	57.1
		Potato flesh and skin, raw	71.4	21.4	7.1
		Potato, white flesh and skin, raw	57.1	28.5	14.3
Radish (n=10)	USDA	Radish, oriental, dried	28.6	42.9	28.6
Cabbage	China	Bok choi, white	57.1	42.9	0
(n=3)	USDA	Cabbage, Chinese (pe-tsaï), raw	42.9	57.1	0

This table compares the investigated mineral composition between Tibetan foods and similar foods listed in the national FCTs. Equality of means was assessed using one-sample Student t-tests for each cluster (Non-Endemic Area, Endemic Area winter and Endemic Area spring) and for each mineral (Ca, P, Mg, Fe, Zn, Cu, and Mn). All the results (i.e. p-values) of a comparison between two foods were brought together. Percentages by category of p-value – $p \geq 0.05$; $0.001 < p < 0.05$; $p < 0.001$ – were calculated and are listed.

4. Conclusions

As an endemic and chronic osteochondropathy affecting hundreds of thousands of people, Kashin-Beck disease remains misunderstood and little studied. Evidence indicates environmental causes, including a potential dietary imbalance, mainly of minerals and micronutrients. A more in-depth analysis of the mineral intake of people living in endemic areas might represent a further step towards understanding the disease.

In anticipation of a forthcoming nutrition survey, this pilot-study was focused on the adequacy of the mineral composition of Tibetan foods in comparison with values shown in food composition tables. Main staple Tibetan foods were sampled once in Rinpung (non-endemic area, summer 2011) and twice (winter and spring 2010) in Nimo and Lhundrop (endemic area). The results revealed a very limited number of food /constituent pairs with a variation in mineral composition during a single year of testing in a given region. In addition, results showed significant differences in mineral content between the endemic area and the non-endemic area, particularly for wheat flour.

The mineral content of sample Tibetan foods was statistically compared with similar foods listed in two food composition tables: the USDA National Nutrient Database (USDA Food Search for Windows, Version 1.0, database version SR23), and the China Food Composition Table (book 1, 2nd edition). More than 50 to 60% of p-values < 0.05 were highlighted, suggesting the inappropriateness of using FCTs as part of nutrition surveys in rural Tibet, and emphasizing the need for analysis of traditional foods. The difference between the mineral content of the sampled Tibetan foods and official listed values was the most marked for barley flour. In this particular case, it is important to note that there is no equivalent in the China Food Composition Table. Moreover, in Tibetan households, barley grains are first roasted before being ground into flour. This could partly explain the difference in mineral composition of the Tibetan samples compared to that of *barley flour or meal* as listed in the USDA National Nutrient Database (which probably undergoes industrial processing). Other reasons, which apply to all foods, may explain the differences between the mineral composition values of our Tibetan food samples and those listed in the two FCTs. The first reason is geographical distribution: the People's Republic of China is a huge country with a vast and diverse landscape and an uneven distribution of population; hence there is a possibility of variation in the composition of foods across the region (Greenfield et al., 2003). The second reason is the fact that the cultivar, physiological state and maturity of plants when harvesting can also vary in Tibetan agricultural practices and can cause variation in mineral composition (Greenfield et al., 2003).

It is clearly difficult to determine a cut-off point when considering the matching of food mineral content in specific food samples against food composition tables. Although it is obviously too early for definite conclusions to be reached (our analysis was carried out on only approximately 30 samples of each food), it seems that the present pilot-study indicates significant discrepancies between the values we measured and those listed in the food composition tables. In the present case, the results show that more food analyses are required to establish reliable nutritional reference values. Combined with nutrition surveys, these could contribute to an accurate calculation of the nutritional quality of food intake of Tibetan children.

Table 4 General comparison between Tibetan foods and two national FCTs for each investigated mineral expressed as relative proportions (in%) of p-values obtained from applying one-sample t-tests

FCTs	Category of p-value	Ca	P	Mg	Fe	Zn	Mn	Cu
China	≥ 0.05	5	52	38	38	33	24	19
	0.001<p-value<0.05	43	24	14	39	43	29	33
	< 0.001	52	24	48	24	24	48	48
USDA	≥ 0.05	21	53	44	15	53	26	21
	0.001<p-value<0.05	36	36	33	44	44	59	59
	< 0.001	44	12	24	41	3	15	21

Table 4 shows a general comparison between Tibetan foods and national FCTs for each investigated mineral. Equality of means was assessed using one-sample Student t-tests for each cluster (Non-Endemic Area, Endemic Area winter and Endemic Area spring) and for each mineral (Ca, P, Mg, Fe, Zn, Cu, and Mn). All the results (i.e. p-values) of a comparison between Tibetan foods and two national FCTs (China and USDA) were brought together. Percentages by category of p-value – p≥0.05; 0.001<p<0.05; p<0.001 – were calculated per mineral and are listed.

Acknowledgements

This study was made possible thanks to the close collaboration and the support of the Center for Disease Control and Prevention of Tibet Autonomous Region (P.R. China). We are in debt to CLSA Hong Kong who supported financially this study.

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Minerals and trace elements in traditional foods of rural areas of Lhasa prefecture, P.R. China

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Adapted from the reference: Dermience, M., Li, X. W., Mathieu, F., Claus, W., De Maertelaer, V., Yangzom, D., & Lognay, G. 2014. Minerals and trace elements in traditional foods of rural areas of Lhasa Prefecture, Tibet Autonomous Region (P.R. China). *Journal of Food Composition and Analysis,* **35**(2), 67-74.

Abstract

Traditional foods play a major role in the diet of rural people living in the Tibet Autonomous Region of the People's Republic of China. Because these foods are mainly derived from local agriculture as well as artisanal production, their mineral composition may show significant discrepancies when compared with food composition data. This study aims at providing relevant data on the mineral composition of the main Tibetan foods. Sixteen different foodstuffs were sampled, including water, concentrated brewed black tea, chang, *tsampa*, wheat flour, dried cheese, dried yak meat, dried mutton, blood sausage, dried wild peaches, dried Chinese radish, and dried nettles. They were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) for 19 minerals: Na, K, P, Ca, Mg, Fe, Zn, Mn, Cu, Ni, Se, Mo, Al, As, Cr, Co, Cd, Pb, and V. The validity of the results was ensured by the use of standard reference materials. A statistical comparison of the mean mineral contents of the analyzed foods against food composition data from the China Food Composition (CFC) table was carried out. It revealed significant discrepancies, emphasizing the importance of food analysis for nutritional assessment in Tibet autonomous region. To the best of our knowledge, the mineral compositions of some traditional Tibetan foods are here reported for the first time.

Keywords

Food composition data; traditional foods; minerals; trace elements; mineral intake; nutritional assessment; Tibet Autonomous Region; dietary assessment; food analysis; food composition.

1. Introduction

In areas as remote as rural Tibet, traditional foods occupy an important place in the diet. To the best of our knowledge, there is little information available on the composition of these traditional foods, which originate mainly from local agriculture and artisanal production. It is well known that multiple factors influence the mineral composition of foods: agricultural practices, climate, altitude, geochemistry, the variety of cultivars, physiological state and maturity, and post-harvest treatments can affect the mineral composition of cereals and vegetables (Greenfield and Southgate, 2003; Rodríguez et al., 2011; Yada et al., 2013). Processing methods, handling, and food preparation are also likely to modify the mineral concentrations of foods (Greenfield and Southgate, 2003; Lisiewska et al., 2009; Campo et al., 2013). The mineral profile of a food product can also be used for identification of a cultivar or for authentication of geographical origin (Rodrigues et al., 2011; Rodríguez et al., 2011).

McCance and Widdowson, cited by (Greenfield and Southgate, 2003), stated: "*A knowledge of the chemical composition of foods is the first essential in dietary treatment of disease or in any quantitative study of human nutrition*". It is especially true that mineral deficiencies or excesses play a role in the etiology of numerous diseases throughout the world (Martin, 2000; Mudgal et al., 2010). In the particular environment of rural T.A.R., a rampant disease called Kashin–Beck disease (KBD) affects thousands of people (Mathieu and Hinsenkamp, 2008). Although it is unclear exactly what causes this disease, this endemic and chronic osteochondropathy may find its roots in multiple socio-environmental causes, among which diet minerals seems to play a part (Goyens et al., 2008). Apart from iodine deficiency, very common in inland human populations, soil selenium – and even molybdenum – deficiency areas have been identified in T.A.R. and other provinces of China (Li et al., 2009; Zhang et al., 2010), implying deficiencies in human and animal nutrition (Tan et al., 2002; Chasseur et al., 2008). On the other hand, the specific diet of rural Tibetan, based on cereal and cereal products, is extremely monotonous (Goyens et al., 2008) and results in severe stunting (Harris et al., 2001; Rooze et al., 2012); it may also likely lead to various imbalances in mineral intakes such as calcium, phosphorus, and manganese.

In conducting a nutrition survey in an area as remote as T.A.R., the use of food composition tables (FCTs) is potentially inadequate to assess accurate mineral intakes (Moon et al., 1996; Dermience et al., 2013). Trace elements are especially sensitive, considering the low levels of dietary reference intakes and the possibly significant discrepancies between the food composition data and the composition of local food (Gibson, 1990; Gibson and Ferguson, 2008). This study aims at providing relevant data on the mineral composition of the main Tibetan food products for nutritional assessment. In order to determine the mineral composition of different traditional Tibetan foods, sampling was carried out in parallel to a nutrition survey undertaken in rural areas of Lhasa Prefecture. Because children living in these areas suffer from underweight, stunting, and clinical rickets (Rooze et al., 2012), the food samples were analyzed for most of the mineral elements known to play a role (positive or negative) in bone metabolism.

2. Materials and methods

2.1 Samples and sample preparation

Traditional foods were sampled in 250 families from 65 villages of 8 communities – Nimashangri, Nyanang, Thanggo, Theumba, Paco, Nyemo, Tharong, and Shume – located in three counties – Lhunzhub, Maizhokungar, and Nyêmo – of Lhasa Prefecture. The families were selected according to principles of nutritional assessment by stratified random sampling in the context of an ongoing nutrition survey (Willett, 1998; Gibson and Ferguson, 2008). Traditional home-grown or homemade foods were sampled in each family when available, including: *tsampa* (roasted barley flour, n = 238), wheat flour (n = 55), dry cheese (n = 215), dried meat (n = 19), dried peaches (n = 10), dried radish (n = 13), and dried wild vegetables, namely nettles, colza leaves, and edible wild herbs (total n = 120). Solid samples were collected in single-use ''food quality'' plastic bags (Toppits1 3L, Melitta1 Group, Minden, Germany) and stored in a place free of humidity. Liquid samples were collected in centrifuge tubes (Medical grade, CentriStarTM, Corning Inc., Tewksbury, USA): water (n = 245), concentrated brewed black tea (used to make butter tea and black tea, n = 239), and chang (homemade beer, n = 155). For the sake of conservation, a Micropur Classic MC 1T pill (Katadyn Products Inc., Kemptthal, Switzerland) was added to each tube. The silver ions from the pills preserved the samples from bacterial growth without interfering with the targeted minerals. The potential interferences induced with Micropure pills were assessed by spiking the blanks with these pills.

All mineral analyses were performed in the China National Center for Food Safety Risk Assessment (CFSA, Beijing, P.R. China). In order to reduce the number of analyses, wheat flour, dried cheese, and dried rapeseed leaves samples were pooled by villages. Five grams of each sample, weighed with a Mettler AE50 analytical balance (Gemini B.V., Apeldoorn, The Netherlands), were thoroughly homogenized together. All reagents were of ICP-MS grade. The laboratory glassware and Teflon containers used were soaked overnight in a 15% nitric acid bath (Beijing Institute of Chemical Reagents, Beijing, China) and rinsed with ultrapure water; ultrapure water was also used for all of the analyses. Sampling portions ranging from 0.5 g to 0.8 g for solids samples, and about 1 mL for liquid samples, were digested by microwave assisted oxidative acid digestion. Six milliliters of 65% nitric acid (Beijing Institute of Chemical Reagents, Beijing, China) were added to the sample in a Teflon container, and the entire solution was submitted to the following microwave program in a Mars Lp5 microwave system (CEM, Matthews, USA): from room temperature to 120 °C in 5 min; hold at 120 °C for 5 min; from 120 °C to 160 °C in 5 min; hold 5 min; from 160 °C to 190 °C in 5 min, hold 20 min; cooling for one hour. Digested solutions were quantitatively transferred into 50 mL centrifuge tubes (medical grade, CentriStarTM, Corning Incorporated, Tewksbury, USA) and diluted to approximately 25 mL with ultrapure water (MilliQ plus, Millipore, Billerica, MA, USA). The dilution ratio was ensured by weighing the tared centrifuge tubes after dilution with a Mettler AE50 analytical balance.

2.2. Analytical determination

The concentrations of mineral elements – sodium, potassium, phosphorus, calcium, magnesium, iron, manganese, zinc, copper, nickel, selenium, aluminum, molybdenum, vanadium, chromium, cobalt, arsenic, cadmium and lead (Na, K, P, Ca, Mg, Fe, Mn, Zn, Cu, Ni, Se, Al, Mo, V, Cr, Co, As, Cd, and Pb) – were measured by inductively coupled plasma mass spectrometry (ICP-MS) on an Agilent 7700 Series ICP-MS (Agilent Technologies, Santa Clara, CA, USA). Agilent solutions were used for tuning the mass (5184- 3566), the calibration (5183-4682 and 8500-6942), and for the internal standard (5188-6525).

2.3. Quality control

Several parameters were assessed in order to ensure the reliability of the results. Calibration curves were considered to be linear when the coefficient of determination (R^2) was above 0.996. Limits of detection (LODs) and limits of quantifications (LOQs) were calculated for each element on several blanks based on the average blank value plus, respectively, three and ten times the standard deviation of blanks (Miller and Miller, 2005). Outliers were highlighted and set aside via the Grubbs' test (Miller and Miller, 2005). At least two blanks and two standard reference material (SRMs) replicates were included in each digestion batch. Different SRMs were used to ensure the validity of the digestion procedure and the ICP-MS analyses. They were selected according to their similarity with our sample matrices: Tea Leaves (GBW 08513), Wheat Flour (GBW 08503), and Water (GSBZ 50009-88) from the National Research Centre for Certified Reference Materials (NRC-CRM, Beijing, P.R. China); and Infant/Adult Nutritional Formula (1849a) from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). The SRMs were processed according to the supplier's instructions and analyzed following the same procedures as for the samples. Measurement of each element was performed as described above in at least 8 replicates. Dry matter content was determined. Statistical comparison between measured results and certified values was carried out according to an appropriate procedure (Linsinger, 2005). This procedure aims at comparing Δm – the difference between the certified value (C_{SRM}) and the mean measured value (C_m) – against the expanded uncertainty U_Δ. The expanded uncertainty U_Δ is calculated from the uncertainty of the certified value (u^2_{SRM}) and the uncertainty of the measurement result (u^2_m) according to: $U_\Delta = (\sqrt{(u^2_{SRM} + u^2_m)})*k$. The coverage factor k = 2 is applied to correspond to a confidence interval of approximately 95%. The uncertainties u_{SRM} and um are obtained by dividing the respective standard deviations by the square root of the number of measurements. There is no significant difference between the values of the SRMs and the measurement results when Δm is found to be lower or equal to U_Δ.

2.4. Statistics

All the statistics were calculated using the R software (v. 2.2.1) for Windows. The Shapiro–Wilk test was applied to detect possible non-normal distributions of the variables. When the statistical distribution was not normal, a logarithmic transformation of the variable was performed. The Levene test was applied to detect possible non-homogeneity of the variances. Mean values obtained for the analyzed minerals in the different foods were compared by analysis of variance (ANOVA) with two fixed factors, counties and communities, with 3 and 8 levels, respectively. The function aov of the package stats in R software (v 2.2.1) was applied (Chambers et al., 1992). In case of statistical significance, multiple comparisons with the Tukey test were then applied to identify means which differed from the others. One-sample t-tests were also applied to compare the mean minerals contents of the analyzed foods against food composition data from the China Food Composition Table (CFC) (National Institute of Nutrition, 2009), which is probably the most relevant for nutrition assessment in China. When a food item was missing a comparison was attempted with the most similar food item from the USDA Nutrient Database for Standard Reference (NDSR, V1.0, SR23) (US Department of Agriculture, 2010). A p-value lower than 0.05 was considered for assessing significant statistical effects.

3. Results and discussion

3.1. Quality control

The following values of LODs and LOQs, respectively, were expressed in mg kg^{-1}: Na: 206 and 557; K: 175 and 508; P: 56 and 156; Ca: 238 and 635; Mg: 19 and 49; Fe: 29 and 77; Mn: 0.28 and 0.72; Zn: 12 and 34; Cu: 0.6 and 1.7; Ni: 0.3 and 0.8; Se: 0.13 and 0.37; Al: 12 and 30; Mo: 3.3 and 9.2; V: 0.16 and 0.48; Cr: 0.6 and 1.5; Co: 0.04 and 0.10; As: 0.2 and 0.5; Cd: 0.04 and 0.13; and Pb: 0.3 and 0.82. All the coefficients of determination of the calibration curves were above 0.996. The results of SRM mineral determinations are presented in Table 1, which lists the certified values (C_{SRM}) and associated uncertainties (u_{SRM}) of the SRMs and mean measured values (C_m) and associated uncertainties (u_m) of the laboratory measurements. A statistical comparison was carried out following the appropriate procedure described in Section 2.3, ‘‘Quality control’’ (see above), and the results are presented in Table 2 (Linsinger, 2005). Except for Se in the tea leaves SRM (GBW 08513), Cu in Water (GSBZ 50009-88), and Mn in the wheat flour SRM (GBW 08503), results shown in Table 2 revealed all Dm values lower than UD values, indicating the appropriateness of the laboratory protocol and the accuracy of the results. A list of all the Tibetan foods analyzed in this study is presented in Table 3, which includes the English and Tibetan names in phonetic transcription with the corresponding Wylie transcription. The Wylie transliteration is a standard method for transliterating Tibetan script using only the letters available on a typical English language typewriter (Wylie, 1959). The results of the mineral analyses of the Tibetan traditional foods are listed in Tables 4–7. The data are expressed as mean values plus standard deviation on a fresh weight basis. The different foods were sorted in the tables according to their similarities. A comparison of the mean values, according to the geographical origin of the samples (two factors: Counties and Communities) revealed some significant differences. The factor county (3 levels: A: Lhunzhub; B: Maizhokungar; C: Nyêmo) was selected to summarize the data without losing too much information.

Table 1. Certified values (C_{SRM}) and associated uncertainties (u_{srm}) of the Standard Reference Materials and mean measured values (C_m) and associated uncertainties (u_m) of the laboratory measurements, expressed in mg/100 g[a] or µg/100 g[b]. The uncertainties are obtained by dividing the standard deviations by the square root of the number of measurements.

	Tea Leaves (GBW 08513) (n=23)		Wheat Flour (GBW 08503) (n=16)		Infant/Adult Nutritional Formula (NIST SRM 1849a) (n=8)		Water (GSBZ 50009-88) (n=10)	
	$C_{SRM} \pm u_{SRM}$	$C_m \pm u_m$	$C_{SRM} \pm u_{SRM}$	$C_m \pm u_m$	$C_{SRM} \pm u_{SRM}$	Cm ± um	$C_{SRM} \pm u_{SRM}$	$C_m \pm u_m$
Na[a]	13.9 ± 1.9	10.9 ± 0.7	10[c]	9.1 ± 0.5	4265 ± 42	4282 ± 12	ND	ND
K[a]	863 ± 63	884 ± 68	198 ± 14	198 ± 4	9220 ± 55	9930 ± 54	ND	ND
P[a]	148 ± 8	159 ± 15	150[c]	170 ± 4	ND	ND	ND	ND
Ca[a]	800 ± 66	863 ± 64	441 ± 22	465 ± 11	5253 ± 26	5261 ± 57	ND	ND
Mg[a]	276 ± 25	285 ± 2.6	551 ± 21	573 ± 13	1648 ± 18	1650 ± 8	ND	ND
Fe[a]	34.7 ± 1.2	32.9 ± 0.4	39.8 ± 8	40.1 ± 0.7	176 ± 1	175 ± 1	ND	ND
Zn[a]	2.26 ± 0.15	2.1 ± 0.06	2.27 ± 0.20	2.44 ± 0.79	151 ± 3	149 ± 3	5.05 ± 0.11	4.9 ± 0.02
Mn[b]	217 ± 11	228 ± 16	196 ± 10	220 ± 4	50 ± 0.5	50 ± 0.1	ND	ND
Cu[b]	896 ± 59	811 ± 43	440 ± 31	455 ± 5	20 ± 0.1	20 ± 0.2	1.02 ± 0.2	1.09 ± 0.5
Ni[b]	509 ± 76	512 ± 3.5	ND	ND	ND	ND	502 ± 11	521 ± 2
Se[b]	40 ± 7	93 ± 3	ND	ND	812 ± 1	816 ± 1	ND	ND
Mo[b]	180 ± 49	177 ± 3	220 ± 20	200 ± 5	ND	ND	ND	ND
Al[a]	2000[c]	2007 ± 30	ND	ND	ND	ND	503 ± 12.5	509 ± 2
As[b]	23 ± 4	29 ± 1	31 ±2	31 ± 2	ND	ND	102 ± 3	101 ± 0.6
Cr[b]	1000 ± 50	916 ± 10	350 ± 80	414 ± 22	ND	ND	1020 ± 20	1048 ± 3

[c] uncertified value. ND = not determinable due to absence of data for this element.

Table 2. Comparison of Δm, the differences between the certified values and the mean measured values, against the expanded uncertainties UΔ. The expanded uncertainty corresponds to a confidence interval of approximately 95% and is obtained by calculation of a combined uncertainty of results and certified values**.**

	Tea Leaves (GBW 08513) (n=23)		Wheat Flour (GBW 08503) (n=16)		Infant/Adult Nutritional Formula (NIST SRM 1849a) (n=8)		Water (GSBZ 50009-88) (n=10)	
	Δm	U_Δ	Δm	U_Δ	Δm	U_Δ	Δm	U_Δ
Na	3.0×10^{4}	3.8×10^{4}	ND	ND	17	85	ND	ND
K	2.1×10^{5}	12.7×10^{5}	2.7×10^{3}	292×10^{3}	110	154	ND	ND
P	1.1×10^{5}	1.6×10^{5}	ND	ND	ND	ND	ND	ND
Ca	6.4×10^{5}	13.3×10^{5}	2.4×10^{4}	4.9×10^{4}	8	107	ND	ND
Mg	8.5×10^{4}	50.3×10^{4}	2.2×10^{4}	4.9×10^{4}	2	38	ND	ND
Fe	1.8×10^{4}	2.5×10^{4}	0.3×10^{-3}	5.4×10^{-3}	0,5	3,1	ND	ND
Zn	2.0×10^{3}	3.2×10^{3}	1.7×10^{3}	4.3×10^{3}	2	6	201	225
Mn	1.1×10^{5}	2.2×10^{5}	2.4×10^{3}	2.2×10^{3}	0,7	1	ND	ND
Cu	0.9×10^{-3}	1.2×10^{-3}	146	628	0,1	0,5	68	41
Ni	34	1522	ND	ND	ND	ND	19	22.5
Se	60	15	ND	ND	4×10^{-3}	30×10^{-3}	ND	ND
As	3	98	20	41	ND	ND	ND	ND
Cr	7	ND	ND	ND	ND	ND	6	25.3
Cd	6	8	0,2	5,3	ND	ND	1	6
Pb	84	102	64	166	ND	ND	28	40

ND = not determinable due to absence of data for this element.

Table 3. List of the analyzed Tibetan foods, with their English names, and their Tibetan names in phonetic and standard Wylie transcription.

English name	Phonetic transcription	Wylie transcription
Water	Chu	chu
Concentrated brewed black tea (tea juice)	Jadang	ja dwangs
Homemade beer	Chang	chang
Roasted barley flour	*Tsampa*	rtsam pa
Wheat flour	Drozhip	gro zhib
Dried cheese	Churwakampo	phyur ba skam po
Dried yak meat	Yakshakampo	g.yag sha skam po
Dried mutton	Lukshakampo	lug sha skam po
Blood sausage	Gyuma	rgyu ma
Dried wild peaches	Kyurkhamkampo	skyur kham skam po
Dried Chinese radish	Gyalapkampo	rgya lab skam po
Dried rapeseed leaf	Pelokampo	Pad lo skam po
Dried Chinese radish leaf	Gyalaplomakampo	rgya lab lo ma skam po
Dried nettles	Zapokampo	zwa po skam po
Dried *Malva verticillata*	champahalo	lcam pa ha lo
Dried *Angelica sp.*	Chawa	lca ba

The Wylie transliteration is a method for transliterating Tibetan script using only the letters available on a typical English language typewriter (Wylie, 1959).

3.2. Mineral composition of beverages

Table 4 summarizes the mineral contents of liquids. As mentioned in Section 2.1, above, samples and sample preparation, the blanks were spiked with Micropure pills in order to assess matrix effects. Relatively high sodium concentration was found, which prevented a proper determination of Na in liquid samples. Although the concentrations were higher than Na concentrations of liquid samples, there were not out of the calibration range. Thus no particular interference is to be suspected. Water is not included in the CFC. However, the mineral content of the analyzed samples does not present significant difference with the item ‘‘Water, tap, drinking’’ from NDSR. Although river water remains a source of drinking water for some families, today more and more Tibetan households of rural areas around Lhasa benefit from tap water, ensuring a better quality of the drinking water.

Tea is essential in the Tibetan diet and culture. Due to its specific brewing method, it must be considered as an ingredient in its own. A whole tea brick (made of compressed black tea leaves) is generally brewed in several liters of boiling water for more than one hour. The resulting liquid, a kind of concentrated brewed black tea called ‘‘tea juice’’, was sampled and analyzed. To our knowledge, there is no equivalent of this beverage in any FCT. Subsequently diluted with water (from 10 to 30 times), tea juice is used to make not only traditional butter tea, but also salted black tea and other dishes. Given the concentrations listed in Table 4, and considering the dilution ratio, the tea juice food item is not likely to contribute significantly to mineral intakes. Exception can be made for manganese, which can be provided in substantial amounts by tea juice diluted even up to 30 times.

In Tibetan culture, a homemade beer called ''*chang*'' is commonly drunk even by young children. It can be made from different cereals such as rice, wheat, corn or barley. In the rural area around Lhasa, roasted barley grains are used as the main ingredient. They are fermented in boiled water with yeast for about two days. Despite the unusual process, no significant difference can be reported for Ca, Fe, Zn and Cu contents compared to the average Chinese beer composition (item 17-1-101 in the CFC). However, ''*chang*'' presents some significant discrepancies for other minerals. For example, the potassium content is significantly higher than the CFC value in the three investigated areas (A: $p < 0.001$; B: $p < 0.05$; C: $p < 0.001$). The same is observed for phosphorus, magnesium and manganese (A: $p < 0.001$; B: $p < 0.01$; C: $p < 0.001$ for the three elements). These differences may be explained by the fact that ''*chang*'' is consumed without settling yeast. On the other hand, selenium content is significantly lower than the CFC value ($p < 0.001$).

3.3. Mineral composition of staple foods

The mineral compositions of two Tibetan staple foods, namely ''*tsampa*'' which is a flour made of roasted barley grains, and wheat flour, are listed in Table 5. Wheat cultivation shows poor yields in the two counties of Lhunzhub (A) and Maizhokungar (B) because of the high altitudes. The wheat flour consumed in these areas is mostly imported from the lower regions of T.A.R or from mainland China. Wheat flour was thus sampled only in Nyêmo county.

Barley flour composition is not specified in the CFC although it is perhaps the most important food in the Tibetan diet. Moreover, the specific process of roasting the grains before milling may considerably affect the composition of the flour (Watzke, 1998; Lund, 2003; Cubadda et al., 2009). Thus these data might be of essential help for the assessment of mineral intake of Tibetan populations in rural areas around Lhasa. Compared to the ''barley flour or meal'' item from the NDSR, K, P, Ca, Fe, Mg, and Mn levels were significantly higher ($p < 0.001$) in our study. In addition, the selenium content was significantly lower ($p < 0.001$), which is most probably due to the soil Se deficiency in these zones (Li et al., 2009). The aluminum mean level can be considered as normal for this kind of food (European Food Safety Agency, 2008), although some samples present higher concentrations of up to 31 mg/100 g.The wheat flour samples in Nyêmo county showed lower levels of Na, Zn, Mn, Cu, and Se ($p < 0.001$) than the "wheat flour, standard grade" (01-1-201) from the CFC.

Table 4. Mineral and trace element concentrations (mean ± standard deviation) of liquid samples, expressed in mg/100 g[a] or µg/100 g[b].

	Water			Concentrated brewed black tea (tea juice)			"*Chang*" (homemade beer)		
	A (n=69)	B (n=25)	C (n=153)	A (n=69)	B (n=24)	C (n=106)	A (n=21)	B (n=8)	C (n=128)
K[a]	< 1	< 1	< 1	27.8 ± 16.9	38.1 ± 16.9	36.8 ± 19.6	82.3 ± 33.8	97.2 ± 45	76 ± 26
P[a]	< 1	< 1	< 1	3 ± 2	3 ± ± 1	3 ± 2	36 ± 14	56 ± 34	30 ± 10
Ca[a]	1 ± 0.5	2 ± 1	2 ± 0.5	2 ± 1	3 ± 1	2.5 ± 1	8 ± 4	11 ± 7	8 ± 2
Mg[a]	0.2 ± 0.1	0.4 ± 0.2	0.2 ± 0.1	3 ± 2	4 ± 2	3 ± 2	18 ± 6	22 ± 9	16 ± 5
Fe[a]	< LOD[1]	< LOD[1]	< LOD[1]	0.09 ± 0.08	0.12 ± 0.13	0.11 ± 15	0.40 ± 0.17	0.64 ± 0.35	0.28 ± 0.19
Zn[a]	0.01 ± 0.02	< LOQ[2]	< LOQ[2]	0.03 ± 0.03	0.02 ± 0.01	0.02 ± 0.03	0.26 ± 0.12	0.38 ± 0.31	0.20 ± 0.11
Mn[a]	<0.00	<0.00	<0.00	1.25 ± 1.02	1.77 ± 0.72	1.69 ± 1.06	0.19 ± 0.08	0.24 ± 0.16	0.14 ± 0.1
Cu[b]	2 ± 3	7 ± 21	5 ± 11	18 ± 29	24 ± 22	20 ± 23	20 ± 30	14 ± 7	30 ± 90
Ni[b]	< LOD[3]	0.1 ± 0.2	< LOD[3]	18.6 ± 12.5	26.5 ± 13.7	23.3 ± 14.0	1.4 ± 1.2	1.9 ± 1.3	1.1 ± 3.5
Se[b]	< LOD[4]	< LOD[4]	< LOD[4]	< LOD[4]	< LOD[4]	< LOQ[5]	0.22 ± 0.08	0.20 ± 0.12	< LOQ[5]
Mo[b]	< LOD[6]	< LOD[6]	< LOD[6]	< LOQ[7]	< LOD[6]	< LOQ[7]	6.7 ± 3.6	5.7 ± 3.8	11.8 ± 8.5
Al[a]	0.01 ± 0.02	0.02 ± 0.06	0.01 ± 0.03	2.5 ± 1.7	3.2 ± 1.7	3.3 ± 2.2	0.18 ± 0.15	0.16 ± 0.16	0.16 ± 0.23
As[b]	0.05 ± 0.06	0.06 ± 0.15	0.61 ± 1.56	1.2 ± 2.4	0.8 ± 0.5	1.8 ± 2.3	0.43 ± 0.14	0.53 ± 0.34	0.86 ± 1.30
Cr[b]	< LOD[8]	< LOD[8]	< LOD[8]	0.66 ± 1.03	0.26 ± 0.47	0.65 ± 1.01	1.13 ± 0.29	1.16 ± 0.49	0.53 ± 0.39
Co[b]	< LOD[9]	0.01 ± 0.04	< LOD[9]	0.72 ± 0.42	0.87 ± 0.51	0.75 ± 0.43	0.39 ± 0.18	0.42 ± 0.23	0.19 ± 0.39
Cd[b]	< LOD[10]	< LOD[10]	< LOD[10]	0.02 ± 0.03	0.05 ± 0.02	0.05 ± 0.04	0.03 ± 0.03	0.03 ± 02	0.02 ± 0.02
Pb[b]	< LOD[11]	< LOQ[12]	< LOD[11]	1.7 ± 2.8	1.5 ± 1.2	1.7 ± 1.9	0.54 ± 0.35	0.10 ± 0.14	0.37 ± 2.37
V[b]	< LOQ[13]	< LOQ[13]	0.44 ± 0.55	0.21 ± 0.24	0.23 ± 0.29	0.30 ± 0.41	0.31 ± 0.23	0.27 ± 0.16	0.41 ± 0.36

A: Lhunzhub ; B: Maizhokungar ; C: Nyêmo ; DM = dry matter ; [1] 0.003 mg/100 g; [2] 0.003 mg/100 g ; [3] 0.03 µg/100 g ; [4] 0.01 µg/100 g ; [5] 0.04 µg/100 g; [6] 0.3 µg/100 g; [7] 0.9 µg/100 g; [8] 0.06 µg/100 g; [9] 0.004 µg/100 g; [10] 0.004 µg/100 g; [11] 0.03 µg/100 g; [12] 0.08 µg/100 g; [13] 0.048 µg/100 g

Table 5. Mineral and trace element concentrations (mean ± standard deviation) of flours, expressed in mg/100 g^{a} or µg/100 g^{b} on a fresh weight basis.

	"*Tsampa*" (roasted barley flour, DM = 90.6%)			Wheat flour (DM=86%)
	A (n=69)	B (n=25)	C (n=144)	C (n= 19)
Na^{a}	11 ± 7	15 ± 7	14 ± 5	1.2 ± 1
K^{a}	608 ± 34	576 ± 52	555 ± 41	199 ± 64
P^{a}	385 ± 45	440 ± 66	369 ± 37	209 ± 90
Ca^{a}	49 ± 6	49 ± 7	49 ± 7	26.1 ± 7.1
Mg^{a}	126 ± 7	120 ± 15	119 ± 12	40.8 ± 19.5
Fe^{a}	9.14 ± 3.78	8.21 ± 1.83	9.14 ± 3.45	3.97 ± 1.67
Zn^{a}	2.26 ± 0.26	2.04 ± 0.30	2.12 ± 0.55	0.87 ± 0.39
Mn^{a}	1.53 ± 0.29	1.41 ± 0.15	1.73 ± 0.26	0.65 ± 0.33
Cu^{b}	349 ± 90	337 ± 76	415 ± 107	235 ± 76
Ni^{b}	9.7 ± 4	8.8 ± 3.2	7.4 ± 5.1	3.9 ± 2.2
Se^{b}	1.18 ± 1.42	0.84 ± 0.68	0.19 ± 0.32	0.39 ± 0.30
Mo^{b}	125 ± 36	106 ± 35	255 ± 187	99 ± 87
Al^{a}	5.1 ± 3.5	3.9 ± 1	6.4 ± 5.1	1.6 ± 0.7
As^{b}	6.8 ± 2.9	5.6 ± 2	8 ± 6.1	3.1 ± 1.5
Cr^{b}	7.7 ± 5.7	8.9 ± 4.1	9.3 ± 9.6	6.5 ± 3.2
Co^{b}	2.64 ± 1.64	2.44 ± 1.06	2.79 ± 1.32	1.45 ± 0.97
Cd^{b}	0.28 ± 0.12	0.31 ± 0.13	0.38 ± 0.59	0.45 ± 0.22
Pb^{b}	9.4 ± 9.1	10.5 ± 7	5.2 ± 7.4	2.5 ± 1.2
V^{b}	8.5 ± 6.6	8.3 ± 3.8	14 ± 8	4.4 ± 2.4

A: Lhunzhub ; B: Maizhokungar ; C: Nyêmo ; DM = dry matter

3.4. Mineral composition of dairy and meat products

In rural Tibet, cheese is traditionally made with the buttermilk left over from making butter. After boiling and cooling down, the curd is separated from the whey. The curd is then consumed as is ("*chura loenpa*"), or shaped into various forms and dried ("*chura kampo*") (Dorje, 1985). For reasons of perishability, the fresh curd was not sampled. With respect to "*chura kampo*", which we called "dried cheese" in Table 6, the item most resembling this in the CFC is "Cheese, milk lump, sour, dried" (10-4-004). Although similar in water content, the mineral profiles are highly dissimilar, especially for major elements such as Na, K, P, Ca, and Mg ($p < 0.001$). Indeed, the levels measured in the samples from Lhunzhub and Maizhokungar counties were up to two or three times higher than CFC values. It is worth noting that there are also significant differences between the counties. The mineral profile of dried cheese from Nyêmo county is significantly lower ($p < 0.001$) than in the two other counties for Na, K, P, Ca, Mg, Fe, Mo and Cd, while Cu and Mo levels are significantly higher. These differences may arise from a different milk composition or some differences in the production process. As a rule, selenium concentrations are particularly low in the three locations. Cu levels are also significantly low compared to the CFC value ($p < 0.001$). Aluminum mean levels may be considered as normal for this foodstuff, but some samples reached levels up to 20 mg/100 g, with a maximum of 64 mg/100 g.

Dried yak meat is eaten uncooked as a snack or added as an ingredient to a mixed dish. To the best of our knowledge, very little information, if any, exists about the mineral composition of dried yak meat. Because yak meat is a potentially important source of bioavailable minerals such as Fe or Cu, data about this food might be useful in assessing mineral intake in rural Tibet. However, dried yak meat is an expensive commodity, and very few samples were obtained from the families. The calculated variances are often high, probably due to the different cut of meat, fat content, or drying process involved in production. The same applies for dried mutton. Compared with “Mutton, dried” (8-3-306) from CFC, the mineral profile was not very different, and only Ca and Se were found to be significantly lower ($p < 0.01$ and $p < 0.05$, respectively). It is to be noted that the water content seems to be lower in Tibetan dried mutton than in the same CFC food item (5.5 g/100 g vs. 9.1 g/ 100 g). This could be explained by the very dry air due to high altitude.

Blood sausage is traditionally made from yak or sheep intestine filled with yak or sheep meat, fat, and blood. A little *tsampa* is generally added for texture, as along with salt and wild green onions – sometimes dried – for taste. Blood sausages are boiled and can be eaten as such or sun-dried before consumption. Table 6 shows the results for sun-dried blood sausage. Because of its particular composition, this food item is not really comparable to any other item in the usual FCTs. A high level of iron is worth noting. Furthermore, a substantial part of this iron must be in the form of heme iron, resulting in high iron bioavailability (Martin, 2000). Blood sausages also appear to be rich in copper and manganese, and molybdenum is particularly plentiful.

Table 6. Mineral and trace element concentrations (mean ± standard deviation) of dairy and meaty products, expressed in mg/100 g^{a} or µg/100 g^{b} on a fresh weight basis.

	Dried cheese (DM=88.9%)			Dried yak meat (DM=93.5%)			Dried mutton (DM=94.5%)	Blood sausage (DM=92%)
	A (n=21)	B (n=6)	C (n= 39)	A (n=9)	B (n=3)	C (n=3)	C (n=2)	C (n=2)
Na^{a}	271 ± 43	347 ± 151	136 ± 47	1195 ± 2370	1110 ± 1646	241 ± 141	184 ± 57	1122 ± 168
K^{a}	1101 ± 286	1156 ± 335	322 ± 66	710 ± 287	1240 ± 484	911 ± 553	703 ± 399	388 ± 3
P^{a}	1040 ± 84	977 ± 93	831 ± 67	483 ± 172	685 ± 305	497 ± 293	488 ± 351	317 ± 10
Ca^{a}	1278 ± 284	1435 ± 313	375 ± 80	33 ± 16	20 ± 7	18 ± 7	22 ± 1	63 ± 0.2
Mg^{a}	104 ± 22	115 ± 25	32 ± 8	53 ± 21	71 ± 32	54 ± 30	54 ± 42	92 ± 13
Fe^{a}	27.59 ± 21.81	21.32 ± 8.42	8.54 ± 11.20	13.64 ± 6.70	13.22 ± 6.80	15.24 ± 4.08	8.43 ± 2.94	53.7 ± 29.4
Zn^{a}	5.51 ± 3.24	5.29 ± 0.85	4 ± 3.78	11.48 ± 5.16	19.65 ± 13.82	9.42 ± 8.39	10.72 ± 6.06	2.07 ± 0.12
Mn^{a}	0.263 ± 0.108	0.223 ± 0.073	0.179 ± 0.203	0.202 ± 0.149	0.243 ± 0.294	0.170 ± 0.135	0.087 ± 0.051	1.441 ± 0.206
Cu^{b}	96 ± 64	64 ± 20	191 ± 106	276 ± 141	269 ± 113	214 ± 114	193 ± 88	331 ± 25
Ni^{b}	16 ± 6.7	12.1 ± 3.7	15.3 ± 15	12.2 ± 10.1	7.2 ± 4.6	7.9 ± 5.4	5.6 ± 3.5	20 ± 11
Se^{b}	3.7 ± 1.2	4.5 ± 1.5	6.2 ± 3.9	7.7 ± 2.4	6.7 ± 0.8	6.1 ± 2.6	3.9 ± 0.2	3.3 ± 0.8
Mo^{b}	22.2 ± 5.2	18.4 ± 6	44.5 ± 16.7	6.5 ± 5	2.8 ± 0.6	13.4 ± 5.6	17 ± 19	500 ± 104
Al^{a}	9.2 ± 5.9	3.8 ± 2.4	12.2 ± 11.3	3.9 ± 2.6	3.5 ± 4.1	4.5 ± 4.1	1.8 ± 1.9	9.7 ± 2.5
As^{b}	6.8 ± 3	5.1 ± 2.3	7 ± 10.4	10.3 ± 3.3	11.4 ± 7.21	21.7 ± 6.4	14 ± 11.7	9 ± 2.8
Cr^{b}	15.5 ± 5.9	12.8 ± 4.9	25.6 ± 21.4	19.8 ± 21	16.9 ± 16.4	12.7 ± 4.3	7.7 ± 5.1	28 ± 19
Co^{b}	4.5 ± 2.8	4 ± 1.7	3 ± 4.5	2.3 ± 1.2	2.4 ± 2.2	3 ± 2.1	1.9 ± 0.6	4.1 ± 0.3
Cd^{b}	0.40 ± 0.24	0.57 ± 0.23	0.17 ± 0.12	0.44 ± 0.23	0.97 ± 1.01	0.46 ± 0.03	0.28 ± 0.23	0.59 ± 0.13
Pb^{b}	7.1 ± 4.4	7 ± 6.7	10.9 ± 14.4	23 ± 29.6	27.3 ± 27.4	19.8 ± 10.7	4.8 ± 3.3	15.4 ± 5
V^{b}	6.8 ± 3.1	4.8 ± 2.4	12.8 ± 16.1	6.2 ± 3.7	7 ± 8	9.7 ± 9.4	3.6 ± 3.7	14.9 ± 2.2

A: Lhunzhub ; B: Maizhokungar ; C: Nyêmo ; DM = dry matter

3.5. Mineral composition of fruits, vegetables and edible wild herbs

Table 7 lists the mineral compositions of locally grown vegetables, fruits and edible wild herbs. The Tibetan dried wild peaches look nothing like the commercially available dry peaches. A whole wild peach is dried with its kernel, without any sugar added. It results in a very hard, small dry peach of about 6 g (including the kernel) that is eaten as a snack or a candy. The concentrations reported are related to the edible part, i.e. without the kernel. To our knowledge, there is no counterpart to this foodstuff in any FCT. The potassium content is remarkably high even for a dried fruit. The iron and copper contents are also relatively high but bioavailability might be low (Martin, 2000).

Chinese radishes are dried during summertime for preservation and then consumed during winter. This food item does not exist in the CFC, and compared to "Radishes, oriental, dried" in the NDSR, the composition differs significantly. In spite of similar water content (17.3 g/100 g for the Tibetan radish vs. 19.68 g/100 g for the NSDR Oriental radish) the K, Mg, and Cu concentrations are significantly lower in the measured samples ($p < 0.001$), while P, Fe and Mn concentrations are significantly higher ($p = 0.009$ for Fe and $p < 0.001$ for P and Mn). Surprisingly, the mean Se content is higher than the table value, but no significant difference is highlighted because of the high standard deviation. This standard deviation is due to two high values (10.9 and 8.6 mg/100 g). These values can be considered as outliers and excluded via the Grubbs test. The new calculated mean Se concentration would then be 1.45 ± 0.88 mg/100 g, which is significantly higher than the NSDR table value ($p = 0.014$).

Dried rapeseed leaves and dried Chinese radish leaves are sometimes eaten as vegetables in mixed dishes. These foodstuffs are particularly rich in calcium, magnesium, and iron, but their bioavailabilities are probably low, as often the case with leafy green vegetables (Martin, 2000; Uusiku et al., 2010). Dried rapeseed leaves seem also very rich in copper and molybdenum. Results for dried Chinese radish leaves need to be confirmed by further analysis as only one sample was obtained for this study.

Wild nettles are commonly eaten in rural Tibet either fresh or dried, in the form of a soup called "suptuk" (Malaisse et al., 2008b). Nettles are known to be rich in minerals (Rutto et al., 2013). Although fresh and dried nettles are consumed, only dried nettles were sampled in this study, for preservation issues. Fresh nettles are not included in the CFC but they are mentioned in the NSDR tables, while dried nettles are missing in any table. The mineral composition of dried nettles presented in the Table 7 might be of great use in the case of mineral intake assessment in rural Tibet, for it is very likely that their composition varies according to the soil composition. Dried nettles present an interesting Ca-to-P ratio (about 8) that may contribute to counterbalance an unfavorable ratio in a diet rich in cereals. They are also rich in trace elements such as iron, manganese, copper and nickel, but the high standard deviations suggest significant variability.

Several wild edible potherbs are used by Tibetans as aromatic herbs, spices or medicinal plants (Malaisse et al., 2008a). Two of them are listed in Table 7 namely "*champa halo*", namely *Malva verticillata*, and "*jia"*, or *Angelica sp.* A few dried samples were analyzed. These potherbs seem notable for their high content in Ca (with a high Ca-to-P ratio), Mg, Cu and Mo. Further investigation is needed to assess the beneficial impact of wild edible potherbs considering their chemical, nutritional and toxicological properties (Flyman and Afolayan, 2006). No selenium was detected in the leafy vegetables and the wild edible herbs listed in Table 7. This probably reflects the significant soil selenium deficiency in these regions of the Tibetan plateau (Li et al., 2009).

4. Conclusion

There is a growing need for relevant and accurate data for nutritional assessment all over the world. There are knowledge gaps especially for traditional foods in remote regions, where food originates from local agriculture and artisanal production. The need for food composition data is all the more important in that populations living in remote areas frequently suffer from dietary deficiencies. In this study we investigated the mineral composition of sixteen traditional foods and beverages of rural areas in the Lhasa (Tibet Autonomous Region) Prefecture. We highlighted significant discrepancies between the mean minerals contents of the analyzed foods and the food composition data from the China Food Composition Table. Furthermore, the mineral compositions of some food items had not yet been reported, to the best of our knowledge. We assume that these data might be very useful for nutritional assessment in rural central T.A.R. The data will undoubtedly become fully meaningful in the context of investigating the minerals deficiencies hypothesis as a possible factor of etiology for Kashin–Beck disease, which needs further study.

Table 7. Mineral and trace element concentrations (mean ± standard deviation) of fruits, vegetables, and edible wild herbs, expressed in mg/100 g[a] or µg/100 g[b] on a fresh weight basis.

	Dried wild peaches (DM=87.8%)	Dried Chinese radish (DM=82.8%)	Dried rapeseed leaf (DM=88.3%)	Dried Chinese radish leaf (DM=89.8%)	Dried nettles (DM=89.2%)		“*Champa halo*” (dried Malva verticillata, DM=90.2%)	“*Jia*” (dried Angelica sp., DM=90.3%)
	C (n=9)	C (n=12)	C (n=20)	C (n=1)	A (n=29)	B (n=10)	A (n=3)	A (n=1)
Na[a]	3.8 ± 3.9	214 ± 83	518 ± 399	1166	23 ± 23	27 ± 53	445 ± 394	1
K[a]	2579 ± 403	2477 ± 324	3046 ± 611	1882	3144 ± 958	2942 ± 942	3482 ± 739	5866
P[a]	134 ± 57	424 ± 79	348 ± 74	340	654 ± 152	558 ± 163	483 ± 73	748
Ca[a]	109 ± 27	602 ± 174	3974 ± 1283	3384	4569 ± 1782	5153 ± 1374	2659 ± 535	1651
Mg[a]	68 ± 20	110 ± 15	484 ± 107	468	565 ± 95	647 ± 75	371 ± 62	281
Fe[a]	25.96 ± 13.03	15.62 ± 10.35	32.51 ± 14.28	38.68	36.66 ± 24.12	33.40 ± 24.39	26.14 ± 5.64	24.5
Zn[a]	1.44 ± 2.74	2.14 ± 0.70	2.47 ± 0.529	3.39	4.25 ± 0.89	4.58 ± 1.16	6.07 ± 2.80	6.86
Mn[a]	1.03 ± 0.30	1.02 ± 0.28	6 ± 3.08	8.70	12.94 ± 10.99	12.73 ± 4.72	9.96 ± 5.02	5.76
Cu[b]	392 ± 115	224 ± 60	1028 ± 521	5485	846 ± 188	860 ± 248	788 ± 290	612
Ni[b]	86.9 ± 25.3	21.1 ± 11.4	37 ± 16.7	94.1	103.8 ± 49.4	123.5 ± 47.9	40.7 ± 20.2	40.6
Se[b]	1.2 ± 0.5	2.8 ± 3.4	< LOQ[1]	< LOD[2]	< LOD[2]	< LOD[2]	< LOD[2]	< LOD[2]
Mo[b]	40.5 ± 10.8	823 ± 664	1373 ± 1453	222	175 ± 258	220 ± 151	933 ± 619	616
Al[a]	20.5 ± 8	10.7 ± 10.8	30.3 ± 46.2	14.6	32.1 ± 24.3	32.1 ± 22.2	13 ± 6.2	14.9
As[b]	17 ± 6.8	35.3 ± 24.3	29.5 ± 37	< LOD[3]	18.4 ± 20.1	29.3 ± 27.1	18.1 ± 12.7	4.6
Cr[b]	45.7 ± 19	19.3 ± 9.5	47.2 ± 15.9	203.5	69 ± 42.3	72.7 ± 85.5	39.7 ± 25.9	34
Co[b]	12.4 ± 6.4	10.1 ± 4.2	21.3 ± 26	16.9	14.8 ± 9.3	16.6 ± 13.7	8.4 ± 5.9	7.3
Cd[b]	0.33 ± 0.14	8.63 ± 5	13.35 ± 12.19	25.8	8.87 ± 7.74	10.97 ± 5.86	32.5 ± 24.2	5.84
Pb[b]	26.3 ± 8.8	17.5 ± 18.2	0.3 ± 1.2	< LOD[4]	23 ± 60	61 ± 83	< LOD[4]	< LOD[4]
V[b]	42.1 ± 29.6	24.4 ± 17.9	79.8 ± 134.4	36.5	43.6 ± 37.2	47.8 ± 46.8	20.1 ± 13.2	19.2

A: Lhunzhub ; B: Maizhokungar ; C: Nyêmo ; DM = dry matter; [1] 0.04 µg/100 g; [2] 0.01 µg/100 g; [3] 0.02 µg/100 g; [4] 0.03 µg/100 g

Acknowledgments

This study was made possible thanks to the close collaboration and the support of the Center for Disease Control and Prevention of Tibet Autonomous Region, P.R. China.

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Chapter V.

"L'aliment n'est pas aliment s'il ne peut nourrir; ce qui n'est pas considéré comme aliment est aliment s'il est capable de nourrir"

Hippocrate

Introduction

In chapter III, we highlighted thirty elements impacting bone metabolism. We were able to immediately put aside seven elements, as they are not relevant to the present study. In chapter IV, we evaluated the relevance of the existing food composition tables for nutritional assessment in rural Tibet. We concluded that although of good quality, the values listed in the China and USDA tables were not close enough to the composition of Tibetan foods. Furthermore some food items specific to T.A.R. were not indexed. We therefore elaborated a specific table of mineral composition of staples and vegetables grown in T.A.R. This table also included traditional processed products such as beverages, various dried meat, dried cheese, and several dried vegetables and edible wild herbs.

At this stage, some more elements were excluded from the study for several reasons. Most of the elements exclusively toxic, such as Hg, Pb, Cd, and U were no more considered. The main reason is that symptoms related to their toxicity are often characteristics, and none have been identified in people suffering from KBD. Another reason is that although we analyzed them, the China and USDA FCTs do not include data with regards to these elements. For the same reason of scant data available, Al, As, B, Cr, Mo, Si, and V were discarded. The list of remaining elements to be investigated included: Na, K, Ca, P, Mg, Fe, Zn, Cu, Mn, and Se. Apart from aluminum, all are essential, in a certain range of intake, for a healthy bone and joint metabolism.

We had the tools and the targeted elements, only the design of the study was to be decided. In nutritional epidemiology, designs of study can be classified in two categories: observational and experimental studies (Willett, 1998). Only the first category is of interests to us, and it can be subdivided into three types: cross-sectional, case-control, and cohort studies. Cohort studies extend over a long time and are not relevant in this context. A case-control study, as indicated by its name, aims to compare a group of individuals with a specific characteristic, with a sample of the rest of the population. It is very practical, but presents some disadvantages, as it is a "retrospective" study. This means that we have to select children diagnosed for KBD without doubt, and constitute a control group of children absolutely disease free. Because the clinical symptoms of KBD are not always manifest at this early age (Mathieu et al., 2008), it would have been very difficult to clearly define the two groups within the targeted population.

A cross-sectional survey differs from case-control studies in that it aims to provide data on the entire population under study. It is then possible to compare people with, and without a disease in relation with some variable that are supposed to be associated with the disease. The disadvantage is that it is not possible to establish a cause and effect relationship between the variable and the disease, because it is not possible to be sure of what came first: the disease or the variable observed. A difficulty in cross-sectional study is to define the sample size. It has to be large enough to be representative of the targeted population. It is usually defined according to the study objectives using probability methods, defining the desired significance level and according to the expected variance of the results, which suppose previous data. In our case, due to the lack of existing data related to mineral intakes in these populations, the sample size has been defined according the best of our possibilities, and according to the agreement with local authorities. Hence, 250 preschool children aged 3 to 5 years old from three rural counties around Lhasa were interviewed twice, at six month of interval, via the 24-hour recall method. Although limited in time and space, the outcomes of this study provide an accurate insight into the dietary mineral status of young Tibetan children living rural areas endemic for KBD.

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Minerals and trace elements intakes and dietary patterns of young Tibetan children living in rural areas endemic for Kashin-Beck disease: a cross-sectional survey

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Adapted from the reference: Dermience, M., Mathieu, F., Li, X. W., Vandevijvered, S., Claus, W., De Maertelaere, V., Dufourny, G., Bing, L., Yangzom, D., Lognay, G. 2016. Minerals and trace elements intakes of young Tibetan children living in rural areas endemic for Kashin-Beck disease: a cross-sectional survey. In preparation.

Abstract

Objectives: Kashin-Beck disease (KBD) is an endemic and chronic osteochondropathy whose etiology remains unclear. There is evidence that environmental factors are involved, among which selenium and iodine deficiency are frequently cited. KBD only affects poor and rural populations. Diet is one important factor that differentiates these populations. This survey aimed to assess the daily intakes of ten minerals and trace elements (Na, K, Ca, P, Mg, Fe, Zn, Cu, Mn, and Se) involved in bone metabolism among young Tibetan children living in rural areas around Lhasa endemic for KBD.

Design: A cross-sectional survey was designed. The 24-hour food recall method was used to collect the intakes for two days, during two different seasons (September 2012 and April 2013). Because Tibetan foods are mainly derived from local agriculture and artisanal production, a specific food composition table was compiled.

Subjects: 250 pre-school children aged 3-5 years living in rural areas.

Results: The Chinese dietary recommended intakes are not met for most of the minerals investigated. Intake of sodium is much too high, while mean intakes are too low for K, Ca, Mg, Zn, Cu, and Se. Bioavailability of Ca, Fe, and Zn may be of concern due to the high phytic acid content in the diet.

Conclusion: These nutrient imbalances may impact growth and bone metabolism of young Tibetan children. The advantages of the implementation of food diversification programs are discussed as well as the relevance of supplements distribution. The relation between the observed imbalances and KBD deserves further investigation.

Keywords

24-hour food recall, cross-sectional survey, minerals, trace elements, dietary reference intakes, preschool children, Kashin-Beck disease, Tibet Autonomous Region

1. Introduction

In the Tibet Autonomous Region (T.A.R.) and in several western provinces of the People's Republic of China, the populations are affected by a little known disease called Kashin-Beck disease (KBD). Although its prevalence is decreasing in some provinces, it is estimated that KBD affects between 0.74 million and 2.5 million people in China and other parts of Asia, and between 10 and 30 million people are at risk (Yamamuro, 2001; Mathieu and Hinsenkamp, 2008). KBD is an endemic and chronic osteochondropathy characterized by short stature and skeletal deformities especially of long bones and joints. The pathophysiological feature of KBD is a focal chondronecrosis of mature chondrocytes in the deep zone of the growth plate cartilage and the articular cartilage (Mathieu and Hinsenkamp, 2008; Wang et al., 2008, 2009). Many studies have been conducted and many others are still underway. Yet, the etiology of the disease remains unclear. A multifactorial hypothesis that is currently proposed is one which involves selenium and iodine deficiency, combined with high concentration of organic matters in drinking water (fulvic acids), and mycotoxin poisoning by fungi infecting cereals (Chasseur et al., 2008a, 2008c; Haubruge et al., 2001; Li et al., 2009; Moreno-Reyes et al., 1998; Suetens et al., 2001; Yang et al., 1993). The implication of genetic factors that predispose to the disease cannot be excluded either (Shi et al., 2011; F. Zhang et al., 2015).

According to several authors (Haubruge et al., 2000; Malaisse et al., 2008a), Tibetan populations share four macro-ecosystems: i) the urban zones; ii) the suburban zones; iii) the agricultural zones; iv) the pastoral zones. KBD is only encountered in the agricultural group, and the endemic areas are limited to poor, isolated and rural communities. Diet is a major difference among these different groups. Several studies focusing on anthropometric measurements and nutritional status assessment have been conducted (Chasseur et al., 2008b; Goyens et al., 2008; Harris et al., 2001; Kolsteren et al., 1995). They invariably observed clinical signs of malnutrition and concluded on a poor nutritional status of the rural Tibetans, with a diet low in vegetables, meat and dairy products, but rich in cereals. A comparison of mineral intakes between endemic areas and non-endemic areas was undertaken in the Shanxi Province and Inner Mongolia Autonomous Region and some significant differences in mineral intake – mainly selenium – were highlighted (Liquiang et al., 1991). Nevertheless these results date back twenty years ago and these areas are quite far from T.A.R. Only one study conducted by Wang et al. (2010) was found to assess the mineral intakes of rural Tibetans. They investigated calcium, iron, and zinc intakes (among other nutrients) of rural Tibetan mothers with help of food frequency questionnaires and China food composition table (FCT). Although they did not confront their results to the Chinese Dietary Reference Intakes (DRIs), they concluded that the intakes were close to sufficient intakes compared with the *2002 Chinese National Nutrition and Health Survey*. Many studies can be found on the association between KBD and iodine and selenium deficiencies, but they are mostly prevalence studies, or supplementation clinical trials (Li et al., 2009; Moreno-Reyes et al., 2003; Tan et al., 2002a; Tan and Huang, 1991; Zhang et al., 2011). Although a strong negative correlation was established between selenium concentration in soil and prevalence of KBD, neither selenium nor iodine has been definitely established as cause of the disease. Iodine and selenium are important for a healthy growth but they are not the only elements playing a role in bone and joints metabolism. Several minerals are intimately related to bone health (Dermience et al., 2015). Some major elements are required in substantial amounts, while others trace elements are needed in very small quantities, but they are not less essential to a healthy bone metabolism.

The main objective of this study is to assess the daily intakes for ten minerals and trace elements of dietary relevance (Na, K, Ca, P, Mg, Fe, Zn, Cu, Mn, and Se) among preschool children living in rural areas endemic for KBD and to compare them against the Chinese DRIs. To our knowledge, it is the first time that mineral and trace element intakes have been assessed by the 24-hour food recall method in young Tibetan children living in areas endemic for KBD. Because a previous study highlighted significant discrepancies between minerals concentration in the China FCT and in local Tibetan foods (China CDC, 2009; Dermience et al., 2013), and traditional Tibetan foods are missing, a unique food composition table was compiled from local foods collected and analyzed (Dermience et al., 2014) together with data from the China FCT and from the USDA Food search for Windows (US Departement of Agriculture, 2010). The results of this study will provide an overview of the dietary mineral status of the Tibetan children and could give some leads to further investigate the etiology of KBD. It will also help guiding the ongoing strategies set up by the government and by non-governmental organizations to improve the nutritional quality of the rural Tibetan diet.

2. Material and methods

2.1 Participants and ethical considerations

For this study, 250 preschool children aged 3 to 5 years old from 8 communities – Nimashangri, Nyanang, Thanggo, Theumba, Paco, Nyemo, Tharong, and Shume – located in 3 counties – Lhünzhub, Maizhokungar, and Nyêmo – of the Lhasa prefecture were randomly selected among the civil registries owned by the community leaders, proportionally to the size of the targeted population of each county.

2.2 Design of the study

As it is quantitative and implementation is achievable considering field constraints, the 24-hour food recall method has been chosen for this study. Two climatic seasons can be identified in this part of the Tibetan plateau (Malaisse et al., 2008a), and two dietary patterns intimately related to the seasonal variations can be observed. While fruits and vegetables are more available during summertime (May-October), an increased consumption of meat and dairy products is characteristic of wintertime (November-April). Given these information, two rounds of investigation have been conducted, the first one in September 2012, and the second one in April 2013. With a few exceptions, the same 250 children were interviewed for one day of food recall for each season. All days of the week were equally represented in the final sample.

The care-takers of the children were interviewed by six interviewers. The interviewers were local people, with a high educational level, fluent in local language and in English. They underwent an intensive two-week training inspired by the training program of Gibson & Ferguson (2008). During the campaigns, they followed a three-day workflow pattern. The first day, the interviewers met the selected children and their care-taker. They explained the study and gave them a picture chart depicting the foods most often eaten in rural Tibet. The second day, the caretakers were asked to mark the chart to indicate the foods consumed by the children in order to facilitate the recall on the next day. The third day, the caretakers and the children were interviewed together. Children were also weighed and measured. In a purpose to recall children's exact food intake during the preceding day, the interviewers implemented a four-stage, multiple-pass interviewing method described by Gibson & Ferguson (2008). An indirect approach has been recommended, and leading questions and judgmental comments were avoided. Quantities of foods consumed were estimated principally by measuring household utensils (with water and graduated cylinder), by direct weighing, by modeling play dough molded into the correct size and shape of the food, or by using measured standard weight of food items. The interview protocol was standardized and was tested prior to the study. Adherence to the interview protocol and accuracy of food coding was checked by the field supervisor.

2.3 Food composition data

The choice of a specific FCT is critical to achieve a good quality of results in nutritional assessment, especially when dealing with mineral and trace elements (Gibson and Ferguson, 2008). The China Food Composition Table (2nd edition, 2009)(China CDC, 2009) appears to be the most appropriate for this study as it is the national FCTs. Moreover, it scored a quality index of 2 on 3, according to the ratings for quality control criteria of Gibson & Ferguson (2008), reflecting a good reliability of food composition values. However, for the reasons mentioned in the introduction, the China FCT was not enough and was completed by food composition data from Dermience et al. (2014)(Dermience et al., 2014), from the USDA Food search for Windows (US Departement of

Agriculture, 2010), and from product labels. The relative percentages of foods from the different tables were 56, 29.3, 13.3, and 1.4% respectively.

2.4 Converting portion sizes to weight equivalents

Several procedures were used in order to convert the portion sizes into weight equivalents: i) direct weighing of actual food or replicas by interviewers, using dietary scales; ii) recording the volume of water that is equivalent to the volume of food and beverage; iii) recording household measures volumes. The recorded volumes were converted into weight equivalents by: i) using published specific gravity data (if available); ii) compile specific gravity data. For this purpose, five to eight samples of the desired food product were purchased and weighed. Then the volumes were determined by water displacement, and specific gravities (g/ml) were calculated.

2.5 Calculation of dietary intake

Online software called *Planning Alimentaire Nubel PRO* was used to compute mineral and trace element intakes. Two days of daily intakes per child were computed for the following nutrients: energy (Kcal), water (g), sodium (mg), potassium (mg), calcium (mg), phosphorus (mg), magnesium (mg), iron (mg), copper (mg), zinc (mg), manganese (mg), and selenium (µg).

2.6 Statistical analysis

All the statistics were calculated using the SPSS software (v. 22.0) and Minitab 15 for Windows. A p-value lower than 0.05 was considered for assessing significant statistical effects. In accordance with the central limit theorem and considering the important sample size, assessing the normality of the distributions was not mandatory. The Levene test was applied to detect possible non-homogeneity of the variances. Mean daily intakes between counties (3 levels) and seasons (2 levels) were compared by analysis of variance (ANOVA with two fixed factors), in order to have a general overview of the significant differences and to assess the interactions. Subsequent one fixed factor ANOVA analyses were performed for each season to compare the mean daily intakes between the counties. In case of statistical significance, multiple comparisons tests were applied to identify means which differed from the others. In case of homoscedasticity, the Tukey test was used. In case of non-homogeneity of the variances, the Dunnett's T3 test was used. Usual intakes for the group have been computed with the IMAPP software (Carriquiry *et al.*, 1999) and have been compared to the Estimated Average Requirements (EARs) and Upper intake levels (ULs) to assess the risks of inadequate intakes.

3. Results and discussion

3.1 Dietary pattern

Table 1 shows the relative consumptions, in percentages, of different foodstuff across the three counties and the two seasons investigated. These data confirms the still very traditional character of the rural Tibetan diet, but it also gives a glimpse of some secondary influence of the significant growth of the infrastructures in T.A.R. The main beverage is the famous yak butter tea, which is made of yak butter, tea juice or concentrated brewed black tea (Dermience et al., 2014), and salt, all mixed together with boiled water. It appears that margarine is mixed with yak butter to prepare the butter tea in 4% to 56% of the families depending on the county (table 1). The reason is that margarine is quite cheap even for rural people, while yak butter is much more valuable and is often sold in the cities to generate income. Apart from yak butter tea, black tea and sweet tea are frequently consumed. Black tea is made of black tea leaves brewed with hot water and salt. Sweet tea is made of milk powder, sugar, tea leaves, and hot water. Besides, a significant percentage of the children, up to 30-50% in Nyêmo County, consumed a homemade barley beer called *chang*. Parents are probably not aware of the harmful effects of alcohol on the children's brain (Jacobus and Tapert, 2013; Nixon, 2013; Squeglia et al., 2014), and *chang* is given to the children to warm them up and maybe to calm them when excited. To the best of our knowledge, the consumption of soda drink by rural people of T.A.R. was never reported before in the literature. Here we can notice that sodas are part of the diet for a considerable number of children. This new habit of consumption may be correlated to the recent development of infrastructures in T.A.R., which results in easier access to various consumer goods (Popkin, 1999).

Tsampa is the Tibetan name of the flour obtained from roasted barley grains. It is the main staple with 66 to 85% of the children consuming it. If it is frequently eaten under the form of *tsamtuk*, a kind of soup sprinkled with *tsampa*, it is above all consumed daily under the form of *pag*. In this traditional recipe, *tsampa* is mixed with yak butter tea and eaten as such, without cooking. Sugar, butter or dried cheese depending on availability and tastes may also be added. Although not locally grown, rice is well established in the food habits with remarkable consistency throughout the counties and the seasons. Wheat flour is also frequently consumed, mostly under the form of a traditional Tibetan noodle soup called *thukpa*, or under the form of *momo*, another traditional Tibetan dish. The latter can be plain or stuffed, steamed or fried, and depending of the amount of water incorporated into the dough, it may be compared to a bun or to a kind of ravioli. Potatoes are widely consumed by the rural Tibetan children, who eat them either as a snack between meals, or as vegetable in a dish.

The consumption of meat is generally low across all counties. Yak meat is the most consumed. Yet, according to local people, purebred yak have become rare in the region. Most of the livestock is the result of hybridization between a bull and a female yak, which is called *dzo* (Malaisse et al., 2008a). A remarkably constant consumption of pork meat throughout seasons and counties is noticeable. Contrary to yaks, goats, or mutton, raising pigs is not common in these rural areas. The consumption of pork is maybe also a consequence of the infrastructural development, though pigs are also historically brought from Russia (Daniggelis, 1995). The consumption of chicken meat is very low because of cultural reasons discussed below.

Yak or cow butter represents the main source of lipids. It is principally consumed in the butter tea, and it is more and more frequently mixed with margarine. Yak and cow fats are collected and dried when animals are slaughtered, and it is used for flavoring some traditional dishes (stuffed *momo*, *tsamtuk*¸*thukpa*, and *suptuk*, a nettle soup). Because it is locally produced, rapeseed oil is almost the only vegetable oil available, and is traditionally used for cooking meat and vegetables, or for frying bread. Dairy products are generally integral part of the diet of mountain people. Nevertheless, the children's consumption seems relatively low in the investigated areas. Less than 50% of the children have been drinking milk during the day of recall. Furthermore, these figures several children who only consumed small amounts of milk powder, which is used to make sweet. Dried cheese, which is called *chura kampo*, is made of dried curd (Dorje, 1985). It is also a very traditional food in rural Tibet. Very hard, it is chewed during hours as a snack. The consumption of other dairy products like yogurt and *chura loenpa*, a kind of cottage cheese, is anecdotal.

Very few vegetable enter the dietary pattern of the rural children. Chinese cabbages (*Brassica rapa*, subspecies *pekinensis* and *chinensis*), Chinese radish (daikon), and green onions (scallions) are by far the most consumed vegetables. Wild nettles are sometimes eaten either fresh or dried in a traditional dish called *suptuk* or *satuk* (Dorje, 1985; Malaisse et al., 2008c), which has a consistency between the soup and puree, depending on the addition of *tsampa* or not. It can include yak meat or fat and salt. However, the consumption of nettles tends to decrease since it is associated with a low socio-cultural level. Other vegetables, such as tomato, cucumber, and zucchini are seldom consumed.. Several edible mushrooms are recognized by people (Malaisse et al., 2008b), but few seem to have been consumed during the study, perhaps because it was not the season. Except for apples, eating fruits is also very uncommon in the investigated areas. The reason of the poor diversity of vegetables and fruit consumption probably lies in the harsh climate of the Himalayan plateau which does not allow their cultivation (Chasseur et al., 2008b; Malaisse et al., 2008a), and in the lack of incomes which prevents the rural people to buy imported foods.

Table 1. Relative consumption (%) for different foodstuffs across regions and seasons

	September 2012			**April 2013**		
Foodstuff	**L (n=70)**	**M (n=27)**	**N (n=153)**	**L (n=70)**	**M (n=27)**	**N (n=153)**
Beverages						
Butter tea	69	93	82	83	96	82
Black tea	16	4	8	36	11	12
Sweet tea	1	0	16	7	15	12
Chang (local beer)	16	4	34	14	4	52
Various soft drink	24	56	15	37	26	10
Staples						
Tsampa	66	78	80	74	85	78
Rice	50	52	56	50	59	57
Wheat flour	29	33	41	67	22	42
Wheat noodle (fresh or dried)	6	15	13	13	22	15
Potato	33	37	59	43	37	58
Meat						
Dried yak	5.7	3.7	2.0	7.1	7.4	9.2
Fresh yak/beef	21	37	11	43	41	20
Mutton/goat	1.4	7.4	24	2.9	0.0	13
Pork	10	11	11	11	11	7.8
Chicken	2.9	0.0	2.0	4.3	3.7	2.0
Egg	5.7	15	9.2	2.9	3.7	6.5
Fats						
Yak fat	11	7	23	20	19	29
Butter	77	93	86	89	96	84
Margarin	4	11	5	36	56	42
Rapeseed oil	46	56	50	59	37	58
Dairy products						
Milk (fresh or powder)	53	44	42	40	41	41
Dried cheese	20	56	48	53	59	29
Cottage cheese	1.4	3.7	3.9	1.4	0.0	5.9
Yogurt	13	33	6.5	1.4	11	3.9
Vegetables						
Cabbages (all included)	19	33	17	23	3.7	17
Chinese radish (daikon)	10	15	25	20	19	26
Onion (scallion)	11	11	8.5	10	11	15
Green chili	0.0	0.0	3.9	0.0	0.0	0.7
Tomato	0.0	7.4	1.3	0.0	0.0	0.0
Cucumber	7.1	3.7	0.0	0.0	0.0	0.0
Zucchini	4.3	3.7	0.7	0.0	3.7	0.0
Spinach	1.4	0.0	0.7	1.4	0.0	0.7
Cauliflower	1.4	0.0	0.7	1.4	0.0	0.7
Carrot	0.0	0.0	0.7	0.0	3.7	2.0
Asparagus lettuce	2.9	0.0	0.0	1.4	7.4	0.7
Nettles	4.3	0.0	0.7	14.3	0.0	2.6

Table 1. (continued)

	September 2012			April 2013		
Foodstuff	**L (n=70)**	**M (n=27)**	**N (n=153)**	**L (n=70)**	**M (n=27)**	**N (n=153)**
Mushroom	0.0	0.0	0.7	0.0	7.4	0.0
Fruits						
Apple	17	11	13	13	0	8.5
Peach (fresh or dried)	1.4	0.0	11	2.9	0.0	2.0
Orange	0.0	0.0	0.7	0.0	0.0	0.0
Pear	0.0	0.0	0.0	0.0	0.0	1.3
Hawthorn	1.4	0.0	0.0	0.0	0.0	0.0
Watermelon	0.0	3.7	0.0	0.0	0.0	0.0
Banana	1.4	7.4	0.7	4.3	7.4	0.7
Plum	0.0	0.0	0.0	1.4	0.0	2.0
Various						
Salt (added)	71	85	88	97	89	95
MSG (sodium glutamate)	5.7	7.4	29	5.7	3.7	37
Instant noodle	30	41	37	37	30	37
Chips	0.0	0.0	0.7	1.4	3.7	1.3
Mixed candy	33	15	21	54	26	35
Chocolate	0.0	0.0	2.0	1.4	11	0.0
Biscuit and cake	21	15	14	24	19	28

The relative consumption is calculated based on the number of children who consumed a given foodstuff divided by the total number of children enrolled in the county, expressed in percentage. The letters L, M, and N stand for Lhünzhub, Maizhokungar, and Nyêmo respectively.

A new category of food products has appeared in the Tibetan dietary pattern. Industrialized foods may be the flip side of the coin of the better accessibility to more diversified food products thanks to the infrastructural development. A relative high proportion of children consumed chips, candies, chocolate, biscuit, and cakes during the days of recall. It was also noticed that around 30 to 40% of the children ate instant noodles either as a meal or as a snack. This phenomenon rises some concerns because if industrialized foods – which are sometimes of poor nutritional value and rich in salt – are already a problem in many developed countries, they may have an even greater negative impact on populations with low incomes and low educational level like rural Tibetans (Du et al., 2004; Tzioumis and Adair, 2014).

3.2 Dietary Intakes

The descriptive statistics by counties are presented in table 2. For both seasons (identified by the number following the variable: “1” for September 2012, and “2” for April 2013), the number of data (N), the mean value and the standard deviation are presented for several variables. They include: age, height, weight, energy, water and alcohol intakes, as well as the mean intakes of the investigated minerals.

Table 3 shows the p-values of the ANOVA with two fixed factors. While few informative significant differences are highlighted for the season factor, two thirds of the variables present a p-value below 0.05 for the county factor. There are also significant interactions reflecting an interesting trend. The mean intakes of energy, water, and manganese tend to increase from September 2012 to April 2013 in Lhünzhub and Nyêmo, while it decreases in Maizhokungar. On the other hand, the mean intakes of potassium, magnesium, and selenium tend to decrease from September 2012 to April 2013 in Lhünzhub and Nyêmo, while it increases in Maizhokungar. A possible explanation to this tendency may lie in different dietary habits according to the season between the counties.

In order to highlight the significant differences between the three counties, ANOVAs with one fixed factor (counties) were performed on every variable for both seasons, followed by multiple comparisons (results not shown). In September 2012, several significant differences were observed between Lhünzhub and Nyêmo counties for the following variables: energy, water, Mg, Fe, Zn, Mn, and Se. There is also a significant difference between Maizhokungar and Nyêmo counties for the weight, and the water, Zn, and Mn intakes. In April 2013, Cu and Se intakes differ significantly between Lhünzhub and Nyêmo counties. Energy and Se intakes differ significantly between Maizhokungar and Nyêmo counties. These recurrent differences between Nyêmo and the two others counties could find its roots in its location. Lhünzhub and Maizhokungar are located at the north and north-east of Lhasa with relatively difficult access, while Nyêmo is at the south-west with easier access from Lhasa.

Table 2. Descriptive statistics by county

		Lhünzhub (n = 70)		Maizhokungar (n = 27)		Nyêmo (n = 153)	
	Period	Mean	SD	Mean	SD	Mean	SD
Age	1[a]	4.0	0.8	4.0	0.7	4.0	0.8
	2	4.5	0.7	4.6	0.8	4.8	0.9
Weight	1[b]	14.3	2.6	15.1	2.5	13.6	2.5
	2	16.4	3.6	16.5	2.6	15.4	2.6
Height	1[b]	97	9	94	9	95	8
	2[c]	103	10	102	7	102	8
Energy	1	1026	428	995	535	848	455
	2	1074	509	791	320	956	441
Water	1	811	386	798	334	606	354
	2	857	397	705	354	792	350
Alcohol	1[d]	2.6	1.5	1.5	-	1.9	1.9
	2[e]	3.5	2.7	4.4	-	6.8	5.0
Na	1	1945	1152	1739	944	1580	1327
	2	1838	1366	1361	818	1432	1337
K	1	998	571	981	511	821	533
	2	933	436	813	552	936	497
Ca	1	361	314	394	252	251	374
	2	330	276	266	158	269	235
P	1	575	306	588	272	487	387
	2	564	295	533	348	555	314
Mg	1	157	71	143	72	116	57
	2	156	70	130	89	154	75
Fe	1	14.7	10.1	12.0	6.0	11.2	10.4
	2	13.7	6.2	10.5	7.0	12.1	7.0
Zn	1	4.9	2.2	5.2	4.2	3.6	2.2
	2	5.0	2.5	4.7	3.4	4.2	2.1
Cu	1	0.67	0.28	0.72	0.37	0.79	1.10
	2	0.77	0.47	0.80	0.98	1.14	0.75
Mn	1	2.47	1.06	2.29	1.04	1.68	0.70
	2	2.85	1.50	2.00	1.06	2.29	1.08
Se	1	12.5	8.2	13.6	12.2	9.1	9.3
	2	12.3	8.8	8.9	7.2	10.6	10.0

Period 1 correspond to September 2012, period 2 correspond to April 2013. SD = standard deviation. [a] n = 152 for Nyêmo; [b] n = 148 for Nyêmo; [c] n = 69 for Lhünzhub; [d] n = 11, 1, and 52 for Lhünzhub, Maizhokungar, and Nyêmo respectively; [e] n = 10, 1, and 80 for Lhünzhub, Maizhokungar, and Nyêmo respectively. Age is expressed in year, weight in kg, height in cm, energy in Kcal, water and alcohol in g, and all elements in mg, except selenium which is expressed in µg.

Table 3. P-values of ANOVA with two fixed factors, counties (3 levels), and seasons (2 levels)

	Season	County	Season * County
Age (y)	<0.001	0.265	<0.001
Weight (kg)	<0.001	0.005	0.442
Height (cm)	<0.001	0.449	0.720
Energy (kcal)	0.735	0.011	0.038
Water (g)	0.224	0.004	0.007
Na (mg)	0.107	0.024	0.753
K (mg)	0.430	0.339	0.030
Ca (mg)	0.138	0.021	0.187
P (mg)	0.984	0.383	0.241
Mg (mg)	0.269	0.022	0.001
Fe (mg)	0.576	0.018	0.365
Cu (mg)	0.042	0.013	0.202
Zn (mg)	0.823	<0.001	0.117
Mn (mg)	0.036	<.001	0.006
Se (µg)	0.227	0.052	0.044

Even when significant differences are present between the counties, all the mean intakes are often far from the Chinese DRIs. Thus, the comparisons with the Chinese recommendations were performed considering the overall mean intakes, calculated upon the three counties and for both seasons. Table 4 presents the Chinese DRIs, the harmonized Estimated Average Requirements (hEAR), the Upper intake levels (UL), usual intakes, intakes at 5th and 95th percentiles, and risks of inadequate intakes. Except for Chinese DRIs, the other values have been obtained or computed with IMAPP software. All the children were considered as belonging to the category of age between 4 and 8 years old.

With 66% of the recommended nutrient intake (RNI), the usual intake of these Tibetan children is too low in calories. Although the energy required for growth is estimated to account for 3% of the total energy requirement (Institute of Medicine, 2005b), such low intakes may not be without consequences on the growth of these children. Not surprisingly, it has been reported that the weight-for-age and height-for-age P50 growth curves of Tibetan children are below those of the World Health Organization (Rooze et al., 2012). Another aspect of the energy requirement should be raised. Because people generally adapt their clothing and their environment to maintain thermoneutrality, there is no need of additional energy intake for thermoregulation (Institute of Medicine, 2005b). However, it is possible that in the context of the very low temperatures of the Tibetan plateau and the scarce resources for heating and cooking, an increased need of energy for thermoregulation purposes becomes a relevant question. Another possible source of underestimation of the RNI is the physical activity level (PAL) of the children. Although it has neither been measured in this study, nor has it ever been reported to the best of our knowledge, several studies revealed a high exercise capacity of the Tibetan children (Bianba et al., 2014; Curran et al., 1998). Anyway, this energy deficit most probably results in underweight, growth retardation, and stunting, which are widely observed among Tibetan children (Dang et al., 2004; Harris et al., 2001; Rooze et al., 2012).

Table 4. Chinese DRIs (China CDC, 2009), hEARs, ULs, usual intakes, and risks of inadequate intakes of the rural Tibetan children (n = 250, mean for: age = 4.4 y.o.; weight = 14.9 kg; height = 99 cm)

	Chinese AI/*RNI*	hEAR	UL	Usual intake	Intake 5th	Intake 95 th	% of inadequacy	% > UL
Energy (kcal)	*1430*[*]	ND	ND	943	389	1795	ND	ND
Water (g)	-	1700	ND	742	229	1436	98.3	ND
Alcohol (g)	-	ND	ND	4.6[†]	ND	ND	ND	ND
Na (mg)	900	900	1900	1619	375	3565	26.3	27.6
K (mg)	1500	3800	ND	910	310	1880	99.9	ND
Ca (mg)	800	640	2500	292	48.7	784	92,0	0.2
P (mg)	500	405	3000	539	197	1145	40.7	0,0
Mg (mg)	150	110	ND	141	58.6	278	39.5	ND
Fe (mg)	12	ND	40	12.3	4.6	26.8	ND	0,0
Zn (mg)	*12*	4.0	12	4.3	1.6	9	55.5	1.5
Cu (mg)	1.00	3.4	30	0.87	ND	ND	ND	ND
Mn (mg)	-	1.2	3	2.19	0.87	4.31	15.9	18.8
Se (µg)	*25*	23	150	10.72	ND	ND	ND	ND

Chinese recommended nutrient intakes (RNIs) are in italic type and adequate intakes (AIs) are in ordinary type. [*] RNI for energy is the mean RNI for children from 3 to 5 years old, both gender included. Harmonized Estimated Average Requirements (hEAR), Upper intake levels (UL), Usual intakes, Intakes at 5th and 95th percentils, and risks of inadequate intakes have been computed with IMAPP software. ND = not determined. [†] Consumption data for alcohol corresponds to mean intake and not usual intakes.

No recommendation for water intakes is listed in the Chinese DRIs (China CDC, 2009). The US Institute of Medicine recommends an intake of total water of 1.7 L/day for children aged 4-8 years

(Institute of Medicine, 2005a). With a usual intake of 0.74 L/day, which represents 45% of the adequate intakes (AI), more than 98% of the children present inadequate intakes. Even if the metabolism of the Tibetan population may be adapted to their harsh environment, a so low intake of water is susceptible, on the long term, to lead to severe dehydration with deleterious consequence on the metabolism. Traditionally, Tibetan peoples drink tea or homemade beer (*chang*) but little water. Until recently, the main sources of water for the population used to be well water or surface water (Zhang et al., 2011), with all the risks related to chemical, microbial, and biological contaminants. During the study, only 5% and 12% of the children consumed river water (used in the preparation of tea drinks or soup) in September 2012 and April 2013 respectively. Most of the enrolled families have access to tap water which is expected to improve their water potability criteria. Hence, a higher consumption of water should be encouraged among the rural Tibetans.

Alcohol is known to have deleterious effects on adolescent's brain (Hiller--Sturmhöfel and Swartzwelder, 2004; Jacobus and Tapert, 2013; Zeigler et al., 2005). In Western cultures, the age of first drinking in a parentally supervised environment is usually around 13 to 14 years old (Newbury-Birch et al., 2009). If it has been reported that 3-4 year old children could be able to recognize alcoholic drinks (Lang and Stritzke, 1993), it is uncommon to report alcohol consumption. With as much as 50% of the children having drinking alcohol in Nyêmo county during the recalls of April 2013, and a mean consumption of 6.8 g (table 3), it cannot be without consequences on these children's health. In addition to the adverse physical consequences of early alcohol consumption, there is a risk of a vicious circle involving an increase in consumption with age and a repeat of the educational scheme (Newbury-Birch et al., 2009). This is a potential public health problem that must be fought through education (Guo et al., 2008; Schnohr et al., 2004; Tang et al., 2013). Although it has never been reported nor denied in T.A.R., alcohol consumption by pregnant mothers might also be a problem. Indeed, *in utero* alcohol exposure is susceptible to cause, *inter alia*, developmental abnormalities, growth retardation and abnormal gene expression (Abbott et al., 2016). The question of alcohol consumption by Tibetan women during pregnancy in T.A.R. deserves further attention.

Usual intake for sodium in this study represents 180% of the Chinese AI. No upper limit (UL) is entered in the Chinese table for sodium, while the Institute of Medicine set up the UL at 1900 mg/day for a 4-8 year child. More than 25% of the children exceed the UL, and the 95th percentile is above 3500 mg/day. Besides the traditional foods, the high sodium intakes observed comes mainly from two sources. The first is the added salt. In the recalls of September 2012, 83% of the children had salt added in their meals. In April 2013, it concerns 95% of the children. The problem comes from the amount of salt added. It was common to observe during the study some families adding up to 20 to 30 g of salt in the cooking pot. Another important source of sodium intake arises from the consumption of instant noodles. As seen in table 1, from 30 to 40% of the children consumed at least one serving size of instant noodles during the study, and it was not uncommon for some to have two. A classic serving size is about 77 g. According to the China FCT, it represents an intake of 880 g of sodium, which consist in 98% of the Chinese AI. Overconsumption of sodium is a global health issue (World Health Organization, 2007), and with his fast economic growth, its relentless urbanization, and a shift in its traditional diet, China is particularly exposed to this problem (Zhai et al., 2014; R. Zhang et al., 2015). Experiencing the development of the modern infrastructures, with new access to industrial foods, combined with low education levels, the Tibetan populations are even more at risk and should raise the concerns of the competent authorities. Notwithstanding, thanks to the iodized salt program started in 1995 by the Chinese government, the majority of the rural families have access to this essential element for the growth that is iodine (Lu, 2012). In the recalls of September 2012 and April 2013, 75% and 89%, respectively, of the families who added salt in their meals used iodized salt. Historically, rural people of T.A.R. trade lake salt with nomadic people for *tsampa*. Lake salt was still consumed by 14% of the children in the recall of September 2012, and by 15.6% in April 2013. In parallel to iodized salt, the consumption of lake salt may present some benefits. As it is unrefined, lake salt is richer in magnesium and in several trace elements, notably iron.

The usual intake for potassium in this study appears to account for only 60% of the Chinese RNI. Yet, the Chinese RNI for potassium only represents 40% of hEAR. Comparing to this last, 99.9% of the children are having inadequate intakes. Severe potassium deficiency due to low dietary intakes in healthy subjects is rare (Martin, 2000), but it is increasingly evident that high sodium diet combined with low potassium intakes plays a major role in the pathogenesis of hypertension and cardiovascular diseases (Aaron and Sanders, 2013; Adrogué and Madias, 2014, 2007). Although no ideal has yet been defined, recent studies suggest that a high sodium-to-potassium ratio is associated with significantly increased risk of cardiovascular diseases (Yang et al., 2012). In the US population, where heart disease is the leading cause of death (US CDC, 2015), the median sodium-to-potassium ratio of suckling is below 1, and is increasing with age (Institute of Medicine, 2005a).The adult population (>20 y/o) presents an average ratio of 1.41 (Zhang et al., 2013). In this study the average ratio of the 3-5 years old children is 1.59. The Tibetans are no exception to the rule as it appears that more than 40% of the agricultural populations around Lhasa suffer from hypertension (Yao et al., 2015). In addition to being a risk factor for cardiovascular disease, potassium deficiency is likely to increased bone turnover resulting in reduced bone formation and increased bone resorption (Institute of Medicine, 2005a). The problem of imbalance in sodium and potassium intakes is exacerbated by the low level of awareness of the population. While the considerable sodium intakes come mainly from substantial amount of added salt and processed foods (instant noodles), the low intakes of potassium are a consequence of the low consumption of fruits and vegetables. As suggested by Xu et al. (2015)(Xu et al., 2015), there are urgent needs for implementation of an efficient policy to control these risk factors. A rapid preventive measure could be a modification of the iodized salt program in which the sodium chloride could be partially replaced by potassium chloride (Levings and Gunn, 2014). Nevertheless, on the long-term, an efficient policy, integrated, in the cultural and socio-economic environment, must pass through the education of the population in order to rise their awareness and modify they dietary habits (Yao et al., 2015). To our opinion, one of the most promising actions, currently implemented by the Chinese government and some NGOs, is the greenhouses construction, combined with seed banks (China Daily USA, 2015; KBD Fund, 2012). This will allow the population to grow a wider variety of fruits and vegetables in a sustainable way, and so diversify their food intakes.

With a usual intake representing less than 40% of Chinese adequate intake of the hEAR in calcium, and more than 90% of the children presenting inadequate intakes, the situation is challenging. Sufficient amount of this mineral is essential for a normal and healthy growth, and maintenance of the skeleton, especially during childhood (Institute of Medicine, 1997). Such long-term calcium deficiency inevitably leads to lower bone mineral content and bone mineral density, inducing stunting and even rickets, with higher risks to develop osteomalacia and osteoporosis in more advanced stage of life (Marieb, 2005; Martin, 2000). Although altitude is believed to play a role in growth retardation in Tibet (Argnani et al., 2008; Bianba et al., 2015; Dang et al., 2008), undernutrition unequivocally has its share of responsibility in stunted growth and signs of rickets which are widely observed in the Tibetan population (Dang et al., 2014, 2004; Harris et al., 2001; Rooze et al., 2012). The situation may be even worse if we take into account the relatively high intakes in phosphorus. Despite of little relevance in adults, the Ca:P ratio may be useful in assessing the quality of the diet in growing children (Institute of Medicine, 1997). According to the Chinese DRIs, the ideal Ca:P ratio (in mass) is 1.8. With a ratio of 0.55, calculated on the basis of the mean intakes, the balance is clearly unfavorable. It has to be reminded that the etiology of rickets is complex and not limited to calcium deficiency. There is more than one cause but it seems that all types of rickets share hypophosphatemia as a common symptom (Tiosano and Hochberg, 2009). Hypophosphatemia in the Tibetan children could be, *prima facie*, surprising considering the more-than adequate intakes in phosphorus; 108% of the Chinese AI and 130% of hEAR. However, secondary hypophosphatemia may result from inadequate absorption of phosphorus due to a calcium deficient diet or because of vitamin D deficiency which enhance calcium absorption (Allgrove and Mughal, 2014). In the present case, it is demonstrated that the diet is poor in calcium. The vitamin D status of the Tibetans has been poorly studied and results are conflicting. Indeed, a study of Norsang et al. (2009)(Norsang et al., 2009) found that nomad people where predominantly deficient but farmers presented normal vitamin D serum levels. On the contrary, analysis performed by the Kashin-Beck Disease Fund asbl-vzw revealed low vitamin D levels among farmers of the investigated area (unpublished results). Another factor has to be taken into account in this problematic, the bioavailability of minerals. The main source of calcium and phosphorus in the Tibetan diet is cereals and cereals products which contain substantial amounts of phytic acids (Ma et al., 2005). During a preliminary study (unpublished data), we measured the phytic acid content in staples by a method adapted from Latta and Eskin (1980)(Latta and Eskin, 1980). The samples were collected in five families from Lhünzhub county and in five families from Nyêmo county. We found a mean phytic acid content of 372 ± 311 and 180 ± 50 mg/100g (expressed as mean ± standard deviation, on a wet weight basis), for wheat flour (n=19) and rice (n=14) respectively. These results are in line with the values of Ma et al. (2005)(Ma et al., 2005). The phytic acid content of *tsampa* (n=20) was found to be much higher with a mean value of 1105 ± 177 mg/100g. In another study, Ma et al. (2007) (Ma et al., 2007) concluded that the Chinese dietary intakes of phytate were high with risk of impairment of the bioavailability of calcium, iron, and zinc. It is highly probable that Tibetan people, with high consumption of *tsampa*, richer in phytic acid than wheat flour and rice, have higher phytate intakes and present even more unfavorable molar ratios of phytate-to-calcium, phytate-to-iron, phytate-to-zinc, and phytate x calcium/zinc. Several strategies are possible to remedy the situation. First of all it would be appropriate to confirm as accurately as possible the vitamin D status of the Tibetan population. Depending on the results, a supplementation in calcium and eventually in vitamin D would be a short-term solution. Yet, as Fang and Li (2014)(Fang and Li, 2014) rightly recall: “Hereby, the dietary calcium deficiency, inadequate calcium intake, usually refers to failure to consume the full recommendation for calcium intake, which does not necessarily mean a true dietary deficiency for most of individuals”. The Tibetan diet is based on secular traditions and the people may have undergone physiological adaptation to low calcium intake. In this case, we have to be certain that long-term calcium supplement does not do more harm than good. It has recently been suggested that it may enhance the risk of cardio-vascular disease (Daly and Ebeling, 2010; Reid, 2013), a factor that should not be added to a population already at risk without the certainty of beneficial health effects. If calcium deficiency should be confirmed by subsequent studies, a sustainable strategy could aim at decrease the phytic acid intake by decreasing the consumption of foods rich in phytates, and to diversify the dietary sources of calcium, which brings us back to support food diversification programs.

With its implication as a cofactor in more than 300 enzymatic reactions, and the storage of 50 to 60% of the body content into the skeleton, magnesium is indubitably essential for normal growth and healthy bones (Institute of Medicine, 1997). Magnesium is probably not of concern as the usual intake is ranging between the Chinese AI and the hEAR. However, there is no consensus on how to evaluate the magnesium status. If severe deficiency can easily be detected, low chronic deficiency are much more difficult to characterize (Martin, 2000). While the decrease in bioavailability of magnesium by phytic acid chelation is still controversial, the main source of dietary magnesium remains paradoxically whole cereal products, which are rich in phytate (Bertinato et al., 2014; Lopez et al., 2002). Besides, *tsampa* is richer in magnesium than wheat flour (Dermience et al., 2014). Other traditional Tibetan foods – such as dried cheese, dried wild peaches, dried nettles, and various dried leaves – are also rich in magnesium (Dermience et al., 2014). Regrettably, they are under consumed for cultural reasons, being associated with a low social level. The same discussion applied for copper, for which the mean intake is slightly, but significantly, below the Chinese AI. Copper is also essential for bone and joints growth (Dermience et al., 2015), and its decreased bioavailability due to phytic acid is as well controversial (Lopez et al., 2002). High zinc and iron intakes may decrease copper absorption (Martin, 2000), but it is unlikely in the diet of the enrolled children considering the present results. Alcohol has also been reported to decrease copper absorption, which could be a problem for some children who present high *chang* consumption (Martin, 2000). Investigating on biological markers would be advisable in order to conclude about the copper status of the Tibetan children (Olivares et al., 2008).

Iron usual intake is very close to the Chinese AI. However, such DRI is made assuming a balanced intake between heme iron and non-heme iron. In a western diet – in which approximately 75% of iron is supposed from heme iron sources – it is estimated that approximately 18% of the total ingested iron is absorbed, while it fall down to 10 to 5% in a vegetarian diet (Institute of Medicine, 2001). Adequate iron intake in case of vegetarian diet is therefore recommended to be doubled. With a low consumption of meat products, the Tibetan diet is closer to the vegetarian diet. The major source of dietary iron in the Tibetan diet is the cereal products, including *tsampa*, wheat flour, and rice. Phytic acid is well known to decrease the bioavailability of iron (Kumar et al., 2010). Tea drinks – including yak butter tea, black tea, and sweet tea – are abundantly consumed and even mixed with the flours and are also known to negatively affect iron absorption due to their polyphenol and tannin contents (Martin, 2000). On the other hand, some components known to enhance iron absorption, such as ascorbic acid, are not much present in the Tibetan diet. Investigating the iron status of the Tibetan population would also deserve some attention, especially since signs of anemia have already been observed (Dang et al., 2004; Xing et al., 2009). Iron is also essential to bone integrity (Dermience et al., 2015), and we can wonder if a strong mobilization of the body iron pool in order to maintain a sufficient hemoglobin concentration in a high altitude context could cause deleterious effects on bone health.

If iron intakes may deserve further investigation because of a possible low bioavailability, zinc intakes are much more debatable. Indeed, zinc has also its bioavailability strongly affected by foods high in phytic acid (Kumar et al., 2010), but the usual intake calculated in this study provides only 36% of the Chinese RNI. The recommended dietary allowance (RDA) of the US institute of Medicine for zinc is 5 mg/day for the same category of age, which represent 40% of the Chinese RNI and the hEAR is about 4 mg/day. This discrepancy probably arises from the difference in dietary pattern between western and eastern diets, the first being richer in meat products. It is in accordance with the suggestion of a 2-fold greater RDA in case of a vegetarian diet (Institute of Medicine, 2001). It is important to consider that zinc recommendations are formulated as recommended nutrient intake (RNIs) and not adequate intakes (AIs). Contrary to AIs which are not based on sufficient scientific evidences, RNIs can be associated to the RDAs and are calculated based on estimated average requirement so that the risk of inadequacy to an individual is very small (FAO/WHO, 2002; Institute of Medicine, 2001). With a usual intake of 4.3 mg/d, and cereals products as the main source of dietary zinc, it would not be surprising that the majority of children have a zinc deficiency. Considering its implication in many enzymatic reactions and the numerous effects of zinc deficiency on bone metabolism (Dermience et al., 2015), a possible implication in the etiology of Kashin-Beck disease would deserve some serious investigations. Particularly since zinc deficiency has been reported to increase lipid peroxidation (Shaheen and Abd El-Fattah, 1995) which is a physiopathological sign of KBD (Peng et al., 1992; Sudre and Mathieu, 2001).

An ideal solution to the problem of low intakes in iron, zinc, and copper would be a higher consumption of meat and fish. However, for economic and philosophical reasons, or because of lack of availability, this solution is hardly possible. Fishes are scarce in many rural areas and are never consumed by local people for cultural reason. If yak, sheep and goat are raised, they are very useful in the traditional rural life and hence sparingly butchered. Poultry breeding could be a solution to improve the supply of meat, but the Tibetans prefer to eat large animals in order to sacrifice the least possible lives. (Dorje, 1985). The next best alternative to our opinion has already been mentioned and consists in food diversification. Greenhouses could allow people to growth vegetables rich in iron such as parsley, which is by the way rich in vitamin C, an enhancer of iron absorption (Institute of Medicine, 2001). Lentils or beans could also be a valuable source of iron and zinc (D. Thavarajah et al., 2009). Some genotypes present relatively low levels of phytic acid, and germination or fermentation could further increase the bioavailability of these minerals (Ayet et al., 1997; P. Thavarajah et al., 2009). Nettles, many other wild dried herbs, and dried mushrooms also have high iron and zinc contents (Dermience et al., 2014; Malaisse et al., 2008b). Their consumption should be encouraged despite their difficult acceptation by local people, at least as regards the nettles. Anecdotally eaten as a snack by some children, sunflower seeds are also rich in iron and zinc. Cultivation of *Helianthus* and promotion of seeds consumption would be for sur beneficial. Poultry breeding for egg consumption could also be a possible way to improve the overall quality of the diet.

No recommendation for manganese is provided in the Chinese DRIs (China CDC, 2009). The usual intake represents more than 180% of the hEAR,, and almost 20% of the children are above the UL, set up at 3 mg/day. These high intakes should not be of concern because manganese comes primarily from cereal products for which the intestinal absorption rate is generally low. However, some children have a high consumption of tea drinks and *chang*, relatively rich in Mn, with a higher bioavailability (Dermience et al., 2014; Martin, 2000). If the high intake is recurring, it could be neurotoxic for these children (Aschner and Aschner, 2005).

The usual selenium intake fulfills less than 45% of the Chinese RNI, and it is probably overestimated. Indeed, most of the vegetables consumed by Tibetans are produced locally but have not been analyzed for their selenium content, and the values of Chinese FCT which were used are likely to be overestimated because of the very low level of selenium in the soils of these regions (Chasseur et al., 2008c; Li et al., 2009; Tan et al., 2002b). One example is the Chinese cabbage for which several samples were analyzed in a previous study and whose selenium concentrations were below 0.01 µg/100g while the value found in the Chinese FCT is about 0.49 µg/100g (China CDC, 2009; Dermience, 2010). It is worth emphasizing that selenium recommendations are also expressed as RNIs and not AIs, underlying the importance to meet the daily requirements. Selenium is an essential trace element whose deprivation can have many deleterious health effects (Martin, 2000). With regard to bone and joints metabolisms, selenium deficiency has been reported to induce growth retardation, osteopenia, and chondronecrosis in animal and *in-vitro* models (Downey et al., 2009; Moreno-Reyes et al., 2001; Ren et al., 2007). Selenium deficiency has been clearly observed among the Tibetan populations living in areas endemic for the Kashin-Beck disease (Ge and Yang, 1993; Moreno-Reyes et al., 1998; Peng et al., 2000; Yang et al., 2015). Even if no definite conclusion has been reached, it is widely thought to play a role in the etiology of the disease (Mathieu and Hinsenkamp, 2008; Suetens et al., 2001; Yao et al., 2011; Zou et al., 2009). If the imbalances in the previous minerals could be relatively easily solved through food diversification, the selenium issue is much more challenging. Indeed, selenium dietary deficiency is rooted in a serious deficiency of the soil, and the solution lies in the import of selenium in one form or another. Several studies have been conducted to investigate the influence of the consumption of selenium-enriched salt on the prevalence of Kashin-Beck disease (Jirong et al., 2012; Yu et al., 2015). Beneficial effects have been observed, if not always on the KBD prevalence, at least on the selenium status of the people (Moreno-Reyes et al., 2003; Ning et al., 2013; Zou et al., 2009). But it is clearly not a long-term sustainable solution (Ning et al., 2015), and its implementation on a large scale would be difficult. Besides, given the amounts of salt consumed by the Tibetans, there could also be a risk of selenium overconsumption with an underlying risk of toxicity (Institute of Medicine, 2000). Infrastructure development allows Tibetans to have access to foods rich in selenium coming from other region of China. Positive effects were observed on the selenium status (Sun et al., 2014), but it is not an economically viable solution, especially for the poorest rural people, who are the most affected (Chen et al., 2015; Suetens et al., 2001). Selenium-supplemented fertilizer has also been used in some provinces of China endemic for KBD, and it has been shown to increase the average Se content in total diets (Liquiang et al., 1991). The Se-supplemented fertilizers could also have benefits on the crop yields and animal nutrition (Hartikainen, 2005). This solution has already been implemented at a nation-wide level in Finland and has been proved to be an effective, safe and controlled way of bringing the selenium intake of the population to the recommended level (Aro et al., 1995). It is perhaps the most appropriate solution to the selenium issue in T.A.R. and in the other soil-selenium deficient areas in China.

4. Conclusion

Clinical signs of stunted growth and rickets have frequently been reported among rural populations of T.A.R., and especially amid children. Further, a chronic and endemic osteochondropathy called Kashin-Beck disease plagues these rural populations. The overall prevalence of the Kashin-Beck disease seems to decrease for some time, but it remains high in some areas of T.A.R. Today, its etiology has not been fully elucidated even if the general consensus involves environmental factors and a possible genetic predisposition. Among the potential factor causing the disease, diet is in a good position. A healthy bone metabolism depends on many factors, including balanced intakes in several minerals. This study aimed at calculates the usual intakes of rural Tibetan children for the main elements of dietary relevance. Further studies are mandatory to confirm the results and the trends revealed by the present study. However, in the investigated areas, the diet of the children appears to be very monotonous and poorly diversified. It is mainly based on cereals products, with low consumption of meat and dairy products, and even lower consumption of vegetables and fruits. Local alcohol drinking is not uncommon even in young children. This diet presents several imbalances in mineral intakes, with possible consequences on growth and bone metabolism of the children. Besides, experiencing fast development of its infrastructures, T.A.R. is changing. Although still very traditional, these changes are beginning to influence eating habits of rural people, with positive and negative effects. A better accessibility to food products that cannot be grown at such an altitude – like rice, and many vegetables and fruits – has a beneficial impact on the diversification of the rural Tibetan diet. On the other hand, industrialized foods and beverages of poor nutritional quality are frequently consumed. Yet, rural people, with frequent low education levels, are not aware of the potential risks of non-moderate consumption of these foods. Several solutions can be envisaged to improve the situation, and some are already implemented. An elegant one is the promotion of food diversification programs and education programs to raise awareness among Tibetans about healthy eating practices. The distribution of selenium-supplemented fertilizers, and in the most urgent situations, the distribution of nutritional supplements also deserves consideration. For sure the ideal solution should be integrated, sustainable and fully adapted to the socio-cultural environment of T.A.R. Further studies are mandatory to confirm trends revealed by the present results. Case-control studies should also be implemented to compare mineral intakes between proximate highly endemic areas and non-endemic areas.

Acknowledgement

This study was made possible thanks to the close collaboration and the support of the Center for Disease Control and Prevention of the Tibet Autonomous Region, P.R. China. We also wish to thank all the people who actively participated in this study: Tsewang Lhakdrun, Pema Drolkar, Pema Drolkar, Rinchen Lopsang, Lakpa Wangdu, Tashi Yangzom, Dikki Yangzom, Nima Kanden, Tsewang Rinzen, and Chungdak, the KBD Fund team members; Gelek, Tsering Namgye, Nyima Tashi, Chimy Namgye, Tashi, Dawa, Dorjee, and Tserin Cheuden, the interviewers; Fatma Yasar and Caroline Etienne, who computed the intakes. None of the authors declared a conflict of interest.

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Chapter VI.

“Ce n'est pas celui qui mange le plus, mais celui qui digère le mieux,

qui jouit de la meilleure santé"

Aristippe de Cyrène

Introduction

Although limited in scope, the conclusions of chapter V are challenging. The young rural Tibetan children are exposed to high risks of inadequate intakes for several essential minerals and trace elements. Compared to the Chinese dietary recommended intakes (DRIs) and to the harmonized estimated average requirements, they consume too much sodium and manganese, while their usual intakes in potassium, calcium, zinc, copper, and selenium are far too low. With regard to phosphorus, magnesium, iron, and copper, usual intakes are close to the recommendations. But one has to remember that DRIs are established for a standard population with a balanced and varied diet (Institute of Medicine, 2001). If Chinese DRIs are adapted to the overall Chinese diet, which is without doubt different from most western diets, Tibetans still have a specific and traditional diet that cannot be compared to the standard Chinese diet. Their diet is very monotonous and mainly based on cereals and cereals based products, with few vegetables, meat, and dairy products, while fruits are almost absent. Cereals are known to contain significant amounts of phytic acid, also called inositol polyphosphate, which is the principal storage form of phosphorus in many plants. At intestinal pH, this molecule forms insoluble complexes, called phytate, with several elements such as calcium, magnesium, iron, zinc, copper, etc (Kumar et al., 2010). Once chelated, the elements are no more absorbable by the intestine, resulting in a decrease in absorption rate. DRIs are largely influenced by the absorbability of elements. For example, 18% of iron is estimated to be absorbed if 75% of total iron in the diet is coming from meat (Institute of Medicine, 2001). In a vegetarian diet, this absorption rate falls down to 5 to 10%. It is therefore recommended to double iron DRIs for vegetarian people. It is the same for zinc. One can also cite calcium, which is much more absorbable from dairy products than from vegetable (25-35% versus 5-10%) (Martin, 2000).

And phytic acid is not the only natural compound known to affect minerals' absorbability. Tannins and other polyphenols, but also dietary fiber, decrease iron absorption as well (Martin, 2000). On the other hand, some compounds, such as ascorbic acid, are enhancers of the absorption of minerals (Teucher et al., 2004). However there are not many sources of such compounds in the Tibetan diet.

In light of the previous results, the rural Tibetan diet is not very far from a vegetarian diet. The mainstay of their diet is *tsampa*, a traditional dish made of roasted barley flour mixed with yak butter tea. Because it combines all the factors previously cited, which can largely influence DRIs, the question of bioavailability of the mineral elements in the rural Tibetan diet cannot be ignored. For this reason, we conducted an animal experimentation on a rat model to assess the apparent digestibility, the fecal excretion and the urinary excretion of minerals and trace elements in *tsampa*.

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Assessment of growth performances, apparent digestibilities and apparent retentions of selected minerals and trace elements of dietary relevance in the traditional Tibetan dish called *tsampa pag* on a rat model

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Abstract

Background: Kashin-Beck disease is an osteochondropathy endemic in China. Although unclear, a multifactorial etiology is proposed in which mineral deficiencies is likely to play a role. The Tibetan diet is based on cereal products, and *tsampa* – the traditional roasted barley flour – is probably the main one. It is often mixed to yak butter tea to form *tsampa pag*, and it is eaten as such.

Objective: This experiment aimed at assessing the apparent digestibilities and apparent retentions of selected minerals and trace elements (Na, K, Ca, P, Mg, Fe, Zn, Cu, and Mn) of dietary relevance in this traditional Tibetan dish on a rat model.

Design: Three experimental diets were composed: a reference diet based on the AIN 76 diet; a traditional *tsampa* diet; and a *tsampa* diet supplemented with minerals and vitamins. Thirty-six male Sprague Dawley® rats were divided into three groups. Each group was fed during seven weeks with one of the diets. Each rat spent two periods of five days in individual metabolic cages. Rats were weighted weekly. Quantities of food ingested were precisely recorded. Feces and urine were collected and analyzed for mineral and trace element composition. After sacrifice, femurs and livers were collected for biometric and elemental analysis.

Results and discussion: The three groups of rats constituted presented homogenous mean weights at the beginning of the experiment. After seven weeks fed with their respective diet, the mean weights were all significantly different ($p<0.001$). The rats of the *tsampa* group had the lowest mean weight while rats of the reference group had the highest. Rats of the *tsampa*_supp diet being in-between. Lower diet intakes, but also energy, protein, and fat intakes are presumably the main responsible factors. Reference and *tsampa*_supp groups presented higher bone calcium and phosphorus contents. Despite important amounts of phytic acid in the diet, calcium, phosphorus, and magnesium, apparent digestibilities were significantly higher for these elements in the *tsampa* group. However, the rats of the *tsampa* group presented a significantly higher urinary phosphorus excretion. The high variability with regard to the low apparent digestibilities of Fe, Zn, Cu, and Mn make difficult the interpretation of the data. However the similar or even significantly higher concentrations found in the livers and the femurs of the *tsampa* group did not suggest any limitation with regard to these elements.

Keywords

Kashin-Beck disease; *tsampa*; minerals; trace elements; rat; growth performances ; apparent digestibility; apparent retention

1. Introduction

In the Tibet Autonomous Region (T.A.R.), the Tibetan populations can be roughly divided into four categories according to their macro-ecosystem: nomads, farmers, city dwellers, and suburban dwellers (Haubruge et al., 2000; Malaisse et al., 2008). If lifestyle and dietary habits are undergoing changes because of socio-economic evolution in T.A.R., diet remains very traditional among rural populations (Chasseur et al., 2008b; Dermience et al., 2014). Tibetans are affected by an endemic and chronic disease called Kashin-Beck disease (KBD), that makes victims exclusively among poor, isolated rural populations (Mathieu and Hinsenkamp, 2008; Yamamuro, 2001). KBD is an osteochondropathy affecting long bones and joints, sometimes as soon as of the end of breastfeeding (Mathieu et al., 2008). Although the etiology of this disease is not definitely established, little doubt remains regarding its multifactorial character, and the implication of environmental factors (Chasseur et al., 2008a, 2008c; La Grange et al., 2001; Suetens et al., 2001).

One of the recurrent components of the multifactorial hypothesis is a dietary iodine and selenium deficiency due to very low levels in soils (Li et al., 2009; Tan et al., 2002). Further studies confirmed the very low levels of selenium in cereals and vegetables grown in endemic areas (Dermience et al., 2014, 2013). However, selenium deficiency has never been proved to play a definite role in the etiology of KBD, while iodine deficiency may rather be considered as a risk factor (Moreno-Reyes et al., 2003, 1998; Zou et al., 2009). But these two elements are obviously not the only ones to be related to bone metabolism (Beattie and Avenell, 1992; Dermience et al., 2015), and it cannot be ruled out that other elements may have some impact on bone health of affected Tibetans (Liquiang et al., 1991; Wang et al., 2010). One of our recent studies conducted among young Tibetan children revealed several imbalances in mineral intakes for nine elements, directly or indirectly involved in bone metabolism (Dermience et al., 2016). Although the results need to be confirmed by larger studies, it appears that Chinese dietary reference intakes (DRIs) are not met for potassium, calcium, zinc, copper, and selenium. Despite adequate intakes, iron may also be of concern because of the high level of phytic acid in the diet (Ma et al., 2007, 2005). Phytic acid is known to form insoluble complexes with several elements, and is susceptible to exacerbate calcium, and zinc deficiencies and to induce iron deficiency (Grases et al., 2001).

There are several sources of phytic acid in the Tibetan diet such as rice, wheat flour, or potatoes. But the main source is probably the traditional flour made of roasted barley which is called *tsampa*. This flour contains significant amounts of phytic acid – up to 1.2 g / 100 g –and it is almost daily consumed in large quantities by the majority of the rural Tibetan population (Ma et al., 2007, 2005, Dermience et al., 2016). It is generally prepared according to a traditional recipe called *tsampa pag*, in which *tsampa* is mixed with yak butter tea. It is consumed as such, without cooking. Besides phytic acid, black tea generally is reputed to contain significant amounts of polyphenols, which are also known to influence the absorbability of some minerals or trace elements (Fairweather-Tait and Hurrell, 1996; J. Powell et al., 1998).

The main objective of this study was to measure apparent digestibilities and apparent retentions of minerals and trace elements of dietary relevance in the traditional Tibetan dish called *tsampa pag* on a rat model. The use of rats instead of an *in vitro* model was motivated by the fact that the rat has been validated as an experimental model for studies of nutrition and also for human bone disorders (Frost and Jee, 1992; Seco et al., 1998). Consequently, the growing performances of the rats, and the influence of *tsampa pag* on femur and liver mineral and trace element composition was also investigated. To the best of our knowledge, it is the first time that such results about this traditional Tibetan dish are reported.

2. Experimental section

2.1 Experimental diets

For this study, three experimental diets were prepared. Their compositions are listed in Table 1. The first experimental diet called "*tsampa*" was based on the traditional Tibetan recipe of *tsampa pag*" described above. The roasted barley flour and the black tea leaves were directly imported from Qamdo prefecture (Tibet Autonomous Region, China), an area highly endemic for KBD. For sanitary reasons, yak butter was not imported and cow butter was bought in Belgium (Everyday®, Colruyt, Halle, Belgium). The concentrated brewed black tea was prepared in the traditional Tibetan way: about 174 g of black tea leaves (1 brick, as it is sold) were boiled in 6 L of ultrapure water for 1h (Dermience et al., 2014), and then filtered.

A second experimental diet called "reference" was based on the commonly used purified diet AIN-76A for rats (National Research Council, 1995). This reference diet was prepared by combining casein and methionine protein sources, a mixture of corn starch and sucrose, a mixture of rapeseed oil and peanut oil, pure cellulose (Alba-fiber C-200 Mikro-Technik GmbH & Co, Bürgstadt, Germany), and a mineral and vitamin supplement (AIN-76 mineral mixture and Vitamin Mixture, MP Biomedicals, Santa Ana, CA, USA). The protein content of this diet had intentionally been decreased to be closer to the *tsampa* diet. Apart from protein, this diet was supposed to fully meet the maintenance and growing needs of newly weaned rats (National Research Council, 1995; Potier de Courcy et al., 1989; Reeves et al., 1993).

A third experimental diet, called "*tsampa*_supp" was also prepared according to the *tsampa pag* recipe. The only difference with the *tsampa* diet being that the AIN-76 mineral and vitamin supplement was added in the same proportion than in the reference diet. The purpose of this diet was to assess the effects of a supplementation in minerals and vitamins on the growth of rats, on the apparent digestibility of the diet, on the apparent retention of minerals, and on the mineral contents of the femur.

The three diets have been prepared with a mixer (WP Kemper GmbH, Rietberg, Germany) and distributed in plastic bags (Toppits® 3L, Melitta Group, Minden, Germany) per serving of about 500g. They were stored in a freezer at -18 ° C and defrosted at 4 ° C for daily use.

Table 1. Compositions of the experimental diets (g/100g on a fresh matter basis)

Experimental diet	**Ingredient**		**g/100g**
Reference	Ultrapure water		24.1
	Starch		40.4
	Sucrose		17.9
	Casein		6.9
	Methionin		0.1
	Albafibre		3.7
	Oils (rapeseed and peanut)		3.5
	Mineral supplement		2.8
	Vitamin supplement		0.7
Tsampa			
	Roasted barley flour		50.7
	Yak butter tea		
		Hot ultrapure water	45.3
		Concentrated brewed black tea	2.8
		Cow butter	1.0
		Salt	0.2
*Tsampa*_supp			
	Roasted barley flour		47.2
	Yak butter tea		
		Hot ultrapure water	46.4
		Concentrated brewed black tea	2.8
		Cow butter	1.0
		Salt	0.2
		Mineral supplement	2.0
		Vitamin supplement	0.5

Graphical abstract of the animal experimentation

40 weened Sprague Dawley® male rats

3 experimental diets:	Reference		*Tsampa*_supp		*Tsampa*		
3 groups:	12 rats		12 rats		12 rats		4 spare rats
6 subgroups:	6 rats	6 rats	6 rats	6 rats	6 rats	6 rats	
Week of experiment:							
1 2 3	Adaptation period, collective housing, fed *ad libitum*						
4	Indiv.	Coll.	Indiv.	Coll.	Indiv.	Coll.	
5	Coll.	Indiv.	Coll.	Indiv.	Coll.	Indiv.	
6	Indiv.	Coll.	Indiv.	Coll.	Indiv.	Coll.	
7	Coll.	Indiv.	Coll.	Indiv.	Coll.	Indiv.	

Sacrifice of rats and organ harvesting

Samples:	femurs	livers	diet	feces	urine

biometric measurements

moisture content determination

muffle furnace calcination	microwave-assisted nitric acid and hydrogen peroxide digestion	filtration, nitric acid digestion on hot plate

Elemental analysis: Na, K, Ca, P, Mg, Fe, Zn, Cu, and Mn analyzed on a 4200 MP AES

calculation of apparent digestibility and apparent retention

Indiv.: individual housing; Coll.: collective housing. Rats were weighed weekly. During individual housing in metabolic cages feces, urine and food leftovers were quantitatively harvested daily. Each period of individual housing lasted 5 consecutive days of the week and rats were back in collective housing the week-end.

2.2 Rats and housing

All procedures involving the rats were approved by the Official Animal Care and Use Committee of the University of Liege (Protocol No. 14-1643) before the research began.

Forty weaned Sprague Dawley® male rats were purchased from Charles River Laboratories International, Inc. (Wilmington, Massachusetts, USA). Rats were approximately 21 days old with a weight of 59.7 g ± 6.4 g (mean ± standard deviation). The rats were ranked by weight, and the two heaviest and the two lightest were kept as spare rats. The thirty-six remaining rats were divided into 4 blocks according to their weight: very heavy, heavy, light, and very light. Within a block, 3 rats were randomly assigned to one of the experimental diets, for a total of 12 rats per diet. PICO-ID transponders (UNO BV, Zevenaar, Netherlands) were implanted into each rat for identification purpose. The temperature in the room was 22.5 °C +/- 1 °C, with a light/dark cycle of 16/8 h. The renewal of the room air was provided by controlled mechanical ventilation.

During the first three weeks of the experiment, considered as the acclimation period, rats were randomly placed in groups of six in collective cages according to their diet. They were fed ad libitum with their respective diet and deionized water. Cages were furnished with poplar bedding (Litaspen Premium 6, Carfil, Oud-Turnhout). Bedding was changed every week, but a small amount of soiled bedding was kept to maintain the olfactory environment and reduce stress. No other cage enrichment was provided to avoid possible contamination due to nibbling. Weight of the animals was recorded weekly by dynamic weighing, as well as food intakes of the groups with a PM4600 Mettler-Toledo scale (Greifensee, Switzerland).

During the second phase of the experiment, from week 4 on, each rat spent two periods of five consecutive days housed in individual metabolic cages (Type 304 stainless steel, Tecniplast, Buguggiate, Italy). During these periods, the daily amount of food given was limited to 15g (on a dry matter basis), precisely weighed. Deionized water was provided *ad* libitum. Feces, urine and food leftovers were individually and quantitatively harvested daily, weighed at the end of the period, and stored at -18°C for elemental analysis. Cages were cleaned thoroughly every two-days to avoid cross contamination between feces, urine and food leftovers. Plastic and glass parts of the cages were washed with detergent and rinsed with tap water and deionized water consecutively. They were then soaked overnight in a 10% nitric acid bath (Merck Millipore, Darmstadt, Germany) and rinsed three times with deionized water. Between the periods of individual housing, rats were housed in their respective collective cage. Weight of the animals was recorded weekly as well as food intakes of the groups in collective cages.

At the end of the experiment, rats were anesthetized by inhalation of isoflurane (Isoba®, Schering-Plough Animal Health, Welwyn Garden City, UK), and put to death under carbon dioxide atmosphere. Livers and femurs were directly harvested and stored at 4°C in 50 ml Falcons (Medical grade, CentriStarTM, Corning Inc., Tewksbury, USA) for biometric and elemental analysis.

2.3 Elemental analysis

Ultrapure water (18 megohm) was used throughout the analyses. The laboratory glassware used was cleaned by soaking overnight in a 6 N nitric acid bath and rinsed at least three times with ultrapure water. All reagents were of analytical grade. Several blanks were realized for each digestion procedure.

Moisture content of experimental diets, livers, left femurs, and feces was determined by drying at 105°C until constant weight in an air oven (Memmert GmbH & Co Schwabach, Germany). Before drying, feces were meticulously rid of fur and food leftover. Femurs were also rid of flesh and ligament residues.

Experimental diets, livers and feces were digested with 6 ml of 65% nitric acid and 1 ml of 30% hydrogen peroxide in a high performance microwave digestion unit (MLS 1200 mega, Milestone, Brøndby, Denmark). The following microwave program was implemented: 250 W: 2 min; 0 W: 2 min; 250 W: 6 min; 400 W: 5 min; 600 W: 5 min; ventilation: 10 min. The rotor was then cooled down for a period of 1 h. Digested solutions were quantitatively transferred into 50 ml volumetric flasks and diluted to volume with ultrapure water. For experimental diets sampling portions were about 1 g of fresh matter, weighed with an analytical balance (ALT-250-4b, KERN & SOHN, Balingen, Germany). Livers and feces were finely ground after drying and a sampling portion of about 0.5 g was weighed.

Left femurs were placed in quartz beakers and calcined in a muffle furnace at 450°C during 18 h, and 1 h at 550°C. After cooling, ashes were solubilized by heating during 15 min on a hot plate with 3 ml of 65% nitric acid and 0.5 ml of 30% hydrogen peroxide. Solutions were quantitatively transferred into 25 ml volumetric flasks and diluted to volume with ultrapure water. Femur length, width of the distal epiphysis and width of the middle of the diaphysis were recorded on the fresh right femurs with a caliper (TL-VC02, Toolvizion, Rijswijk, Netherlands).

To get rid of fur, feces and diet residues, urine was filtered (MD 615 ¼, Macherey-Nagel GmbH & Co., Düren, Germany) in a 250 ml Erlenmeyer flask. The flasks were surmounted with a condenser to control vapor release and losses of sample. After 30 min of heating on a hot plate, 5 ml of 65% nitric acid were added to each flask. After 45 min, 5 ml of nitric acid were added again. Then, every 5 minutes, 1 ml of 30% hydrogen peroxide was added to each flask for a total addition of 4 ml. After the last addition, the samples were further heated for 30 min. After cooling, digested solutions were quantitatively transferred into 100 ml volumetric flasks and diluted to volume with ultrapure water.

The following elements were analyzed on a 4200 MP AES (Agilent Technologies, SantaClara, California, USA): Na (588,995 nm); K (769,897 nm); Ca (422,673 nm); P (213,618 nm); Mg (383,829 nm); Fe (371,993 nm); Zn (213,857 nm); Cu (324,754 nm); Mn (403,076 nm). The MP AES was equipped with an SPS-3 autosampler, an inert OneNeb nebulizer and double pass glass cyclonic spray chamber. Calibration standards were prepared from a multi-element solution (02426.2000, Bernd Kraft®, Duisbourg, Germany). All calibration curves were obtained by linear regression and presented a coefficient of determination above 0.998. All calibration errors were below 5%, or below 10% for low concentrations.

Accuracy of the results was ensured by analysis of the following certified references materials whose matrix was the closest possible to those of the samples analyzed: BCR®-679 *White cabbage* and ERM®-BB186 - *Pig Kidney*. Analyses were made in five replicates. Dry matter content was determined according to the provider's instructions. Statistical comparison between measurement results and certified values was also made according to the provider's instructions (Linsinger, 2005).

2.4 Apparent digestibility and apparent retention

Apparent digestibility was calculated for each experimental diet and each mineral according to the following formula:

$$AD = [(C_{diet} * DI) – (C_{feces} * DF)] / (C_{diet} * DI)$$

Apparent retention was calculated for each experimental diet and each mineral according to the following formula:

$$AR = [(C_{diet} * DI) – (C_{feces} * DF) – (C_{urine} * DU)] / (C_{diet} * DI)$$

With AD = apparent digestibility; AR = apparent retention; C_{diet} = average concentration of a given mineral in the diet (µg/g, expressed on a dry matter basis); DI = daily intake, which is the amount of food ingested on one day (g/day, expressed on a dry matter basis); C_{feces} = concentration of a given mineral in the feces of an individual rat (µg/g, expressed on a dry matter basis); DF = daily amount of feces of an individual rat; C_{urine} = concentration of a given minerals in the urines of an individual rat (µg/g); and DU = daily amount of urine of an individual rat (g).

Statistics

Generalized linear mixed models (GLMM) were used to analyze the effect of the diet (reference, *tsampa* and *tsampa*_supp) on biometric measurements (i.e. initial and final weights of rats, weekly growth rates, relative weights of femur and liver as well as length, epiphyseal and diaphyseal width of femur). Prior to these analyses, percentage data were arcsine-transformed to achieve variance stabilization. Normality of the residuals and overdispersion in variance of the data were checked ($p > 0.05$). Data were transformed when assumption violation occurred. Effects of fixed and random factors (i.e. ANOVA and difference of least squares means) were assessed using the step function implemented in the package stats. Similar models (i.e. GLMMs with diets as fixed factor and blocks as random factor) were used to detect a potential effect of the diet on mineral composition of femurs and livers.

To analyze the effect of the diet on the apparent digestibility and retention of minerals, we used GLMMs including the diets and the periods as fixed factors and the weight blocks as random factor. Data transformations were performed when needed (i.e. assumption violation) and GLMMs were conducted separately for each period when significant interaction between diets and periods was detected. In addition, the differences between apparent retention and apparent digestibility were assessed for iron and for the trace elements (i.e. Zn, Cu and Mn) using Wilcoxon Signed-Rank Test. All analyses were performed in R version 3.0.2 (R Core Team 2013). A p-value lower than 0.05 was considered for assessing significant statistical effects in every test.

3. Results

The rat number 30 assigned to the *tsampa*_supp diet behaved abnormally during the experiment and refused to eat during the period of individual housing. All data relating to this rat were therefore discarded. One rat of the *tsampa* group was accidentally killed during the first week of experiment. It was replaced by one of the spare rats which were already assigned to the *tsampa* diet.

3.1 Composition of the experimental diets

Table 2 lists the nutrient composition of the three experimental diets. The diet called reference appears to fully meet, or exceed, the estimated nutrient requirements (ENRs) for maintenance and growth of rats, except for proteins, fat, and calcium (55.8%, 83.6%, and 71.3% of ENRs respectively). The deficiency in protein content was intended. The deficiency in fat probably results from underdosing during the preparation of the diet. Calcium deficiency was not expected in this diet. It is likely to be explained by the decreased content in protein, which is thought to be under the form of calcium caseinate.

The *tsampa* diet fulfills 98% of the ENR with regard to energy. With respectively 32%, 29%, 7.7% and 52.7% of the ENRs, this diet can be considered deficient in protein, fat, calcium and copper. Compared to the reference diet, the *tsampa* diet also has lower content in zinc and manganese.

The vitamin and mineral supplement added in the *tsampa*_supp diet removed calcium and copper deficiencies. It also increased the mineral and trace element levels up to 106 to 220% compared to the reference diet. Yet, the diet remains deficient in protein and fat, and moderately deficient in energy (93% of ENR).

Table 2. Estimated nutrient requirements (ENRs) for maintenance and growth of rats and nutrient composition of the experimental diets (amount per kg of diet on a dry matter basis)

Nutrient	**ENRs**[1]	**Reference**[2]	***Tsampa***[2]	***Tsampa*_supp**[2]
Energy (Kcal/kg)	3800	4058	3722	3543
Energy (KJ/kg)	15900	16769	15365	14628
Proteins (g/kg)	165	92	53	51
Fats (g/kg)	55	46	16	16
Carbohydrates (g/kg)	-	815	839	797
Elements (mg/kg)				
Na	550	1794	1687	3708
K	3960	5140	5307	10360
Ca	5500	3922	426	5103
P	3300	3900	3662	7270
Mg	550	727	1084	1894
Fe	39	60	85	147
Zn	13	51	25	74
Cu	5,5	8.5	2.9	12.5
Mn	11	82	15	103
Phytic acid (mg/kg)	-	-	11670	10400

[1] adapted for dry matter content from *Nutrient Requirements of the Laboratory Rat* (National Research Council, 1995); [2] values of minerals contents were measured according to the elemental analysis section, phytic acid content was calculated based on values found in Dermience et al. (2016) other values were calculated based on food composition tables (China CDC, 2009; US Departement of Agriculture, 2010)

Table 3. Weights of the rats at the beginning and at the end of the experiment (g), and mean weekly growth rates (%)(mean ± standard deviation)

Experimental diet	**Weight (g)**		**Growth rates (%)**						
	W 0	W 7	W 1	W 2	W 3	W 4	W 5	W 6	W 7
Collective housing									
Reference	60.1 ± 6.3 ^{a}	237.9 ± 28.5 ^{a}	44.7 ± 13.2 ^{a}	22.1 ± 5.9 ^{b}	28.2 ± 9.2 ^{a}	24.0 ± 5.0 ^{a}	35.1 ± 6.1 ^{a}	35.9 ± 5.9 ^{a}	30.7 ± 4.4 ^{a}
Tsampa	60.3 ± 6.6 ^{a}	120.1 ± 10.9 ^{c}	34.1 ± 6.9 ^{b}	20.0 ± 5.0 ^{b}	-1.8 ± 3.3 ^{c}	6.1 ± 4.4 ^{c}	14.9 ± 2.1 ^{b}	18.2 ± 4.0 ^{b}	11.5 ± 2.4 ^{b}
*Tsampa*_supp	59.6 ± 5.3 ^{a}	174.4 ± 17.9 ^{b}	35.1 ± 5.8 $^{a.b}$	28.2 ± 5.1 ^{a}	17.1 ± 2.3 ^{b}	13.8 ± 2.2 ^{b}	31.5 ± 4.3 ^{a}	33.4 ± 2.1 ^{a}	15.1 ± 1.7 ^{b}
Individual housing									
Reference						7.7 ± 3.7 ^{a}	1.5 ± 5.1 ^{a}	-2.4 ± 7.1 ^{a}	-1.9 ± 9.6 ^{a}
Tsampa						-0.7 ± 3.7 ^{b}	-1.7 ± 2.9 ^{a}	2.0 ± 4.4 ^{a}	0.4 ± 4.2 ^{a}
*Tsampa*_supp						-1.8 ± 1.4 ^{b}	-4.1 ± 5.2 ^{a}	-1.6 ± 2.0 ^{a}	-2.0 ± 3.1 ^{a}

^{a,b,c} within a column, and according to the housing conditions, means associated to different letters present significant differences

3.2 Animal performances and biometric analysis

The mean weights of the three groups of rats at the beginning and at the end of the experiment, as well as the weekly growth rates, are presented in Table 3. No significant difference between the mean weights of the three groups was highlighted at the beginning, while they are all significantly different at the end of the experiment ($p<0.001$). The reference group was the heaviest, while the *tsampa* group was the lightest. Rats of the *tsampa*_supp group present were in-between. Mean growth rates were calculated week by week, and according to the collective or individual housing. During collective housing, the reference group presented significantly higher growth rates compared to the *tsampa* group, except in week 2. The *tsampa*_supp group also presented significantly higher growth rates compared to the *tsampa* group, except in week 1 and 7. The mean growth rates during the periods of individual housing are close to zero for every group, and for every week, except for the reference group in week 4. The relative standard deviations are higher during the period of individual housing.

Table 4 lists the average daily gains (ADGs), the diet daily intakes (DIs), and the daily feces (DF) and daily urine (DU) excretions for the two periods of individual housing according to the diet. The ADGs are close to zero in the three groups, and for both periods of individual housing. DIs of the *tsampa* group are significantly lower than the two other groups for both periods. Feces excretions of this group are significantly lower compared to *tsampa*_supp group for both periods, and significantly lower compared to the reference group in the first period.

Table 4. Average daily gain (ADG), daily intake (DI), daily feces (DF) and daily urine (DU) excretions for the two periods of individual housing (means ± standard deviation, expressed on a dry weight basis for diet intake and feces excretion)

	Diet	**ADG** (g/day)	**DI** (g/day)	**DF** (g/day)	**DU** (g/day)
Period 1					
	Reference	1.2 ± 1.7 ^{a}	12.1 ± 0.8 ^{a}	0.9 ± 0.1 ^{b}	6.9 ± 3.3 ^{a,b}
	Tsampa	-0.4 ± 0.8 ^{b}	5.9 ± 0.5 ^{c}	0.7 ± 0.1 ^{c}	8.7 ± 3.1 ^{a}
	*Tsampa*_supp	-0.9 ± 1.1 ^{b}	9.4 ± 1.0 ^{b}	1.2 ± 0.2 ^{a}	5.0 ± 1.4 ^{b}
Period 2					
	Reference	-1.0 ± 2.1 ^{a}	11.8 ± 1.7 ^{a}	1.0 ± 0.1 ^{b}	5.3 ± 2.9 ^{a}
	Tsampa	0.3 ± 0.7 ^{a}	7.1 ± 0.7 ^{b}	0.9 ± 0.2 ^{b}	7.7 ± 2.4 ^{a}
	*Tsampa*_supp	-0.6 ± 0.7 ^{a}	10.9 ± 1.0 ^{a}	1.6 ± 0.3 ^{a}	6.4 ± 1.7 ^{a}

^{a,b,c} within a period and a column, means associated to different letters present significant differences

Table 5 summarizes some biometric measurements made on the femurs and the livers of rats. Relative weight of femurs or livers has been obtained by dividing the weight of the femur or liver by the final weight of the rats. The same principle has been applied to the relative measurements of femurs: length, epiphyseal width, and diaphyseal width. The three groups present significant differences with regards to the relative dimensions (length and widths), the rats fed with the *tsampa*_supp diet present similar relative femur weight compared to the *tsampa* group, while it is significantly lower for the reference group. On the other hand the mean dry matter of the femurs of the *tsampa*_supp is similar to the reference group, the *tsampa* group being significantly lower. The mean relative liver weight of the reference group is significantly higher than the two other groups, which present no significant difference between them. The three groups present significant differences in liver dry matter.

Table 5. Biometric measures of femurs and livers according to the diet (mean ± SD)

	Control	*Tsampa*	*Tsampa*_supp
Femur			
Relative femur weight (g/g)	0.265 ± 0.019 ^{b}	0.308 ± 0.020 ^{a}	0.310 ± 0.028 ^{a}
Dry matter (coef.)	0.618 ± 0.021 ^{a}	0.526 ± 0.027 ^{b}	0.622 ± 0.014 ^{a}
Ash weight/dry weight (coef.)	0.612 ± 0.017 ^{a1}	0.440 ± 0.024 ^{c2}	0.594 ± 0.015 ^{b3}
Relative lenght (mm)	0.129 ± 0.013 ^{c4}	0.209 ± 0.017 ^{a}	0.165 ± 0.013 ^{b5}
Relative epiphyseal width (mm)	0.027 ± 0.003 ^{c4}	0.048 ± 0.004 ^{a}	0.036 ± 0.003 ^{b5}
Relative diaphyseal width (mm)	0.014 ± 0.001 ^{c4}	0.020 ± 0.002 ^{a}	0.017 ± 0.002 ^{b5}
Liver			
Relative liver weight (g/g)	4.60 ± 0.48 ^{a}	3.62 ± 0.39 ^{b}	3.58 ± 0.23 ^{b}
Dry matter (coef.)	0.304 ± 0.005 ^{a}	0.276 ± 0.007 ^{c}	0.286 ± 0.007 ^{b}

Relative data: individual weights and measures were divided by the individual final weight of each rat. Weights and dry matters of femurs were recorded on left femurs; measurements and ash weight/dry weight have been performed on right femurs; Sample size by group = 12 except for ^{1} n=8, ^{2} n=11, ^{3} n=9, ^{4} n=10 and ^{5} n=11; ^{a,b,c} within a line, means associated to different letters present significant differences

3.3 Apparent digestibility and apparent retention

Table 6 lists the ingestions and the feces and urinary excretions of minerals and trace elements according to the experimental diet and the period of individual housing. Calculated values of apparent digestibility (AD) and apparent retention (AR) for each experimental diet, for every mineral investigated, and for both periods are listed in Table 7.

Compared to the reference group, the *tsampa* group presents lower ingestion for every element (6% to 81%). With 6% of the amount ingested by the reference group, calcium ingestion is especially low for the *tsampa* group. Conversely, the mineral and trace element ingestions of the *tsampa*_supp are much higher than those of the reference group (106% to 220%). Fecal excretions of Na and K are very low for every group, and urinary excretions of Na and K are high. As expected, ADs are high, above 95%, for sodium and potassium in every group, while ARs are much lower. Fecal excretions of Ca and P are very low compared to the reference group, while they are high in the *tsampa*_supp group. Consequently, for both periods, the *tsampa* group presents significantly higher ADs for calcium than the two other groups (p<0.001). The ARs for this element are very similar to the ADs, reflecting an important retention, presumably to ensure bone growth. Urinary excretions of Ca are low for the three groups. Phosphorus ADs are very similar to calcium ADs, and the rats of the *tsampa* group also present a significantly higher absorption rate for this element (p<0.001). Phosphorus urinary excretions are low for the reference group and the *tsampa*_supp (around 5%), while it is much higher for the *tsampa* group (above 50%). The phosphorus ARs of the reference group and the *tsampa*_supp group are important (around 60-80%), while it fall to 35% for the *tsampa* group. Magnesium urinary excretions are around 50% of the ingested amount for the reference and the *tsampa* groups, and around 25% for the *tsampa*_supp group.

Fecal excretions of Fe, Zn, Cu, and Mn are very high (>85% of ingestion) in the three groups, and especially in the *tsampa* group (>95%). Nevertheless, urinary excretions for these elements are very low. The constantly encountered closeness between ADs and ARs suggests high retention rates with regard to iron and every trace element (Zn, Cu, and Mn). The ADs values between period 1 and period 2 are relatively constant for the reference group. Although it is not significant, ADs values of the *tsampa*_supp group tends to decrease from period 1 to period 2. In period 1, ADs values of the *tsampa* group are close to 0% or even slightly negative, in average and significantly lower compared to the reference group and *tsampa*_supp group (respectively to the groups: p<0.01 and p<0.001 for Fe, p>0.05 and p<0.05 for Zn, p<0.05 and p<0.001 for Cu, p<0.05 and p<0.001 for Mn). In period 2, ADs of *tsampa* group increased up to about the same levels than the two other groups (all p>0.05 except p<0.05 for Zn between *tsampa* and *tsampa*_supp). The relative standard deviations of mean ADs for these elements (Fe, Zn, Cu, and Mn) are important compared to other elements (Na, K, Ca, P, Mg).

Table 6. Ingestions, fecal excretions, and urinary excretions of minerals and trace elements according to the experimental diet and the period of individual housing (mean ± standard deviation, mg/day.rat)

Experimental Diet	**Period**	**Na**	**K**	**Ca**	**P**	**Mg**	**Fe**	**Zn**	**Cu**	**Mn**
Ingestion										
Reference	1	22 ± 1	62 ± 4	47 ± 3	47 ± 3	8.8 ± 0.6	0.72 ± 0.05	0.61 ± 0.04	0.102 ± 0.007	0.98 ± 0.06
	2	21 ± 3	61 ± 9	46 ± 7	46 ± 7	8.6 ± 1.2	0.70 ± 0.10	0.60 ± 0.09	0.100 ± 0.015	0.96 ± 0.14
Tsampa	1	10 ± 1	31 ± 3	2.5 ± 0.2	22 ± 2	6.4 ± 0.5	0.50 ± 0.04	0.15 ± 0.01	0.017 ± 0.001	0.09 ± 0.01
	2	12 ± 1	38 ± 4	3.0 ± 0.3	26 ± 3	7.7 ± 0.7	0.61 ± 0.06	0.18 ± 0.02	0.020 ± 0.002	0.10 ± 0.01
*Tsampa*_supp	1	35 ± 4	97 ± 10	48 ± 5	68 ± 7	17.7 ± 1.9	1.38 ± 0.15	0.69 ± 0.07	0.117 ± 0.012	0.96 ± 0.10
	2	40 ± 4	113 ± 11	56 ± 5	79 ± 8	20.6 ± 2.0	1.60 ± 0.15	0.81 ± 0.08	0.136 ± 0.013	1.12 ± 0.11
Fecal excretion										
Reference	1	0.3 ± 0.2	0.8 ± 0.3	10.6 ± 3.1	8.8 ± 2.1	4.1 ± 0.7	0.63 ± 0.06	0.58 ± 0.06	0.091 ± 0.010	0.91 ± 0.10
	2	0.3 ± 0.2	0.8 ± 0.4	14.6 ± 3.3	11.1 ± 2.7	4.2 ± 1.0	0.63 ± 0.12	0.55 ± 0.09	0.092 ± 0.017	0.90 ± 0.17
Tsampa	1	0.6 ± 0.2	1.5 ± 0.4	0.2 ± 0.1	2.3 ± 0.5	2.5 ± 0.6	0.51 ± 0.07	0.14 ± 0.02	0.018 ± 0.003	0.09 ± 0.01
	2	1.0 ± 0.5	2.4 ± 0.9	0.3 ± 0.1	3.0 ± 0.6	3.3 ± 0.8	0.53 ± 0.11	0.15 ± 0.03	0.019 ± 0.004	0.09 ± 0.02
*Tsampa*_supp	1	1.5 ± 0.5	3.8 ± 1.2	17.6 ± 3.9	19.8 ± 5.0	9.4 ± 2.5	1.11 ± 0.24	0.61 ± 0.13	0.093 ± 0.019	0.80 ± 0.17
	2	2.5 ± 1.2	5.6 ± 2.2	25.8 ± 4.7	27.6 ± 4.3	12.7 ± 1.9	1.42 ± 0.19	0.78 ± 0.10	0.122 ± 0.016	1.03 ± 0.14
Urinary excretion										
Reference	1	17.1 ± 1.7	45.2 ± 5.3	0.7 ± 0.2	1.5 ± 0.2	3.1 ± 0.6	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01
	2	17.4 ± 3.0	45.8 ± 8.7	0.7 ± 0.2	1.9 ± 0.4	3.1 ± 0.7	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01
Tsampa	1	8.1 ± 1.1	24.2 ± 2.9	0.2 ± 0.0	11.8 ± 1.2	3.1 ± 0.4	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01
	2	9.7 ± 1.6	30.4 ± 4.4	0.2 ± 0.0	13.9 ± 1.3	3.6 ± 0.7	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01
*Tsampa*_supp	1	24.9 ± 4.8	79.7 ± 15.3	0.8 ± 0.3	2.5 ± 2.2	4.5 ± 1.0	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01
	2	29.0 ± 5.5	92.6 ± 18.5	0.8 ± 0.2	2.7 ± 3.6	4.7 ± 1.3	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01

Table 7. Apparent digestibilities and apparent retentions of the diet and the investigated minerals in the experimental diets (%)

Experimental diet	**Period**	**Diet**	**Na**	**K**	**Ca**	**P**	**Mg**	**Fe**	**Zn**	**Cu**	**Mn**
Apparent digestibility											
Reference	1	90 ± 3	98 ± 1	99 ± 1	78 ± 6	81 ± 4	53 ± 7	12.9 ± 7.8	5.6 ± 6.0	11 ± 7	7.1 ± 7.5
	2	90 ± 3	98 ± 1	99 ± 1	68 ± 4	76 ± 4	51 ± 8	11.1 ± 8.6	7.3 ± 7.7	8 ± 9	7.1 ± 8.7
Tsampa	1	88 ± 1	94 ± 2	95 ± 1	91 ± 2	89 ± 2	61 ± 7	-0.6 ± 10.6	1.9 ± 9.9	-5 ± 15	-1.7 ± 12.2
	2	87 ± 3	92 ± 4	94 ± 2	89 ± 3	88 ± 2	57 ± 8	12.6 ± 13.2	12.1 ± 10.7	8 ± 13	8.9 ± 13.1
*Tsampa*_supp	1	89 ± 3	96 ± 1	96 ± 1	63 ± 5	71 ± 5	48 ± 10	20.4 ± 10.9	13.1 ± 11.4	21 ± 9	17.7 ± 11.6
	2	88 ± 4	94 ± 3	95 ± 2	54 ± 5	65 ± 3	38 ± 6	11.9 ± 4.7	3.6 ± 4.9	10 ± 4	8.4 ± 5.9
Apparent retention											
Reference	1	-	19 ± 8	26 ± 8	76 ± 6	78 ± 4	17 ± 6	12.7 ± 7.8	5.6 ± 6.0	10 ± 7	7.0 ± 7.5
	2	-	16 ± 12	23 ± 14	67 ± 4	72 ± 3	15 ± 9	10.9 ± 8.6	7.4 ± 7.6	7 ± 9	7.0 ± 8.7
Tsampa	1	-	12 ± 10	18 ± 8	85 ± 3	35 ± 5	13 ± 7	-0.8 ± 10.5	1.6 ± 9.9	-7 ± 14	-1.9 ± 12.2
	2	-	10 ± 9	13 ± 8	84 ± 2	35 ± 3	11 ± 10	12.4 ± 13.2	12.0 ± 10.6	4 ± 13	8.7 ± 13.1
*Tsampa*_supp	1	-	24 ± 10	14 ± 10	62 ± 5	67 ± 4	22 ± 6	20.3 ± 10.8	13.2 ± 11.3	20 ± 9	17.6 ± 11.6
	2	-	22 ± 8	13 ± 11	52 ± 5	62 ± 4	16 ± 4	11.8 ± 4.7	3.7 ± 4.9	10 ± 4	8.4 ± 5.9

Apparent digestibility = [(Ingesta-feces)/Ingesta]; Apparent retention = [(Ingesta-feces-Urines)/Ingesta]

3.4 Mineral contents of femurs and livers

Table 8 lists the mineral content (mg/Kg on a dry matter basis) of femurs and livers, according to the experimental diet. The femurs of the *tsampa* group present significantly lower contents ($p<0.001$) in calcium and phosphorus compared to the two other groups. On the other hand, the levels of potassium, magnesium and zinc are significantly higher in the femurs of the *tsampa* group. Iron content is also significantly higher in the latter compared to the reference group, but not compared to the *tsampa*_supp group. For technical reasons, the copper content in femurs has not been determined properly, and data are not reported.

Livers of the *tsampa* group present significantly lower calcium content ($p<0.001$) and significantly higher phosphorus content ($p<0.001$) compared to the two other groups. Higher levels of magnesium, zinc, and iron ($p<0.001$) are also observed. Unexpectedly, the mean copper content of the livers of the *tsampa*_supp group is significantly lower compared to the two other groups ($p<0.001$). The reference group had the lowest liver manganese content, while the *tsampa*_supp group had the highest ($p<0.001$); the *tsampa* group being in-between.

Table 8. Mineral contents of femurs and livers (^{1} g/Kg, ^{2} mg/Kg, expressed in dry matter)

Diet	Na^1	K^1	Ca^1	P^1	Mg^1	Fe^2	Zn^2	Cu^2	Mn^2
Femur									
Reference	5.26 ± 0.28 ^{a}	2.99 ± 0.39 ^{b}	186 ± 9 ^{a}	119 ± 2 ^{a}	3.17 ± 0.14 ^{c}	75.9 ± 21.2 ^{b}	97 ± 9 ^{c}	- ± -	1.25 ± 0.18 ^{a}
Tsampa	4.78 ± 0.23 ^{b}	4.28 ± 0.50 ^{a}	128 ± 6 ^{b}	89 ± 3 ^{c}	5.34 ± 0.30 ^{a}	97.7 ± 10.2 ^{a}	217 ± 23 ^{a}	- ± -	1.34 ± 0.79 ^{a}
*Tsampa*_supp	4.81 ± 0.33 ^{b}	2.83 ± 0.25 ^{b}	178 ± 11 ^{a}	114 ± 6 ^{b}	3.51 ± 0.21 ^{b}	91.0 ± 17.0 ^{a}	132 ± 12 ^{b}	- ± -	1.16 ± 0.14 ^{a}
Liver									
Reference	1.92 ± 0.12 ^{b}	11.0 ± 0.78 ^{b}	0.110 ± 0.007 ^{b}	8.6 ± 0.4 ^{c}	0.55 ± 0.03 ^{b}	313 ± 29 ^{c}	59 ± 6 ^{b}	14.6 ± 4.3 ^{a}	5.6 ± 0.4 ^{b}
Tsampa	2.58 ± 0.23 ^{a}	12.1 ± 1.06 ^{a}	0.084 ± 0.009 ^{c}	10.6 ± 0.6 ^{a}	0.66 ± 0.05 ^{a}	443 ± 47 ^{a}	96 ± 11 ^{a}	12.1 ± 1.0 ^{a}	6.2 ± 0.6 ^{a}
*Tsampa*_supp	2.50 ± 0.24 ^{a}	11.6 ± 1.30 $^{a.b}$	0.142 ± 0.015 ^{a}	9.4 ± 0.6 ^{b}	0.57 ± 0.02 ^{b}	353 ± 39 ^{b}	65 ± 7 ^{b}	9.2 ± 0.7 ^{b}	6.7 ± 0.5 ^{a}

^{a,b,c} within a period and a column, means associated to different letters present significant differences

4. Discussion

4.1 Animal performances and biometric analysis

The mean weights of the rats at the beginning and at the end of the experiment indicate that the different groups constituted were homogenous at the beginning, and that significant effects of diets on growth are observable after seven weeks (table 3). The always close to zero mean growth rates during the period of individual housing probably reflect a behavioral disturbance of these growing rats. This species is indeed known to be social. The ADGs, also always close to zero in the three groups, further confirm this hypothesis (table 4). All data about the minerals and trace elements ingestions and excretions obtained during the periods of individual housing should therefore be interpreted considering a model of maintenance of metabolism rather that a model of growth.

The mean energy daily intakes (EDIs) can be calculated by multiplying the mean diet daily intakes (DIs, table 4) by the energy contents of the diets (table 2). Both periods included, the mean EDIs are of 48.4, 24.2, and 35.6 Kcal/day for the reference, the *tsampa*, and the *tsampa*_supp groups, respectively. Compared to the reference group, the EDI of the *tsampa* group represents about 50%, and the EDI of the *tsampa*_supp group about 70%. Compared to the latter, the EDI of the *tsampa* group is of about 68%. Even if we are inclined to consider maintenance model rather than a growth model, one can reasonably think that such differences in energy intakes are probably the main factor accounting for the different mean weights at the end of the experiment (Lee et al., 1986). Considering that the three diets had similar contents in carbohydrates, their difference in energy content is mainly accountable to difference in fat contents.

The lower level of dry matter in the femurs of the *tsampa* group compared to the two other groups is likely to be due to a lower bone mineral content (table 5). This hypothesis is further supported by the significantly lower calcium and phosphorus contents of the femurs of the *tsampa* group. Compared to the two other groups, the *tsampa* group presented concentrations in Ca and P of the femurs lower from 30 to 40% (table 8). It is more difficult to explain the higher relative femur weight of the *tsampa*_supp group compared to the reference group. The evolution of the length of the femur of a rat according to its age is following a complex relation in which many factors can interfere (Wunder et al., 1979). One must keep in mind that the reference group was fed a diet deficient in protein, fat, and calcium. And the *tsampa*_supp group is further burdened by energy deficiency, and severe deficiencies in protein and fat. A possible overload in some elements due to high concentrations in the *tsampa*_supp diet should not be excluded.

As to the liver, which is a major actor of lipid metabolism, the higher relative weight of the liver the reference group could be explained first by the higher food intake of this group, but also by the fact that the reference diet contains more fat than the diet of the two other groups. The difference between the *tsampa* and the *tsampa*_supp groups could, for its part, be explained by the higher food intake, and higher energy intake, of the latter, and possibly also by the higher vitamin and mineral content of their diet (Younossi, 2014).

4.2 Apparent digestibility and apparent retention

Sodium and potassium are alkali elements hardly retained by many animal metabolisms. These elements are principally absorbed by diffusion in the intestines, and are mainly excreted in urine (Martin, 2000). As it was expected, their apparent digestibilities were high, and their apparent retentions were low for the three groups.

For both periods, the *tsampa* group had significantly higher ADs for calcium than the two other groups (table 7). Calcium is absorbed by diffusion in the in the ileum, but also by active transcellular transport in the duodenum (Martin, 2000). The active transport allows an effective and reactive absorption mechanism (Morgan et al., 2007). It is likely that the rats of the *tsampa* group had a higher active absorption rate in response to a diet severely deficient in calcium (Table 2). The very low urinary excretions for this element also reflect an important retention, presumably in order to increase the metabolic pool and ensure growth.

As for calcium, the rats of the *tsampa* group present significantly higher ADs for phosphorus compared to the two other groups. But whereas about 3 to 5% of the ingested amount of phosphorus is excreted in urine by the reference and the *tsampa*_supp groups, more than 60% of the ingested phosphorus is excreted by this pathway for the *tsampa* group. One hypothesis is an important excretion of phosphorus consequent to insufficient amounts of calcium ingested, preventing its accretion under the form of hydroxyapatite into bones (Martin, 2000). Secondary hyperparathyroidism is also an attractive hypothesis that can account for some observations: low renal calcium excretion and high intestinal calcium absorption (Marieb, 2005).

Something unexpected hides behinds the differences in ADs for calcium, phosphorus, and magnesium between the three groups. The two *tsampa*-based diets are supposed to contain substantial amounts of phytic acid (table 2), the main storage form of phosphorus in plants (Kumar et al., 2010). Phytic acid is well known to form insoluble complexes with calcium, magnesium, and other elements, like iron and zinc, at the intestinal pH (Kumar et al., 2010). Consequently, one might expect ADs of both *tsampa*-base diets to be lower for Ca, P, and Mg compared to the reference group. One might also expect, at least for magnesium, higher ADs for the *tsampa*_supp group than for the *tsampa* group, considering that the *tsampa*_supp diet has been considerably supplemented in this mineral. But it is not so. A higher active absorption of calcium in the *tsampa* group is a credible hypothesis, even if it does not fit well with the significant amounts of phytic acid. On the opposite, absorption mechanisms for inorganic phosphorus and magnesium are supposed to predominantly rely on passive absorption. The significantly higher ADs for these elements in the *tsampa* group are more difficult to explain (Martin, 2000). More logically, the *tsampa*_supp group presents lower ADs compared to the reference group for both calcium and phosphorus. ARs are also lower, reflecting a higher urinary excretion in the *tsampa*_supp group. This is probably the consequence of higher intakes for these elements (table 4).

The high relative standard deviations of the ADs and ARs for Fe, Zn, Cu, and Mn compared to the other elements make the interpretation of these data difficult. Considering that the performance requirements of elemental analysis are the same for every element, these differences in ADs variability can reasonably be attributed to individual variability and not to analytical uncertainty. The generally high variability observed for these elements is therefore a consequence of the individual variability within the groups of rats. It is likely that all rats do not react the same way to the stress of individual housing. Most of them are probably in the situation of a maintenance metabolism behavior model, as suggested by the close-to-zero average daily gains. Nevertheless, individual data (not shown), suggests that a few rats were able to keep a growth metabolism.

4.3 Mineral contents of femurs and livers

The significantly lower contents in calcium and phosphorus of the femurs of the *tsampa* group compared to the two other groups is clearly supporting our hypothesis about the difference observed in dry matters of femurs between the groups. The lower dry matter level of the femurs of *tsampa*_supp group compared to *tsampa* group is the consequence of a lower bone mineral content. Given the higher level of magnesium in the femurs of the *tsampa* group, one can put forward that the deficiency in calcium is partly compensated by higher accretion of magnesium in bones. This phenomenon seems however limited considering that the urinary excretion of magnesium is also significantly higher in this group, despite the lowest dietary intakes.

The *tsampa* group showed significantly higher levels of magnesium, zinc, and iron in liver (all $p<0.001$) compared to the two other groups. This tendency is also observable in the femurs ($p<0.01$ or lower). As bones and liver are both storage organs for these elements, it could be a good argument to support the hypothesis that the intakes and the absorption of these elements are not limiting factor in the growth of the *tsampa* group in this particular dietary context.

Copper, which is also stored in the liver, was found to be severely deficient in the *tsampa* diet (table 2). The mean daily intakes of the *tsampa* group for this element represent less than 20% of the mean daily intakes of the reference group. Yet, the fecal excretion rate is higher in the *tsampa* group, while its liver content did not present significant difference with the reference group. Further, the mineral supplement added in the *tsampa*_supp diet increased the mean daily intakes up to about 125% and 670% compared to the mean daily intakes of the reference and the *tsampa* groups, respectively. The fecal excretions rates in the *tsampa*_supp group were found to be lower than that of the *tsampa* group, and close to that of the reference group. Yet, the mean copper content of the livers of the *tsampa*_supp group was significantly lower compared to the two other groups ($p<0.001$). In rats, copper is known to accumulate in the liver until the 12th day of life, and bile excretion is blocked. From the 13th day on, bile excretion is unblocked, and ceruloplasmin (the extracellular copper carrier) secretion increase, resulting in a decreased liver content (Zatulovskaia et al., 2015). The high fecal excretion rates observed for the three groups could therefore be explained by an important excretion through bile. Yet, the fact that the *tsampa*_supp, which present the higher copper intakes, and the lower fecal and urinary excretion rates, had the lowest liver concentration is hard to explain. As the results do not suggest any problem with regard to absorption in this group, a possible reason could be a competition with zinc when binding to metallothionein once the element is absorbed, or a higher ceruloplasmin activity compared to the two other groups (Petering and Fowler, 1986, Zatulovskaia et al., 2015).

Although close to the reference group, the *tsampa*_supp group had the highest mean daily intakes in manganese, and the lowest fecal excretion rates. Logically, livers of the rats of this group present a significantly higher manganese content than the reference group ($p<0.001$). Less logically, despite the lowest daily intakes (about 10% of the reference intakes), and the highest fecal excretion rates (>95% of the ingested amount), the manganese content of the livers of the *tsampa* group is also significantly higher than the reference group ($p<0.05$). Moreover, there is no significant difference between the two *tsampa*-based diets. Normally, the manganese body level is finely regulated through gastrointestinal absorption and hepatobiliary excretion (Aschner and Aschner, 2005). Nevertheless, high dietary intakes can result in elevations in tissue of Mn concentrations. The observed results are not in line with the literature, as the *tsampa* group had Mn intakes representing about 10% of the reference intakes, while the *tsampa*_supp group intakes were close to the reference group. Although not conclusive, the beginning of an explanation may lie in the difference of bioavailability of manganese in the different diets. In the reference diet, the manganese is under the form of manganese carbonate, which is of low solubility ($Kps = 2.24 \times 10^{-11}$), whereas in the *tsampa*-based diet, a significant amount of manganese come from the tea, and is reputed of high bioavailability (Greger, 1998; J. Powell et al., 1998). However, fiber and polyphenols, which might be present in significant amounts in the *tsampa*-based diets, are known to decrease the bioavailability of Mn. Another hypothesis would be that both *tsampa* and *tsampa*_supp groups maintain a higher level of manganese content in the tissue for an unknown purpose. The bioavailability of manganese in the traditional Tibetan dish called *tsampa pag* deserve further investigation as manganese excess is likely to have deleterious impact on bone metabolism (Kies, 1987).

5. Conclusion

The three groups of rats constituted presented homogenous mean weights at the beginning of the experiment. After seven weeks fed with their respective diet, the mean weights were all significantly different (p<0.001). The rats of the *tsampa* group had the lowest mean weight while rats of the reference group had the highest. Rats of the *tsampa*_supp diet being in-between. The lower food intakes and energy intakes of *tsampa* group and the *tsampa*_supp group are presumably the first factor accounting for the growth retardation compared to the reference group. The deficiencies in protein of both *tsampa*-based diets compared to the reference diet are also susceptible to play a role, possibly through a decrease in palatability of these diets.

The severe calcium deficiency of the *tsampa* diet is also, in all probability, an important factor in the growth retardation of the *tsampa* group compared to the two other groups. Although known to form insoluble complexes, the important amounts of phytic acid in the *tsampa* diet did not seem to burden the apparent absorption rates of calcium, phosphorus, and magnesium. On the contrary, they were all higher in this group compared to the two others. Yet, the *tsampa* group was found to excrete more than 60% of the apparent amount of phosphorus absorbed, whereas it is about 5% in the two other groups. Biometric analysis and elemental analysis confirm a lower bone mineral content in the femurs of the *tsampa* group.

It is more difficult to draw firm conclusions about iron, zinc, copper, and manganese considering to the important individual variability combined with low apparent digestibilities. Despite high fecal excretion rates, the similar or even significantly higher concentrations found in the livers and the femurs of the *tsampa* group did not suggest any limitation with regard to these elements. When supplemented with the mineral and vitamin mixture, the rats of the *tsampa*_supp group presented lower fecal and urinary copper excretions, but their liver content decreased, which may be a sign of secondary deficiency.

Acknowledgement

The authors thank Christopher Chif for his essential help in the animal experimentation and elemental analysis, as well as Geneviève Jean and the entire staff of the unit of Animal Science for sharing their experience and for providing valuable help.

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Chapter VII.

“The great end of life is not knowledge but action”

Thomas Henry Huxley

Conclusions and perspectives

In Tibet Autonomous Region, rural populations are affected by an endemic and chronic disease called Kashin-Beck Disease. Behind this name hides an osteochondropathy that can affect young children as soon as weaning. Although being the subject of a large number of studies for the last hundred years, its etiology remains unclear. However, the noose is tightening around a multifactorial hypothesis involving environmental factors. Mycotoxins poisoning and a combined iodine and selenium deficiency are two hypothetic factors that had been subject of numerous investigations. Unexpectedly, many studies conducted *in situ*, either epidemiological or randomized controlled trials, produced contradictory results, puzzling scientists who address the question.

Because most of the studies about the mineral deficiencies focused essentially on selenium and iodine, and considering that more than one chemical element is involved in bone and joints metabolism, the present thesis aimed at expanding the scope of investigation of this mineral deficiency hypothesis. The primary objective was to evaluate the mineral dietary status of the young Tibetan children living in rural areas of T.A.R. endemic for KBD.

A first step in this way was to get an overview of the elements, beneficial or toxic, which are known to impact bone metabolism. Not less than thirty elements were identified. Few appear to be exclusively toxic, some are thought to be exclusively beneficial, but most of them are both essential and deleterious, depending on the amounts and relative quantities. For the great majority, the underlying molecular mechanisms are not yet fully elucidated. It is not surprising given the complexity of the bone system. Many of these elements were not deemed relevant for investigation in the context of KBD. Among the eligible elements, a focus has been made on the main essential dietary minerals and trace elements, namely Na, K, Ca, P, Mg, Fe, Zn, Cu, Mn, and Se.

Before deciding the design of the nutrition survey, another essential step consisted in the evaluation of the adequacy of existing food composition tables, which are inescapable tools in every study with nutritional assessment purpose. Indeed, the accuracy of the results of such a study will greatly depend on the closeness between the data of the FCTs, and the actual composition of the foods consumed by the targeted population. In this purpose, a preliminary study compared the mineral content of locally grown Tibetan foods with similar foods listed in two most relevant FCTs: the USDA National Nutrient Database and the China Food Composition Table. The results revealed significant discrepancies between the Tibetan food locally grown and the two FCTs, suggesting a high risk of introducing important bias by using them for nutritional assessment in rural Tibet.

It logically led us to analyze the mineral composition of the traditional foods and beverages of rural Tibet. For this purpose, 1119 samples from sixteen different kinds of food products and beverages were collected in 65 rural villages of the Lhasa Prefecture. Locally grown crops and traditional foods were especially targeted, as their mineral composition is highly depending on the agro-environmental conditions. We also believe the mineral compositions of some food items, such as dried yak meat, was never reported before. Combining data from the China FCT, the USDA National Nutrient Database, and our own results, we had at our disposal reliable food composition data for nutritional assessment in rural T.A.R.

A cross-sectional study involving 250 young Tibetan children aged 3 to 5 was designed and conducted. Food intakes of the children were recorded by interviewing the caretakers using the 24-hour food recall method. Interviews were done twice, in two different seasons, by trained local interviewers. Combining these data with food composition data allowed an accurate calculation of the usual dietary intakes of energy, water, alcohol, and minerals and trace elements (Na, K, Ca, P, Mg, Fe, Zn, Cu, Mn, and Se) of these young rural Tibetan children. The results of this study revealed a challenging dietary context. The diet of these children appeared to be very monotonous and poorly diversified. It is mainly based on cereals products, with low consumption of meat and dairy products, and even lower consumption of vegetables and fruits. Alcohol drinking was not uncommon, even at early ages. Compared to the Chinese dietary recommended intakes, this diet presented several imbalances, with too much sodium and manganese, and conversely far too little potassium, calcium, zinc, copper, and selenium. Compared to the harmonized estimated average requirements, more than 90% of the children present inadequately lower intakes for water, potassium, and calcium. The proportions of children presenting inadequate intakes for copper and selenium are also probably high, but a violation of the normality condition of their distribution prevented their calculation. On the other hand, more than 25% and 18% of the children exceeded the upper intake levels for sodium and manganese, respectively. In the light of these results, one has to consider the probability of consequences of such dietary imbalances on growth and bone metabolism of the rural Tibetan children.

This survey assessed the usual intakes for several minerals and trace elements, and compared them to Chinese DRIs and the harmonized EAR. However, these general recommendations may not be fully relevant to a very specific population such as rural Tibetans. This may be a major limitation to the conclusions of the study. Very monotonous and mainly based on cereals and cereals based products, their diet is rich in phytic acid and other natural compounds, such as polyphenols and fibers, known to affect bioavailability of several minerals. As a matter of fact, the mainstay of the

Tibetan diet, called *tsampa pag*, a traditional dish made of roasted barley flour mixed with yak butter tea, combines all the factors previously cited. Because it is highly susceptible to influence dietary recommendations, the question of bioavailability of the mineral elements in the rural Tibetan diet was not to be ignored. We therefore conducted an animal experimentation on a rat model in order to assess growth performances, apparent digestibilities and apparent retentions of selected minerals and trace elements of dietary relevance in *tsampa pag*.

For this purpose, thirty-six Sprague Dawley® rats were enrolled and divided into three groups. Three experimental diets were made, and each group of rats was assigned to one of the diet during seven weeks. The first experiment diet was prepared following the traditional recipe of *tsampa pag*. A reference diet was made to meet the dietary requirements of the rats, with the exception of decreased protein content, in order to be closer to the *tsampa* diet. A third experimental diet was similar to the *tsampa* diet but it was supplemented with the same minerals and vitamins mixture used in the reference diet. After seven weeks fed with their respective diets, the rats of the *tsampa* group had the lowest mean weight while rats of the reference group had the highest. Rats of the *tsampa*_supp diet being in-between. It was obviously the consequence of lower food intakes and energy intakes in the *tsampa* group and the *tsampa*_supp group. The deficiencies in protein and fat of both *tsampa*-based diets compared to the reference diet are also susceptible to play a role, possibly through a decrease in palatability of these diets. The severe calcium deficiency of the *tsampa* diet is also, in all probability, an important factor in the growth retardation of the *tsampa* group compared to the two other groups. The rats of the *tsampa* group had a lower bone mineral content attributable to low calcium intakes and important phosphorus urinary excretion. Conversely they had higher potassium, magnesium, iron, and zinc contents compared to the reference group. The important amounts of phytic acid in the *tsampa* diet did not seem to burden the apparent absorption rates of calcium, phosphorus, and magnesium. Although low apparent digestibilities and important individual variability which prevent definite conclusions to be drawn, the results did not suggest any limitation in the *tsampa* group with regard to iron, zinc, copper, and manganese. However the mineral and vitamin supplementation seemed to induce a secondary deficiency in copper in the rats of the *tsampa*_supp group.

Tibet Autonomous Region is located at the top of the world, with a unique geological framework and ecoclimatology. Living almost in autarky in a harsh environment for thousands of years, Tibetans are a vivid example of the extreme adaptability of the human species. No doubt that their metabolism has adapted as best possible (Simonson et al., 2010; Yi et al., 2010). However no-one should be charged beyond his capacity. Many nutrients are essential to any living organism, and even the most resilient have their limits. Many studies revealed broad prevalence of stunting and malnutrition in T.A.R. The present thesis gave strong evidence to support imbalanced intakes in several minerals and trace elements essential to healthy growth and bone metabolism. Our study conducted on rats fed with *tsampa* further suggests a challenging situation with regard to minerals and trace elements. But it revealed a remarkable capacity of the metabolism to struggle for survival. The results strengthened the importance of energy, but also calcium deficiency in the growth retardation. Possible secondary deficiencies are to be excluded, as well as excesses.

With regard to results presented here, it seems mandatory:

- To confirm the results by a broader nutrition survey, ideally with a case-control study conducted in highly endemic areas;
- To proceed to a clinical evaluation of the mineral status of the Tibetans, balancing all ethical and medical considerations;
- To not refrain to the main dietary elements. In the light of chapter III of the present thesis, at least six more little studied elements essential or highly beneficial for bone metabolism deserve attention (As, B, Cr, Mo, Si, and V);
- To investigate for possible chronic exposure to toxic elements, such as aluminum or lead, whose levels in some Tibetan foods may not be innocuous.

Although it remains high in some areas of T.A.R., today, the overall prevalence of the Kashin-Beck disease seems to decrease. This may be the result of voluntary actions of engaged people or a consequence of the fast socio-economic development of T.A.R. It is probably both, but where the balance lies is difficult to judge. One thing is obvious for anyone having been in T.A.R., the inevitable changes are beginning to influence eating habits of rural people. A better accessibility to food products that cannot be grown in T.A.R. – such as rice, vegetables, and fruits – may have nothing but beneficial impact on the diversification of the Tibetan diet. On the other hand, industrialized foods and beverages of poor nutritional quality seem to make a significant appearance. Rural people, with frequent low education levels, may not be armed with the knowledge necessary to exercise critical judgment with this influx of new commodities. Several solutions are already implemented or can be envisaged to improve the situation, but above all to prevent it from following paths that would be regretted later. It would be presumptuous for someone to claim he has the solution, but what can be said loud and clear is that any solution considered must be integrated, sustainable and fully respectful of people and socio-cultural environment.

Scientific publications and communications

Peer-reviewed publications

As first author

Dermience, M., Mathieu, F., Barthelemy, J.-P., Maesen, P., Romnee, J.-M., De Maertelaer, V., Denchen, Y., Tsewang, P., & Lognay, G. (2013). The relevance of food composition data for nutrition surveys in rural Tibet: pilot study in the context of Kashin-Beck Disease. *Biotechnology, Agronomy, Society and Environment [=BASE], 17(1), 32-42.*

Dermience, M., Li, X. W., Mathieu, F., Claus, W., De Maertelaer, V., Yangzom, D., & Lognay, G. (2014). Minerals and trace elements in traditional foods of rural areas of Lhasa Prefecture, Tibet Autonomous Region (P.R. China). *Journal of Food Composition and Analysis, 35(2), 67-74.*

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Dermience, M., Mathieu, F., Li, X. W., Vandevijvered, S., Claus, W., De Maertelaere, V., Dufourny, G., Bing, L., Yangzom, D., Lognay, G. (2016). Minerals and trace elements intakes of young Tibetan children living in rural areas endemic for Kashin-Beck disease: a cross-sectional survey. Submitted in *Public Health Nutrition.*

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Dermience, M., Brostaux, Y., Maesen, Ph., Lognay, G. (2016). Study of matrix effects occurring during analysis of food products by microwave nitrogen plasma atomic emission spectrometry. In preparation for *Food Analytical Methods.*

As co-author

Aguedo, M., Fougnies, C., **Dermience, M.**, & Richel, A. (2014). Extraction by three processes of arabinoxylans from wheat bran and characterization of the fractions obtained. *Carbohydrate Polymers, 105, 317-324.*

Tchuenchieu, A., Essia Ngang, J-J., Servais, M., **Dermience, M.**, Sado Kamdem, S., Etoa, F-X., Sindic, M. (2016). Color and nutritional value of orange juice processed at mild temperature in combination with carvacrol. In preparation

Makengo Kafuti, G., Mbemba Fundu Th., Lognay, G., **Dermience, M.**, Sindic M. (2016). Vamine: a food supplement based on local food resources, good nutritional values and free Phytates and cyanides. In preparation for *International Journal of Biotech Trends and Technology (IJBTT).*

Scientific Congress an Symposia

Oral presentations

Rooze, S., de Voghel, P., Mathieu, F., Robert, M., Lobsang, R., Wangdu, L., **Dermience, M.**, Lognay, G., & Goyens, P. (October 14). Food intake of Tibetan children living in Kashin Beck disease endemic areas in Central Tibet. Paper presented at BIT's 1st Annual Congress of Nutrition & Health 2013 (WCNH-2013), Dalian International Convention Center, Chine

Dermience, M., Mathieu, F., Li, X., Vandevijvere, S., Claus, W., de Maertelaer, V., Dufourny, G., Bin, L., Yangzom, D., & Lognay, G. (2015, September 18). Dietary mineral intakes of young Tibetan children living in areas endemic for Kashin-Beck disease: preliminary results of a cross-sectional survey. 36th SICOT Orthopaedic World Congress, Guangzhou, Chine

Mathieu, F., Claus, W., Lobsang, R., Wangdu, L., Sheero, R., **Dermience, M.**, Rooze, S., De Maertelaer, V., Hinsenkamp, M. (2015, September 18). Prevalence study for Kashin-Beck Disease in 6 counties of Lhasa prefecture in Tibet Autonomous Region, P.R.China. 36th SICOT Orthopaedic World Congress, Guangzhou, Chine

Posters

Dermience, M., Maesen, P., Mathieu, F., Goyens, P., Rooze, S., & Lognay, G. (2010,

	October 26). Kashin-Beck Disease: evaluation of mineral intake in young Tibetan children from endemic areas. Poster session presented at 5ème symposium du GCNAS, Bruxelles, Belgique. **Dermience, M.**, Maesen, P., Mathieu, F., De Maertelaer, V., & Lognay, G. (2012, June 01). Comparison of mineral intake between children from endemic and non-endemic areas for Kashin-Beck disease in Tibet Autonomous Region: Pilote study. Poster session presented at 6ème symposium du Groupe de Contact Nutrition Alimentation Santé (GCNAS), Louvain-La-Neuve, Belgique.
Master thesis	**Dermience, M.** (2010). Kashin-Beck disease: Evaluation of mineral intake in young tibetan children from endemic areas. Master thesis, Université de Liège, Gembloux Agro-Bio Tech, Belgique.
Varia	**Dermience, M. (**2012). La nutrition minérale et les carences en oligoéléments. Allocution filmée dans le cadre d'un « Doc'cafés : Des chercheurs à ma table ». **Dermience, M.,** Mathieu, F., Li, X., Vandevijvere, S., Claus, W., de Maertelaer, V., & Lognay, G. (2012). Investigation of the mineral intake of preschool children living in endemic Kashin-Beck disease areas. Study protocol.