Deciduous teeth as a tool to record early life exposure of zinc nutrition

Nieka A. Wahono, BDS, DDS, Paed Dent.

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Institute for Cell and Molecular Bioscience
Newcastle University, UK

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Abstract

Background: Zinc (Zn) is an abundant micronutrient and has essential roles in human growth and development. Zn deposition in human teeth has been reported and related to environmental exposures, such as pollution, disease, and dietary intake. The deciduous tooth lifespan is from the prenatal until childhood period, thus, it may record Zn exposure in children over this critical time frame. With the age-related incremental features of dentine and the development of micro-sampling techniques, Zn deposition may be analysed at different time points using Laser Ablation (LA)-ICP-MS in combination with dental histology. Zn is obtained from the diet and the distribution throughout the body and within cells is regulated by saturable, carrier-mediated transport proteins, known as ZnTs and ZIPs. The precise mechanism by which Zn is transported from the circulatory system of the pulp to the tooth hard tissue, however, remains unclear. The objective of this study therefore, was to analyse the Zn/Ca ratio in dentine of deciduous teeth in individuals consuming different levels of Zn during the pregnancy and infancy period, and elucidate the expression of Zn transporter in pulp tissue at the mRNA level in relation to dietary zinc intake.

Aims: to explore the use of the deciduous tooth dentine to record Zn exposure during pregnancy through to the infancy period, and to analyse the level of Zn transporter expression at the mRNA level in human dental pulp related to dietary intake of Zn.

Methods: Children, who attended Child Dental Clinic at Educational Dental Hospital of Universitas Indonesia for deciduous teeth extraction, paired with their mother were recruited for this study. Dentine from extracted deciduous teeth was prepared for elemental analysis (Zn, Ca and Sr) at different age points using LA-ICP-MS and dental histology. Sr distribution was used as a reference element since it has been studied widely in relation with dietary changes. The pulp from the same teeth was removed and prepared for RNA isolation. Food frequency questionnaires were developed to gather information on daily Zn intake of the mother during pregnancy and children during infancy. RNA from dental pulp of the human deciduous teeth were isolated and Zn transporter expression at the RNA level measured using RT-qPCR. In addition, human odontoblast cells were used as an *in vitro* model to analyse Zn transporter expression at mRNA level in response to high- and low-Zn exposure, using RT-qPCR.

Results: This study showed Zn distribution within dentine of deciduous teeth reflected Zn exposure during pregnancy and infancy period, as well as different feeding regimes during the first six month of life. There were differential patterns of Zn transporter

expression in human pulp RNA and in the human odontoblast cell line, levels of Zn transporter expression were altered in response to extracellular levels of Zn exposure. **Conclusion**: For the first time, we have shown a differential level of Zn distribution within dentine of deciduous teeth in relation to dietary Zn intake during pregnancy and the infancy period. The expression of Zn transporters in dental pulp indicates homeostatic control of Zn uptake into dental tissue, which may be regulated by dietary Zn intake.

Certificate of approval

I confirm that, to the best of my knowledge, this thesis represents an original research carried out by Nieka Adhara Wahono in fulfilment of the requirements for the degree of Doctor of Philosophy according to the regulations of Newcastle University.

Dr Ruth Valentine
Supervisor

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Abbreviations

µg	Microgram
µm	Micrometer
µM	Micromolar
AE	Acrodermatitis Enteropathica
Ba	Barium
BF	Breast Fed
BM	Breast Milk
BMI	Body Mass Index
C	Carbon
Ca	Calcium
CaCl2	Calcium chloride
cDNA	copy Deoxyrribonucleic Acid
cGCShc	Glutamate-Cysteine-Ligase heavy chain
CO2	Carbon dioxide
Cps	Count per Second
Cu	Copper
DNA	Deoxyribonucleic acid
DPC	Dentine Pulp Chamber
DR	Diet Record
DSPP	4′,6-diamidino-2-phenylindole
DSR	Daily Secretion Rate
EAR	Estimated Average Requirement
EDJ	Enamel Dentine Junctino
EDTA	Ethylenediaminetetraacetic acid
EURRECA	European Micronutrient Reccomendations Aligned
EW	Early Weaning
FBS	Fetal Bovine Serum
FF	Formula Fed
FFQ	Food Frequency Questionnaire
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenas
HAP	Hydroxyapatite

HDP-hTERT	Human Dental Pulp transfection with Human Telomerase Transcriptase
HZ	High-Zinc consumer
HZI	High-Zinc Infant
HZM	High-Zinc Mother
IEE	Inner Enamel Epithelium
IgG	Immunoglobulin G
IUGR	Intra Uterine Growth Retardation
IZiNCG	International Zinc Nutrition Consultative Group
KCl	Kalium chloride
KH2PO4	Monopotassium phosphate
LA-ICP-MS	Laser Ablation Inductively Coupled Plasma Mass Spectrometry
LBW	Low Birth Weight
L-FFQ	Long Food Frequency Questionnaire
LZ	Low-Zinc consumer
LZI	Low-Zinc Infant
LZM	Low-Zinc Mother
Max	Maximum
MF	Mixed Fed
Mg	Miligram
MgCl2	Magnesium chloride
Min	Minimum
mM	Milimolar
Mn	Manganese
MRE	Metal-Responsive-Element
mRNA	Messenger Ribonucleic Acid
MT	Metallothioneins
MTF	Metal-Responsive-Element Transcription Factor
N	Nitrogen
Na2HPO4.7H2O	Disodium hydrogen phosphate heptahydrate
NaCl	Natrium chloride
NIST-SRM	National Institute of Standard and Technology-Standard Reference Materials
NL	Neonatal Line

OEE	Outer Enamel Epithelium
Pb	Lead
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDL	Periodontal Ligament
RDA	Recommended Dietary Allowance
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
RT-qPCR	Real-time Reverse-transcription Polymerase Chain Reaction
SCN	Suprachiasmatic Nucleus
SD	Standard Deviation
SEM	Standard Error of the Mean
S-FFQ	Shortened Food Frequency Questionnaire
SILLS	Signal Integration for Laboratory Laser Systems
Sr	Strontium
UHT	Ultra-High Temperature
USDA	United Stated Department of Agriculture
v/v	volume per volume
w/v	weight per volume
ZIP	Zrt-, Irt-related proteins
Zn	Zinc
ZnCl2	Zinc chloride
ZnT	Zinc Transporter
Zrt	Zinc regulated transporter
ZTRE	Zinc Transcriptional Regulatory Element
α-MEM	Alpha modification-Minimum Essential Medium Eagle

Chapter 1 Introduction

1.1 Early life programming event

Early life period spans from prenatal/*in utero* to infancy, when there is a rapid process in growth, development, and maturation of human organs and systems (Langley-Evans, 2014). Exposure and adaptation to environmental factors during early life may encourage permanent changes in human physiology (Gluckman et al., 2008), which have been widely described as a "programming" event and associated with the plasticity of cells and tissues in the embryo and foetus (Langley-Evans, 2014).

The exposure of environmental factors during pregnancy, such as nutrition, infection, drug consumption, pollution, smoking and psychological stress, may affect the child's health in later life. For instance, a psychological stress in pregnant women due to physical alterations, hormonal changes, pregnancy-specific anxiety (such as the pressure to have a 'healthy' child and fear of pain during delivery), may lead to spontaneous abortion, structural malformations, preeclampsia, preterm delivery and low birth weight baby (Mulder et al., 2002).

Extensive studies, in regards to the relationship of nutrition in early life with the health in later life, have been conducted in the last twenty years (Tarry-Adkins and Ozanne, 2011). In 1911, the Hertfordshire project was carried out, where the midwives periodically recorded children's body weight, illnesses, growth, and feeding method from birth until one year old. The mortality rate was then studied in about 15,000 people, who were born during 1911-1930, and the results suggested that the risk of death by cardiovascular disease increased in people with low birth weight history, both males and females, but only in males with a low body weight at one year old (Syddall, 2005).

Furthermore, a series of human studies were carried out to analyse the relationship between undernutrition during early life and some diseases in later life. Birth weight and the body composition at birth, such as skinniness and the size of head circumference in proportion to body length, act as biomarkers to track the satisfactory nutrition of the foetus during pregnancy. An inverse relationship was found between

those biomarkers and the risk of Diabetes Mellitus type 2, increase in blood pressure, insulin resistance syndrome, osteoporosis, sarcopenia, and strokes in later life (Eriksson et al., 2001; Langley-Evans, 2014; Syddall, 2005).

Nutrition is an important energy source during growth and development period, when the rate of cell division increases rapidly. The body responds to undernutrition through the reduction in cell division rates, both directly or by the alteration of growth factors or hormone concentrations (Barker, 1995), which result in permanent changes within cells, tissues, organs, and systems of the human body and increases the risk of disease in later life. Nevertheless, a suboptimal maternal nutrition environment due to pre-existing maternal obesity, excess maternal weight gain during gestation and/or an obesogenic diet during pre-conception may cause overnutrition in the foetus. This may result in adverse effects on the offspring later in life. Additionally, the obesity in children and related unfavourable health problems in adulthood may be preceded by permanent changes in appetite control, metabolism, adipose tissue development and neuroendocrine function, due to the effect of foetal overnutrition condition (Ojha et al., 2013).

Human studies concerning the relationship between nutritional status during pregnancy, infancy, and childhood with a variety of non-communicable diseases, including diabetes mellitus type 2 and cardiovascular disease, still prompt many questions, particularly in correspond to the roles of confounding factors, such as lifestyle. Unfortunately, a study to gain explicit information regarding lifestyle during the human lifespan would take an extended period. Therefore, animal studies have been considered, as beneficial in controlling the genetic and confounding factors, in order to provide an overview about the consequences of undernutrition or excess nutrition during early life, which is known as the programming event, to the health in later life.

The programming events possibly result from changes in nutrition during early life through several mechanisms including: 1) permanent changes in organ structure or remodelling of organs, which involves disruption in the gene signalling during the phases of growth, differentiation, and maturation of organs and tissues; 2) persistent alteration in epigenetic modifications, such as DNA methylation and histone modification, which are memorized within cells and affect gene expression; 3) permanent effects on the regulation of cellular ageing, which increases in oxidative

stress and may encourage macromolecular damage to DNA and more specifically, to telomeres (Langley-Evans, 2014; Tarry-Adkins and Ozanne, 2011).

1.2 Zinc (Zn)

Zn is an essential mineral for human physiology and known as a trace element. The first report of Zn function was confirmed in plants by Raulin in 1869, when he was observing the growth of *aspergillus niger* mold. In animals, it was demonstrated as essential in a study performed in 1933. In the following years, several studies were conducted on Zn and suggested that it is essential for growth, development, and differentiation, as well as for the well-being of humans, microorganisms, plants and animals (McCall et al., 2000).

In 1961, Prasad and colleagues reported Iranian males who suffered from iron deficiency anemia, hepatosplenomegaly, hypogonadism, dwarfism and geophagia. It was discovered that the growth retardation and hypogonadism was due to Zn deficiency related to their dietary habits. At the time, people in Iran and Egypt, particularly those who lived in villages, only consumed bread and beans (Prasad, 2009).

1.2.1 Zn nutrition in early life

Zn is important during the prenatal, lactation and first year of life, when the fastest rate of growth and development occurs. There is a significant vulnerability to Zn deficiency, especially for cells that are rapidly turning over, such as a rapidly growing embryo or foetus (Hambidge, 2000). In humans, the relationship between Zn-deficiency and pregnancy outcomes is poorly understood, however, a mild Zn deficiency during pregnancy is associated with a difficult and prolonged labour, preterm labour, placental abruption and haemorrhage, postpartum haemorrhage, low birth weight (LBW), intrauterine growth retardation (IUGR), pregnancy-related toxaemia (Chaffee and King, 2012; Salgueiro et al., 2002), an adverse effect on neurobiological and behavioural development (Caulfield et al., 1998; Prasad, 1996), and poor development of natural immunity (Shah and Sachdev, 2001).

A recent study using mice, as a model to duplicate the low Zn intake of the mother during pregnancy, was carried out and demonstrated a low birth weight offspring with persistent alterations in epigenetic modification due to Zn deficiency during the

prenatal period, although not in adulthood (Kurita et al., 2013). Tomat et al reported that rats, which had suffered from a moderate Zn deficiency since pregnancy until weaning, showed the risk of cardiovascular disease increased in the adult offspring. It was shown that arterial systolic blood pressure elevated, while the number and glomerular filtration function of nephrons reduced (Tomat et al., 2008, 2013). In addition to this, Zn deficiency in mice during pregnancy decreased IgM concentration in the first, second, and third generation offspring, although all offspring had been fed with adequate Zn diet (Uriu-Adams and Keen, 2010). In humans, there is a limited number of studies on Zn deficiency during early life and the outcomes that happen in later life. A previous longitudinal cohort in Nepal, however, did show the supplementation of Zn, folic acid, and iron during pregnancy, reduced the risk of microalbuminuria and peripheral adiposity in children at 6-8 year of age (Stewart et al., 2009). These essential roles of Zn have been studied over many years to elucidate the involvement of Zn in growth and development, immune function, reproduction, and non-communicable diseases, such as diabetes mellitus, Alzheimer's, and many other illnesses.

1.2.2 Zn bioavailability

Zn is an important micronutrient for human metabolism, which is not provided in human body but recruited from the diet. The highest dietary concentration of Zn is found in animal-source foods, such as the organs and/or flesh of beef, pork, poultry, fish and shellfish, and with lesser amounts in eggs and dairy products. Zn content is relatively high in nuts, seeds, legumes, and whole-grain cereals, and is lower in tubers, refined cereals, fruits, and vegetable (International Zinc Nutrition Consultative Group (IZiNCG) et al., 2004).

Zn bioavailability describes the fraction of Zn intake that is absorbed, retained, and used for physiological functions. The absorption level of Zn increased simultaneously with increase in Zn ingestion, while fractional absorption declined (Cousins and King, 2012). Zn bioavailability has a potency to change when other foods are present and act as an enhancer or inhibitor in Zn absorption. Phytate (myoinositolhexaphospate) is a molecule contained in plant foods such as seeds, roots, and tubers. The interaction between phytate and Zn ions results in insoluble Zn complexes, and thus, inhibits Zn absorption. Zn status may be compromised among different populations in the world as most staple foods, such as rice, corn, cereals and legumes, contain a high level of

phytate. However, the phytate level may be reduced by choosing low-phytate staple foods and/or by certain food preparation methods (Lönnerdal, 2000). Calcium (Ca) sources should be added because of their ability in binding phytate.

The phytate-Zn molar ratio is used to estimate the likely absorption of Zn from a mixed diet. It can be calculated as follows: (phytate content of foods/660)/(Zn content of foods/65.4), where 660 and 65.4 represent the molecular or atomic weights of phytate and Zn, respectively. Plant foods, like seeds and nuts, whole grain cereals, beans and lentils, and tubers, have phytate-Zn molar ratios greater than 15. The lower phytate-Zn molar ratio produces a higher Zn bioavailability (Cousins and King, 2012). Conversely, certain amino acids in Zn-rich foods, such as meat, liver, eggs, and seafood, exhibit their abilities to improve Zn solubility and enhance Zn absorption, thus, they may facilitate Zn uptake by establishing Zn complexes with high stability (Holt et al., 2012).

1.2.3 *Zn metabolism*

Zn absorption is conducted along the entire intestinal tract. The highest rate of Zn absorption is in the proximal jejunum, while another study demonstrated that the highest concentration of Zn appears in the duodenum immediately after a meal (Cousins and King, 2012). Zn is a small, hydrophilic, and a positively-charged ion that may not easily diffuse across the plasma or intracellular organelle membranes. Zn transporters are believed to be responsible for Zn absorption and homeostasis, including transporting, trafficking, and reacting to the availability of Zn (Yu et al., 2007).

Zn transporter proteins are transmembrane transporters that play opposite roles in cellular Zn homeostasis (Yu et al., 2007). Zn transporters with a specific role have been established and divided into two different groups as follows: 1) ZIP, also known as SLC39 has a role in Zn uptake from the extracellular space of the cell into cytoplasm or from the intracellular storage compartment when the Zn level in cytoplasm is low. There are 14 ZIP proteins identified in humans to date, ZIP1 to ZIP14 (Jeong and Eide, 2013); 2). ZnT, also known as SLC30, transfers Zn from cytoplasm to the intracellular compartment. It also transports Zn out to the extracellular space, in order to prevent Zn toxicity. There are ten identified ZnT proteins in humans, ZnT1 to ZnT10 (Huang and Tepaamorndech, 2013). Many studies have explored the role of Zn transporters in specific tissues and organs of the human body. Several diseases in humans have

been detected and associated with the reduction of Zn transporter function (Huang and Tepaamorndech, 2013; Jeong and Eide, 2013).

In the intestinal tract, the endogenous, secreted Zn is derived from the biliary system and the pancreas are available together with Zn from the diet (Krebs, 2000). It is assumed that free Zn ions are transported across enterocytes by Zn transporter protein in the apical membrane of the enterocytes. ZnT5, ZIP4 and ZIP11 have been identified in the apical membrane of the enterocyte and likely, play a role in Zn absorption (Cousins, 2010). Subsequently, Zn is transported into the circulatory system through the basolateral membrane of the enterocytes and binds to albumin in the plasma to be distributed to the cells, tissues, and organs in the human body (Cousins and King, 2012). ZnT1 in the basolateral membrane of the enterocytes appears to regulate Zn efflux to plasma as the Zn release is limited, due to a decrease in the expression of ZnT1 (Cousins, 2010). Ninety five per cent of Zn is stored in body organs, while only 0.1% of total body Zn is comprised in the plasma (Eide, 2006). The unutilized Zn for metabolism will go via the pancreas excretion route and is endogenously secreted into the gastrointestinal tract. Next, along with the unabsorbed dietary Zn, the endogenous secreted Zn will be excreted in faeces (Cousins and King, 2012). Approximately 90-98% of excess Zn is lost in faeces, while only 2-10% is recovered in the urine (Holt et al., 2012).

1.2.4 *Zn function*

The role of Zn in protein and enzyme function was discovered in 1940, when Keilin and Mann observed Zn in carbonic anhydrase enzymes (McCall et al., 2000). The wide utility of Zn in protein is related to the properties of Zn, such as the Lewis Acid characteristic of Zn^{2+} ion, single redox state, the flexibility of its coordination sphere with respect to geometry and the number of ligands associated and combined with the kinetic lability of coordinated ligands (Lim et al., 2005).

Zn has essential roles to play in living organisms, including: 1) catalytic and co-catalytic roles in enzymes, both metalloenzymes and other enzymes; 2) a structural and functional role in several proteins. Zn is involved in DNA replication and reverse transcription, and it performs a crucial role for the function of a number of metalloproteins; 3) a regulatory role in gene expression (Cousins and King, 2012; Stefanidou et al., 2006).

Zn assists the catalytic and co-catalytic role of enzymes, responsible in cell processes including DNA synthesis, normal growth, brain development, behavioural response, reproduction, foetal development, membrane stability, bone formation and wound healing (Stefanidou et al., 2006). More than 300 enzymes, which need Zn in their actions, known as metalloenzymes, have been assigned to six classes of Zn- related enzymes, including oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (Holt et al., 2012). However, the mechanism of Zn donation in apometalloenzymes remains unclear. In complex organisms, the signs of Zn deficiency or toxicity would not affect specific metalloenzymes. The visible physiological defect only appears when Zn levels are involved in a critical biochemical pathway (Cousins and King, 2012).

Furthermore, 2008 human proteins are possibly Zn-binding and are related to 10% of the human proteome, including Zn-finger proteins which are the most abundant class of Zn-binding protein in humans (Andreini et al., 2006). In 1985, a transcription factor called 'Zn-finger', was identified that performs as a tetrahedral Zn^{2+} coordination complex with two to four cysteines and up to two histidines (King and Cousins, 2014, Stefanidou et al., 2006). Zn-finger proteins have a broad cell distribution and facilitate the interaction of protein to other proteins, DNA, RNA and lipids (Holt et al., 2012). Thus, they are responsible for transcriptional and translational control, modulation of those processes and signal transduction (King and Cousins, 2014).

Advanced technology in biomolecular science indicates Zn transporter proteins may function in maintaining the availability of cellular Zn and distribute the essential transition of Zn ions to the whole body. Three main mechanisms of cellular Zn homeostasis are demonstrated by: 1) maintaining cellular Zn levels by importing (ZIP family) and exporting Zn (ZnT family) via membrane cells; 2) Zn-sensing molecules such as metallothionein (MTs) and metal-responsive-element-binding transcription factor-1 (MTF1); and 3) transporter-mediated sequestration into intracellular organelles, for instance endoplasmic reticulum, Golgi and lysosomes, by certain Zn transporters (Figure 1.1) (Fukada et al., 2011; Plum et al., 2010).

Recent studies demonstrated the role of MTF-1 (Metal-Responsive-Element (MRE)-binding Transcription Factor 1) in the regulation of genes, such as metallothioneins

(MTs), which encode metal ion storage proteins; ZnT1 which encodes a Zn efflux transporter protein; and glutamate-cysteine-ligase heavy chain (cGCShc), which encodes an oxidative stress-related protein. MTF-1 is an element which provides Zn responsiveness to many genes and explains the mechanism of Zn-sensing (Laity and Andrews, 2007)

MTF1 participates in chromatin binding via the MREs of the gene promoter when it is translocated to the nucleus due to Zn availability. The response of MTF1 to dietary Zn intakes may vary due to polymorphism in the Zn finger domain of the human MTF1 gene. However, a homologous transcription factor MTF2, which is involved in stem cell development, may act in gene repression during normal Zn status and gene activation in Zn depletion. MTF1, MTF2 and other transcription factors are assumed to play a role in Zn transporter gene expression, in regulating Zn homeostasis to respond to the Zn status (Cousins and King, 2012). Further studies explain the role of Zn transcriptional regulatory element (ZTRE) in regulating a network of Zn-dependent genes at promoter sites through a mechanism of promoter-reporter gene expression(Coneyworth et al., 2012).

1.2.5 *Zn homeostasis*

Zn intake needs to be maintained at an adequate level as Zn has essential functions in metabolism. Almost all the ingested Zn is absorbed when dietary Zn intake is at very low level (less than 1mg/day), and particularly when phytate intake is low. Consequently, pancreatic Zn secretion decreases. On the other hand, Zn absorption decreases with a higher Zn intake, followed by an increase of pancreatic Zn secretions (Cousins and King, 2012). The human body contains 2-3 grams of Zn, and nearly 90% is identified in the muscles and bones. At a cellular level, the nucleus contains 30-40% of cellular Zn, cytosol accommodates 50% Zn, with the remaining Zn located in membranes (Plum et al., 2010). Other organs containing Zn include the prostate, liver, gastrointestinal tract, kidneys, skin, lungs, brain, heart and the pancreas. Nevertheless, Zn is not stored in the body. Therefore, if Zn depletion occurs, there are two kinds of Zn metabolic pools, which divide into rapid and lower Zn turnover. The liver, pancreas, kidneys and spleen have a rapid Zn turnover, respectively. While skeletal muscle, the red blood cells, bone and the nervous system have a slow turnover (Cousins and King, 2012). The Zn level in the body is regulated by this homeostatic mechanism and a regular supply of Zn from the diet (Strain and Cashman, 2009).

One study showed the level of intestinal ZnT-1 mRNA increased by 50%, and that the ZnT-1 protein in intestine was 10% higher in rats, which were treated with dietary Zn supplementation, although this was not seen in the livers of the rats (McMahon and Cousins, 1998). A study in humans and an intestinal cell line, Caco-2, suggested that the ZnT1 mRNA decreased at high levels of dietary Zn along with ZnT5 and ZIP4 proteins in the ileal mucosa, while metallothionein increased at mRNA level. ZnT4 and ZIP1 mRNA revealed no changes with dietary Zn supplementation. However, analysis in the intestinal cell line, showed an increase in expression at higher (still physiological Zn) levels, which were decreased at supra-physiological concentrations. 200 µM extracellular Zn supplementation in the Caco-2 cell line culture demonstrated a reduction in ZnT1, ZnT5 and ZIP4 mRNA, and protein expression compared with the 100 µM Zn supplementation (Cragg et al., 2005).

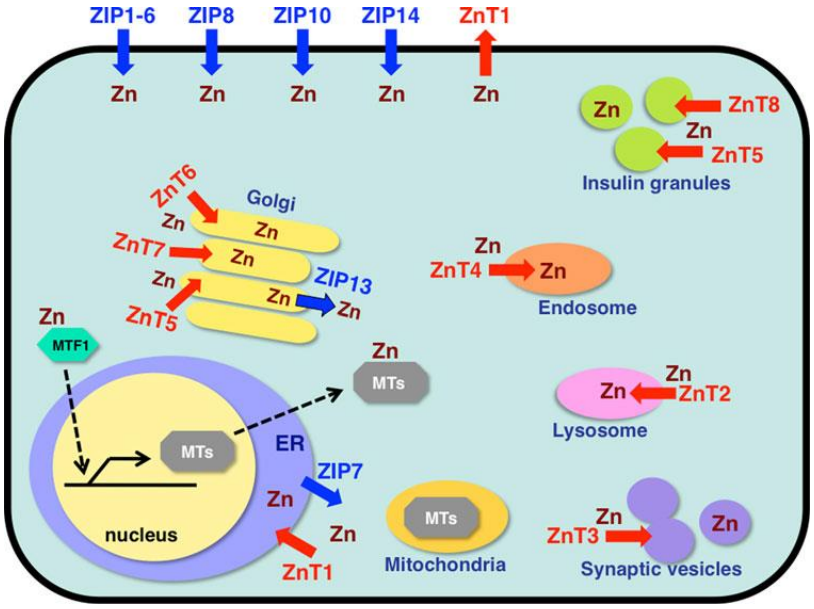

Figure 1.1 Subcellular localization of Zn transporters and metallothioneins (MTs). Localization and potential functions of Zn transporters from the Slc39/Zrt/Irt-like protein (ZIP) (blue) and Slc30/ZnT (red) families, MT and metal-responsive-element binding transcription factor-1 (MTF1) within the cell (adapted from Fukada et al., 2011).

1.2.6 *Zn deficiency*

Zn deficiency may be caused by inherited and acquired factors. Acrodermatitis Enteropathica (AE) has been identified as an inherited disease due to a ZIP4 gene defect, which results in the alteration of Zn absorption. While, acquired factors for example malabsorption syndrome, chronic alcoholism and high consumption of phytate are possible in disrupting Zn absorption (Holt et al., 2012).

Severe Zn deficiency has been reported in the past 50 years, however, it is now less frequent than a mild or marginal Zn deficiency. A tight homeostatic mechanism for Zn may play a role in preventing severe Zn deficiency. It has been noted, however, that marginal Zn deficiency is still prevalent among human populations around the world. A previous study reported Zn deficiency ranged from 9% of the population in developed countries, including Europe, the United States and Canada, to 33% in developing countries, such as South East Asia (International Zinc Nutrition Consultative Group (IZiNCG) et al., 2004). Pregnant and lactating women as well as infants are at risk of marginal Zn deficiency because there is a high demand for Zn to attain the requirement for growth and development (Lamni-Keefe et al., 2008). A study in the mouse placenta demonstrated that Zn transporter expression is responsive to dietary Zn supply, nevertheless, this modulation of expression was insufficient to maintain optimum foetal nutrition at even a modest level of Zn dietary restriction (Helston et al., 2007). Therefore, it is necessary to develop a method to detect Zn deficiency during pregnancy and infancy.

1.3 Teeth

The main functions of human teeth are mastication, speech and aesthetics. In the human digestive system, teeth are in the frontline to cut, tear, and grind foods (Roy and Basu, 2008). There are two types of teeth during human life including deciduous teeth, which are also known as primary teeth, milk teeth, or baby teeth, and permanent or adult teeth. The first deciduous tooth erupts at about 6 months after birth and starts to exfoliate at 5-6 years of age to be replaced with a permanent tooth (Hillson, 2014). The developmental stages and eruption time of each type of tooth is presented in Figure 1.2. The deciduous and permanent teeth have several differences in development rate, morphology, and structure. The deciduous teeth develop faster, their dentinal tubules are larger in diameter, also have thinner enamel and peritubular dentine, as well as a lower concentration of Calcium (Ca) and Phosphorous (P) in enamel than permanent teeth (De Menezes Oliveira et al., 2009; Liversidge and Molleson, 2004; Sumikawa et al., 1999).

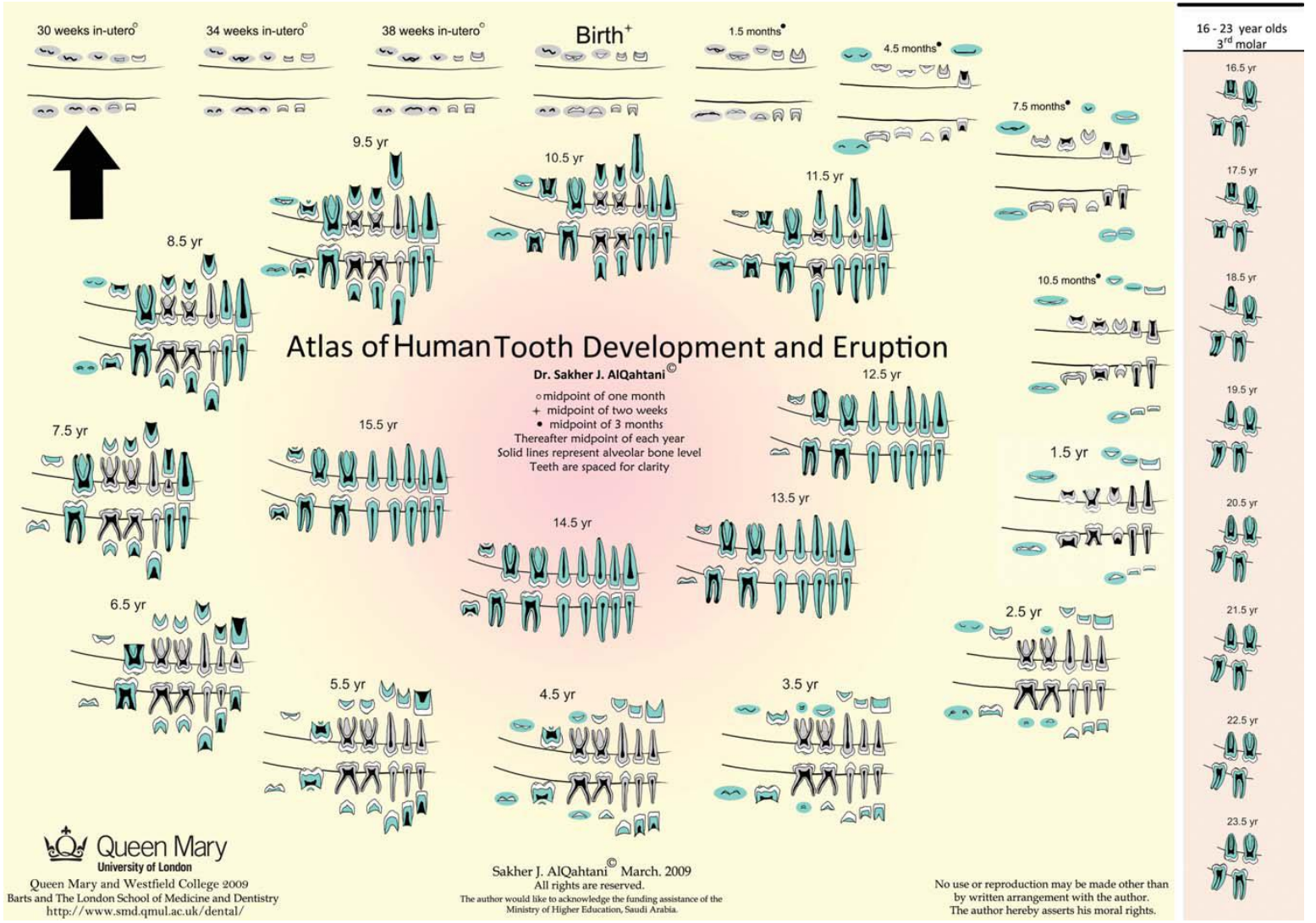

Figure 1.2 The developmental stage and eruption time of deciduous and permanent teeth for each type of tooth (courtesy of Al-Qahtani, 2010).

1.3.1 *Tooth elements*

Dental enamel is the translucent, outmost, hardest and stiffest layer of teeth, which covers the anatomical crowns of the teeth. The enamel contains 95% w/w inorganic constituents or minerals, 1-2% organic material and 3-4% water (Baldassarri et al., 2008; Roy and Basu, 2008). The mineral constituent of enamel consists of hydroxyapatite (HAP) crystal in a hexagonal form composed by Ca and P with the chemical formula $Ca_5(PO4)_3HO$, which build the rods and interrods structure of enamel (Nanci, 2013).

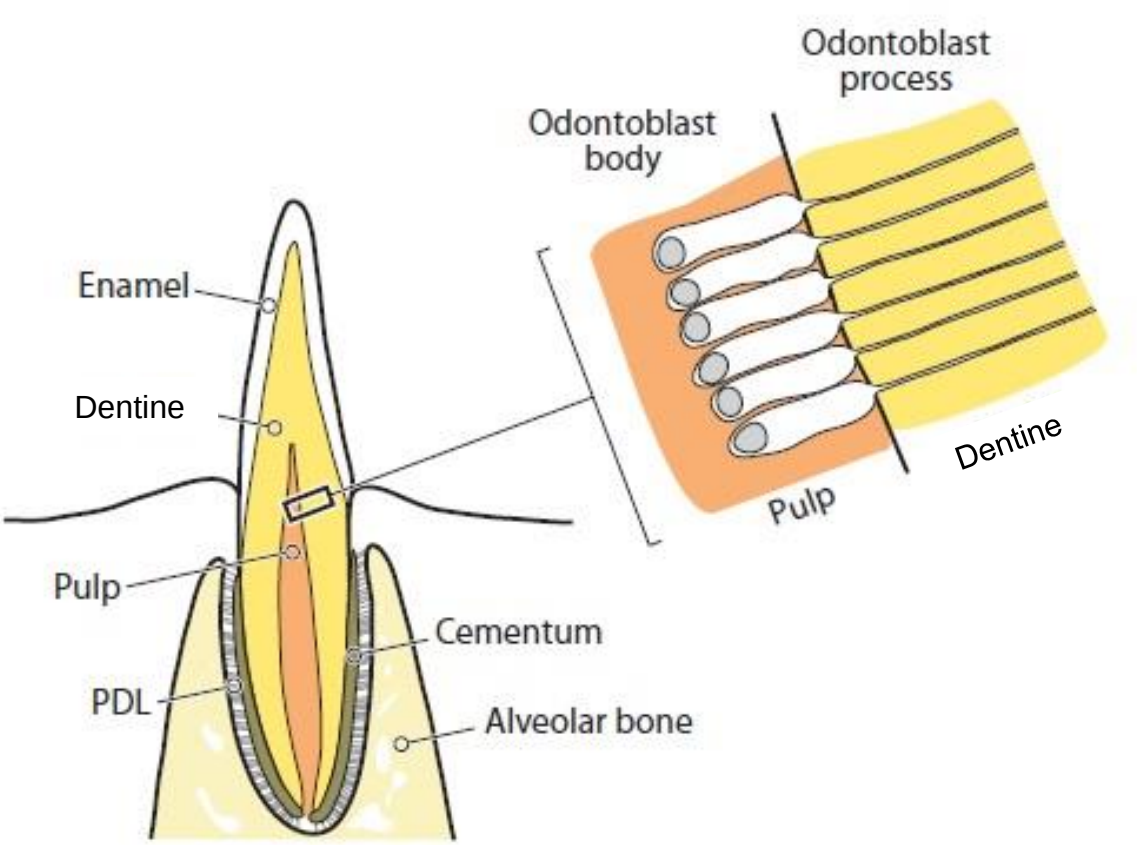

Figure 1.3 The sagittal section of a tooth. Teeth consist of a bulk of dentine covered with inorganic enamel on the crown and cementum on the root surface. The PDL attaches to the cementum at one end and the alveolar bone at the other end. The dental pulp-dentine border is enlarged and separately shown. Odontoblasts extend cytoplasmic extensions (cell processes) into dentine, whereas their cell bodies are aligned along the inner edge of dentine, the dental pulp (adapted from Kawasaki et al., 2009).

The dentine is located beneath the enamel layer and surrounds the dental pulp, including the roots of the teeth. It is resilient and compensates for the brittleness of the enamel, which is effective at preventing fractures to the teeth (Figure 1.3) (Nanci, 2013; Zaslanky, 2008). Dentine is comprised of 70% inorganic material, 20% organic material

and 10% water (Tjäderhane et al., 2009). Depending on its site, function and origin, dentine is divided into various types, such as the mantle and circumpulpal dentine; tubular, peritubular and intertubular dentine; primary, secondary and tertiary dentine (Nanci, 2013). The major part of dentine, which is formed during tooth development, is called the primary dentine.

Two mineralised structures can be distinguished in the primary dentine, which are defined as the mantle and circumpulpal dentine (Linde and Goldberg, 1993). Mantle dentine is atubular and located adjacent to the aprismatic enamel layer. In conjunction, these two layers create a transitional layer from enamel to dentine. The thickness of mantle dentine varies between 5 to 30 µm. In contrast, circumpulpal dentine accommodates dentinal tubules, intertubular and peritubular dentine as shown in Figure 1.4 (Goldberg et al., 2011; Linde and Goldberg, 1993). The dentinal tubules contain the cytoplasm extension of odontoblast and most of the water within the dentine. It is arranged from the dentine-pulp border through the entire dentine, except the outermost layers in the mantle dentine and the enamel dentine junction (EDJ), and adjacent to the cementum (Tjäderhane et al., 2009). When the root formation of the tooth has been completed, the odontoblasts responsible for dentine deposition continue to produce secondary dentine at a slower rate along the edge of the dental pulp (Nanci, 2013). As a protective role, the dentine maintains the pulp vitality and the tertiary dentine is formed due to external stimuli such as chemical irritants, caries, restorative procedures, attrition or other trauma (Linde and Goldberg, 1993).

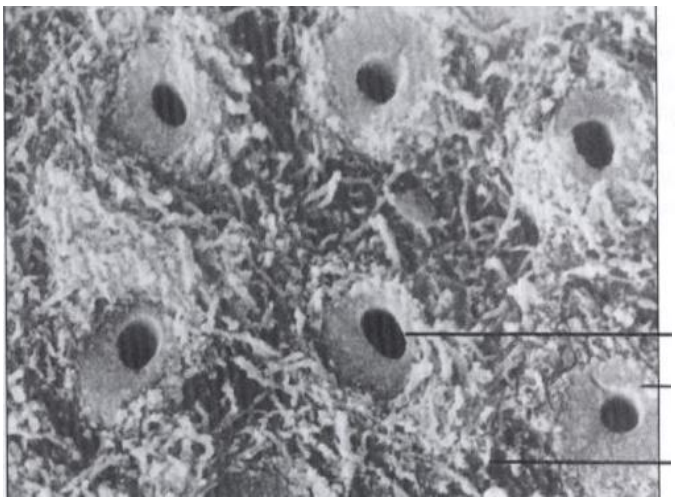

Figure 1.4 The composition of circumpulpal dentine. A transverse section of dentine represents of dentinal tubules, peritubular and intertubular dentine (adapted from Nanci, 2013).

Cementum is an avascular mineralised tissue covering the root surface of the tooth and establishes the connection with the periodontal ligament. In comparison with root dentine, cementum is less mineralised and contains approximately 50% of hydroxyapatite minerals, whereas the remaining part of the cementum contains organic matrix consists of collagens, glycoproteins and proteoglycan (Gonçalves et al., 2005).

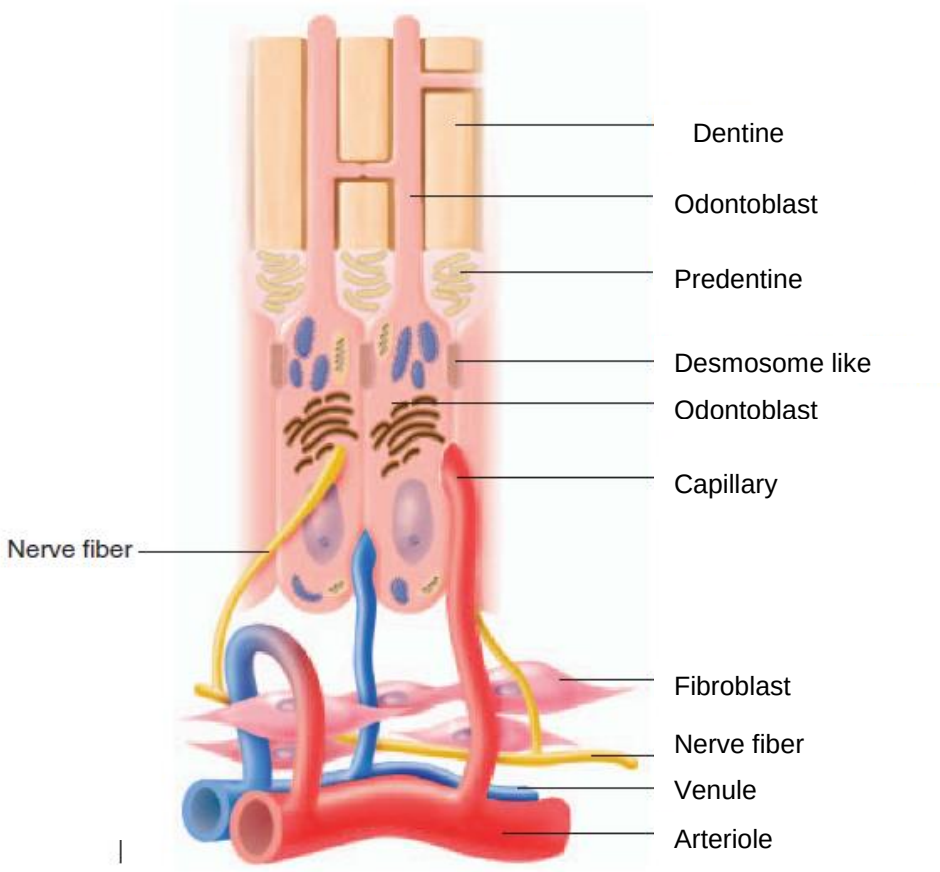

Figure 1.5 Diagrammatic representation of the dental pulp (adapted from Lukko et al., 2011).

Dental pulp is a soft connective tissue that originates from mesenchymal tissues. It acts as a microcirculatory system and consists of nerve fibre, blood and lymph vessels as shown in Figure 1.5. Immediately after the dentine has been developed and began mineralization, the odontoblasts are arranged in the periphery of the dental pulp. In addition to odontoblast cells, dental pulp accommodates other cells, such as sub-odontoblastic and stromal fibroblast cells, as well as neuronal, immune and vascular system cells (Goldberg, 2014). Therefore, it regulates the formation and nutritional status of dentine, protects and provides the innervation of the tooth (Pashley et al., 2002).

1.3.2 *The formation and development of dental hard tissues*

Tooth development is a complex process, that is originated from ectomesenchymal layers, and may be influenced by genes, epigenetic, and environmental factors (Nanci, 2013; Townsend et al., 2012). At initiation stage, the ectoderm thickens and buds into the underlying mesenchymal layer, known as dental lamina, and induces mesenchymal condensation around the epithelial bud, called the dental papilla. The process continues to the morphogenesis stage, where the epithelial bud starts to grow into the shape of tooth crown and forms enamel organ limited by the outer enamel epithelium (OEE) and inner enamel epithelium (IEE). The dental papilla, which is located beneath the IEE, extends to the rim of the enamel organ and forms dental follicle. At the end of this stage, the ultimate shape of the tooth is visible, as described in Figure 1.6. Then, the IEE and dental papilla cells differentiate into ameloblast, which is responsible for enamel formation (amelogenesis), and odontoblast, which forms dentine (dentinogenesis), respectively (Nanci, 2013, 2008; Thesleff, 2006; Townsend et al., 2012).

The ameloblast and odontoblast are then differentiated into the secretory stage as they are ready to secrete extracellular matrix proteins involves in amelogenesis and dentinogenesis. Ameloblast secretes enamel matrix proteins just before the mantle dentine is established, but the enamel matrix protein does not form as a layer until the first dentine layer is developed. The epithelial-mesenchyme signalling mediates the differentiation of ameloblast and odontoblast as well as the matrix protein secretion (Nanci, 2008). The enamel matrix proteins are of a non-collagenous type, such as amelogenin, while dentine matrix proteins, mostly, are collagen type-1.

The enamel formation is divided into three stages, including: 1) the secretory stage, when the enamel matrix is secreted and the earliest stage of enamel apatite crystals (hydroxyapatite) are immediately formed (about 30% mineral by volume). The ameloblast retreats from EDJ to the outermost surface of enamel; 2) the transitional stage, when the ameloblast reaches the outermost surface of enamel, and it is no longer secreting the enamel matrix. Even further, the enamel matrix starts to be replaced by water; 3) the maturation stage, once the initial enamel is completed and the full shape of the tooth enamel layer is formed, enamel maturation starts to occur, when the crystal apatite becomes fully mineralised (about 80-90% mineral by volume) in expense of matrix proteins as well as water, and starts from the cusp edges/tips

toward the cervical area of the tooth (Robinson et al., 1995). Other study, however, suggested the enamel maturation is following the appositional route from the EDJ toward the cusp tips (Smith, 1998). In enamel mineralisation, the mechanism by which mineral ions are taken from the blood vessel and deposited in enamel tissue is still questionable. Two mechanisms have been proposed, especially for Ca transport and distribution, including: 1) a continuous and constant flow of Ca ions occurs in ameloblast cell without the concentration of free Ca ions ever exceeding toxic levels, and or; 2) Ca enters the cell through specific Ca channels (Nanci, 2013).

Dentinogenesis starts in mantle dentine, which is located in adjacent to EDJ. In mantle dentine mineralisation, the initial crystal formation has been shown in membrane-bound matrix vesicles, since odontoblast cells have not been differentiated yet into the secretory-formed cells. The circumpulpal dentine mineralisation, subsequently, occurs when odontoblast cells have been differentiated into the active stage to synthesize and secrete dentine matrix protein and ion minerals, known as the secretory stage, where the cells become columnar and characterised with a well-developed granular endoplasmic reticulum, many mitochondria, pronounced Golgi apparatus, and increased secretory vesicles from the Golgi (Linde and Lundgren, 1995; Simon et al., 2009).

In circumpulpal dentine, the differences between intertubular and peritubular dentine must be addressed. Firstly, the peritubular dentine does not have and or begin with collagen formation, also it is low in organic materials, thus, peritubular dentine contains more minerals, about 40% higher, and more homogenous than the intertubular dentine (Tjäderhane et al., 2009). Secondly, it is assumed that mineralisation in peritubular dentine does not begin with the formation of dentine matrix, as opposed to intertubular dentine, where the dentine matrix is secreted into predentine, then being partly mineralised and transformed into dentine (Goldberg et al., 2011). In intertubular dentine, the mineralisation begins at mineralisation front, and once the predentine is mineralised, the odontoblast cells retreat toward the pulp chamber (Linde and Goldberg, 1993). The intertubular dentine, thus, has more organic matrix, and is mainly collagen (almost 90% by volume) (Tjäderhane et al., 2009). The timing of peritubular dentine formation does not coincide with, but close to, the intertubular dentine formation (Tjäderhane et al., 2009). The formation of both dentine, however, is tightly controlled by the odontoblast cells.

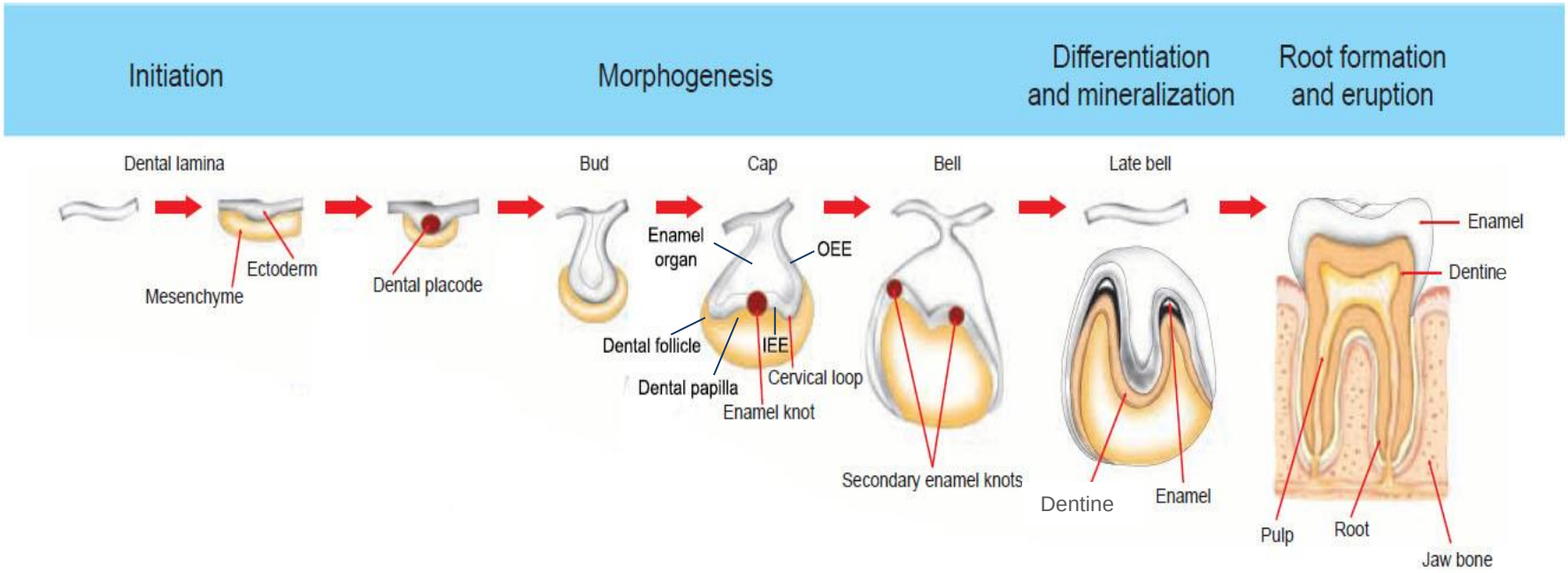

Figure 1.6 A schematic illustration for tooth development stages (adapted from Thesleff, 2003).

In contrast to enamel, dentine is fully mineralised at one time, although peritubular dentine may be continuously mineralised during life (Dean, 2017). The definite location of continual mineralisation in peritubular dentine, however, has not been clear yet. During dentine mineralisation, the ion minerals, especially Ca, is taken from circulatory system into the microcirculatory of dental pulp and transported to the odontoblast cells, which is then distributed into the circumpulpal dentine (Linde, 1995). The odontoblast cells act in Ca regulation and transport through: 1) Ca channels for uptaking Ca into the odontoblast cells; 2) Na/Ca exchanger, possibly plays a reciprocal role in Ca influx and efflux from cytoplasm, along with ; 3) Ca-ATPase for Ca efflux from cytoplasm to extracellular space and or organelle cells; 4) electrophoretic uniporter, along with Na/Ca exchanger, responsible for Ca influx to mitochondria (Linde and Lundgren, 1995).

1.3.3 *Teeth as a biomarker in human life*

The formation of dental hard tissue, both enamel and dentine, is tightly regulated by ameloblasts and odontoblasts at the cellular and molecular level, and spans from prenatal into postnatal period. Thus, the tooth may act as a bio-archive that provide information about development, nutrition, physiological stress, exposure to disease, pollution and residential mobility from early life (Humphrey et al., 2008a; Smith, 2008; Webb et al., 2005). Furthermore, the tooth is gradually developed in circadian rhythms, and dental hard tissue appears as lines that are arranged incrementally. The circadian rhythm is controlled by the body's "central" clock located at the suprachiasmatic nucleus (SCN) in the brain, and is strongly associated with the regulation of ameloblast and odontoblast function in secreting protein matrix and mineralization, and manifests as light/dark lines (Zheng et al., 2011, 2014).

It is possible to carry out age assignment in teeth by measuring the daily secretion rate (DSR) of enamel and dentine using incremental lines as well as accentuated lines in dental hard tissue. In enamel, cross striations, which reflects DSR of enamel, and Retzius lines, that are known as long-period lines and present enamel secretion within 9-10 days, are useful to determine enamel DSR as described in Figure 1.7. While in dentine, von Ebner's lines and Andersen lines correspond with cross striations and Retzius lines (Figure1.8), respectively.

Several methods have been established to obtain DSR in enamel by: 1) measuring the distance between two cross striations (spacing) in random areas within the crown or, as an average of measurement in a specific area or, between fixed-distance points; 2) the proportion of prism length divided by known time between intervals (Dean et al., 1993; Dean, 2006; Reid et al., 1998; Smith, 2008). While, dentine DSR can be determined by: 1) measuring the distance between long-period Andresen lines by their periodicity, which is determined from enamel cross-striations and Retzius lines; 2) using fluorescent-labelled material; 3) direct measurement of daily von Ebners' lines (Dean and Scandrett, 1995; Reid et al., 1998; Smith, 2008). In addition to this, the accentuated lines, such as neonatal line, a distinctive sharp dark band in a longitudinal tooth ground section, that is produced during birth and able to distinguish the part of dental hard tissue developed prenatally and postnatally as shown in Figure 1.9, and or stress accentuated lines, may also be useful in age assignment (Hurnanen et al., 2017; Smith, 2008).

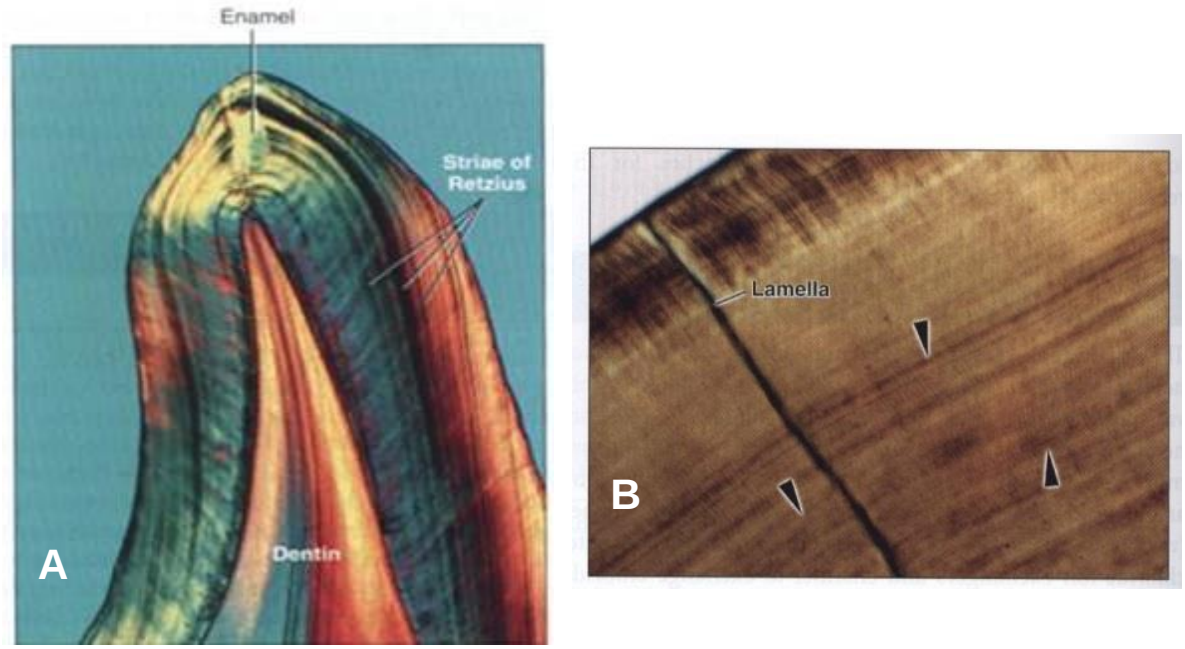

Figure 1.7 Representative images of histological section of enamel: A) Striae of Retzius and B) cross striations, which are appointed by black arrows (adapted from Nanci, 2013).

In addition to the incremental features of dental hard tissue, the minerals deposited within enamel and or dentine, despite Ca and P, as well as organic materials in dentine, may provide valuable information on environmental exposure, such as diets and pollution, as well as migration. Manganese (Mn), lead (Pb), strontium (Sr), barium (Ba),

copper (Cu) and Zn incorporated within dental hard tissue have been studied previously. Using the level of Pb and Mn in human deciduous teeth, the information of the environmental and occupational exposure during early life may be obtained (Arora and Austin, 2013; Gunier et al., 2013). Furthermore, the weaning and feeding practices in early life are possibly tracked using Sr levels deposited in human deciduous teeth (Humphrey, 2014). In addition, stable isotope analysis in organic materials of dentine have also been developed to study nutritional stress and disease, as well as growth and development in humans (Beaumont et al., 2013; Reitsema, 2013).

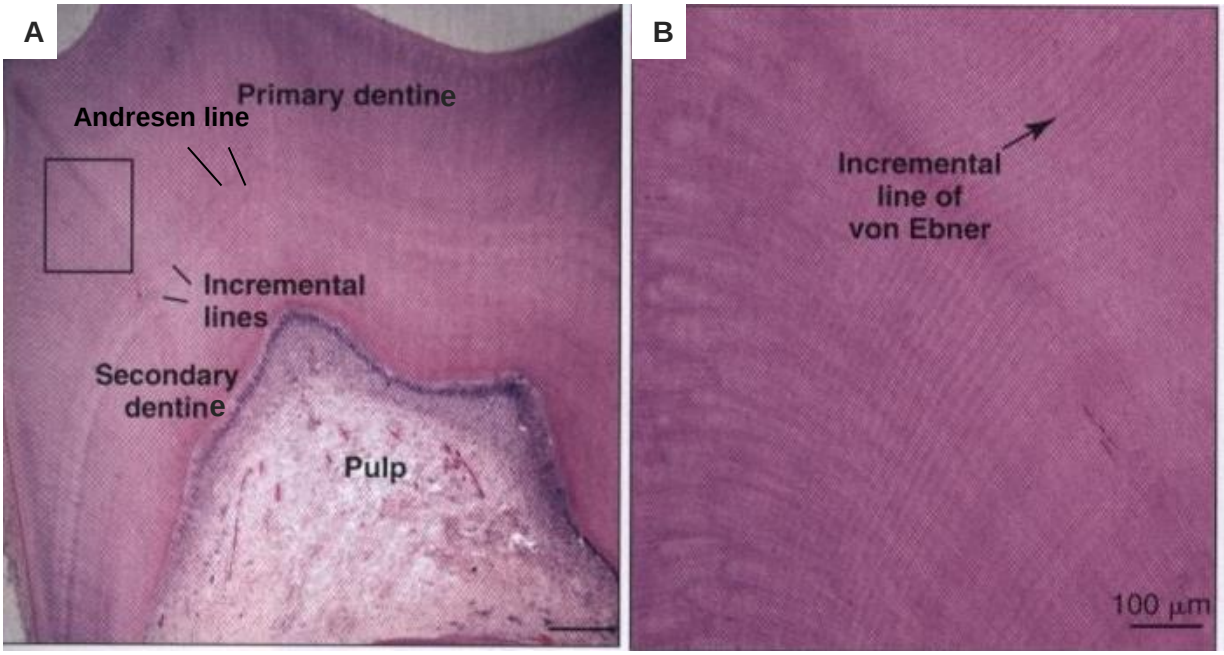

Figure 1.8 Representative images of histological section of dentine. Image A) Andresen lines in histological section of dentine; and B) von Ebner's lines appear as a pair of dark/light incremental line in dentine (adapted from Nanci, 2013).

Nowadays, as the technique of microsampling has been available, including laser ablation and micro drilling, the analysis of trace elements incorporated within dental hard tissue upon the chronological age of tooth allow reconstruction of human life history (Arora and Austin, 2013; Beaumont et al., 2013; Humphrey et al., 2007; Reitznerová et al., 2000; Shepherd et al., 2012). The trace element analysis may carry out using various devices such as atomic absorption spectrophotometry (AAS), atomic emission spectroscopy (AES), inductively coupled plasma atomic emission spectroscopy (ICP-AES), proton induced X-ray emission (PIXE) and inductively coupled plasma mass spectrometry (ICP-MS) (Lane and Duffy, 1996; Reitznerová et al., 2000).

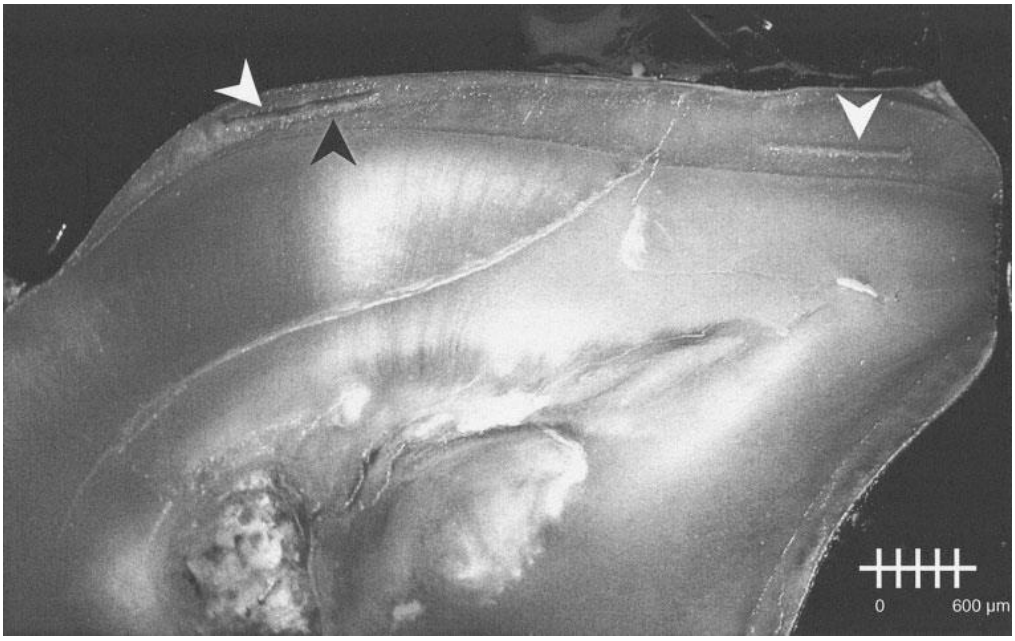

Figure 1.9 A representative image of neonatal line. Location of neonatal line (black arrowhead) and of ablations in prenatal and postnatal regions of enamel (white arrowheads) in a deciduous Solis tooth (adapted from Dolphin, 2005).

1.3.4 *Zn incorporation within teeth*

Zn and other trace elements are deposited in human organs, especially in mineralised organs, such as bones and teeth. The mechanism of mineral deposition in teeth however, is not clear, and is limited to knowledge relating only to Ca (Linde and Lundgren, 1995). Using the enamel and dentine fraction, a large study in Norway compared Zn concentration of ground deciduous teeth between children who lived in different counties as well as archaeological deciduous teeth from medieval era. The study suggested Zn levels in deciduous teeth were related to the increase of Zn absorption due to urbanisation and industrialisation (Fosse and Justesen, 1978). Even though, a more recent study in Norway, using same type of material as the previous study, reported a slight increase of Zn concentration in deciduous teeth compared with the 1970's study as the trend in consuming high-protein foods increased, such as meat, protein-rich cheese, and whole-meal flour (Tvinnereim et al., 1999). The relation of dietary Zn intake and Zn concentration in human teeth was also shown in a previous study as the Zn level of the Ugandan deciduous teeth was lower than UK teeth. The deciduous teeth were recruited from the Ugandan children who lived in poor rural areas and had a Zn deficiency background (Brown et al., 2004). The visible variation on Zn

levels of deciduous teeth from UK children has been reported previously, however, without any information on dietary Zn intake (Shepherd et al., 2012).

A study in women from Solis in Mexico, using the neonatal line in dental enamel along with dietary intake histories, revealed that poor dietary Zn intake in pregnancy correlated with the highest Zn level in enamel in deciduous teeth in children (Dolphin and Goodman, 2009). This may indicate that homeostatic mechanism almost over compensates for deficiency, so Zn level was seen more than minimal levels. In contrary, previous study demonstrated that the expression of Zn transporters in the mouse placenta was influenced by dietary Zn intake, and it was not sufficient to maintain optimal nutrition in the foetus at a very low level of Zn restriction (Helston et al., 2007). Further consideration also needs to be made relating to the direction of enamel maturation, whether it is following the appositional direction from EDJ toward the cusp tips or in a reverse direction, and the timing of enamel maturation, and whether it starts before or after tooth eruption, is still debatable. Thus, the usefulness of enamel in recording Zn nutrition in early life is also questionable. In addition to this, Dolphin and Goodman (2009) suggested enamel is not a suitable material for measuring Zn in relation to the dietary intake during early life.

The tooth is divided into two components of hard tissue, which are enamel and dentine, but the usefulness of dentine in recording early life exposure of Zn nutrition has not been explored yet. On the other hand, Zn incorporation in bone has been previously studied, and was observed in inorganic form as a part of hydroxyapatite structure, and in organic components as Zn involve in the structure of bone matrix protein (Pemmer et al., 2013). Dentine, however, has similarities with bone in term of mineralised tissue (Linde, 1995). A previous study suggested Zn promotes the interaction between collagen molecules, and it should be considered that collagen is a major component of dentine matrix proteins (Rosenberg et al., 1998). Despite of collagen, matrix metalloproteinase (MMP), that is a crucial element involved in collagen degradation during bone mineralisation and a member of Zn-dependent enzymes , is also found in dentine (Goldberg et al., 2011; Pemmer et al., 2013).

Dentine, is likely to haveadvantages in recording the dietary Zn intake in early life as: 1) the tooth has an abundant layer of dentine; 2) the location of dentine is isolated and protected by enamel in coronal part and cementum in cervical part, thus, it may be not

exposed by Zn in oral cavity environment that derives from tooth paste, mouthwash, and or filling materials, and 3) the direction of dentine mineralisation is following the appositional growth, which is running from the EDJ to the pulp chamber, therefore, dentine has a fine structure that is beneficial in analysing element distribution against the chronological age (Dean, 2017; Tjäderhane et al., 2009).

In addition to this, we speculate the regulation of Zn incorporated within dental hard tissue may present within the dental pulp, as this tissue has a connection to the circulatory system of the human body and accommodates the odontoblast cells that responsible in dentine formation. Since Zn transporter proteins play crucial roles in Zn transport, trafficking, and signalling in human body, this study also aimed to elucidate the expression of Zn transporter in pulp tissue at the mRNA level in response to dietary zinc intake.

1.4 Aims and objectives of the study

Overall, this study aimed to evaluate the usefulness of deciduous tooth for recording early life exposure of Zn nutrition, as an essential dietary micronutrient for which early exposure is known to impinge on lifelong health. The objectives for this were to:

1) analyse the relationship between dietary Zn intake during pregnancy and infancy period (early life) with the level of Zn/Ca ratios in dentine of human deciduous teeth.
2) investigate the expression of Zn transporters at mRNA level in human dental pulp related to dietary Zn intake.

Chapter 2 Materials and Methods

2.1 Sample recruitment

An ethical clearance was obtained from the Committee of The Medical Research Ethics of the Faculty of Medicine University of Indonesia (526/UN2.F1/ETIK/2014) at prior to sample recruitment.

Sample size of 38 was calculated for this study using a formula for sample size for difference in means based on zinc concentration (± SEM) in deciduous teeth among UK and Ugandan population, which are 144 ± 24 mg/kg and 124 ± 19.5 mg/kg respectively (Brown et al., 2004), to obtain 80% power at 5% significance level with 10% possibility for drop-off sample (Whitley and Ball, 2002).

Sampling was based on a consecutive sampling method. Children, who attended Child Integration Clinic, Dental Hospital of Universitas Indonesia, for deciduous teeth removal due to dental health reason, were eligible for this study. The inclusion criteria applied to recruiting participants were: 1) the child and mother declared their willingness to participate in the study by signing a consent form which had been explained to them previously; 2) the crown structure of removed teeth was still intact. The clinical assessment was established by dental health personnel. Teeth extraction were carried out by the dental health personnel, following normal procedures.

2.2 Dietary Zn intake

The recruited participants were interviewed to complete a general questionnaire and a shortened FFQ (S-FFQ) for Zn intake during pregnancy, infancy, and at-present (*see appendix A and Chapter 3 for specific method*). Portion sizes were estimated using food photos developed for this population (*see appendix B and Chapter 3 for specific method*). The amount of Zn for each food was obtained from USDA Nutrient Database for Standard Reference (United State Department of Agriculture, 2013) and Nutrisurvey 2007 for Indonesian food database (http://www.nutrisurvey.de), and previous studies (Chan et al., 2007; Norhaizan and Nor Faizadatul Ain, 2009). The dietary Zn intake was estimated on daily basis by multiplying the frequency of food consumption in a month with the portion size using Microsoft Excel software.

2.3 Human dental hard tissue analysis

The removed teeth were cleaned using PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4, pH 7.3) solution to eliminate blood and debris. Immediately, the teeth were preserved in RNAlater® Stabilization Solution (Invitrogen) at 4˚C overnight and stored at -20˚C in Oral Biology Laboratory, Faculty of Dentistry, Universitas Indonesia. The collected teeth were then sent to Oral Biology Laboratory, School of Dental Science, Newcastle University, in RNALater.

2.3.1 *Preparation of tooth ground sections*

Immediately after the removal of dental pulp (Section 2.3), the teeth were rinsed using distilled water to remove any residuals of the RNAlater solution and disinfected using 5% (V/V) Chloramine-T for 5 days. The teeth were then stored in 1% (V/V) Chloramine-T until they were sectioned. The teeth were sectioned using a low speed saw to produce 200 µm thickness ground section from the 1/3 middle in mesial distal direction for incisors and at least 2 cusp tips were included in buccal lingual direction for molars (as described in greater detail in Chapter 4). Photographs were taken for each tooth section, and they were then mounted on a microscope slide, 8 teeth per slide. Under the light microscope, each section was marked by hand using a tip of flame-shaped diamond bur to determine the path for the laser ablation, which followed the dentinal tubules and included neonatal line (NL) (Figure 2.1).

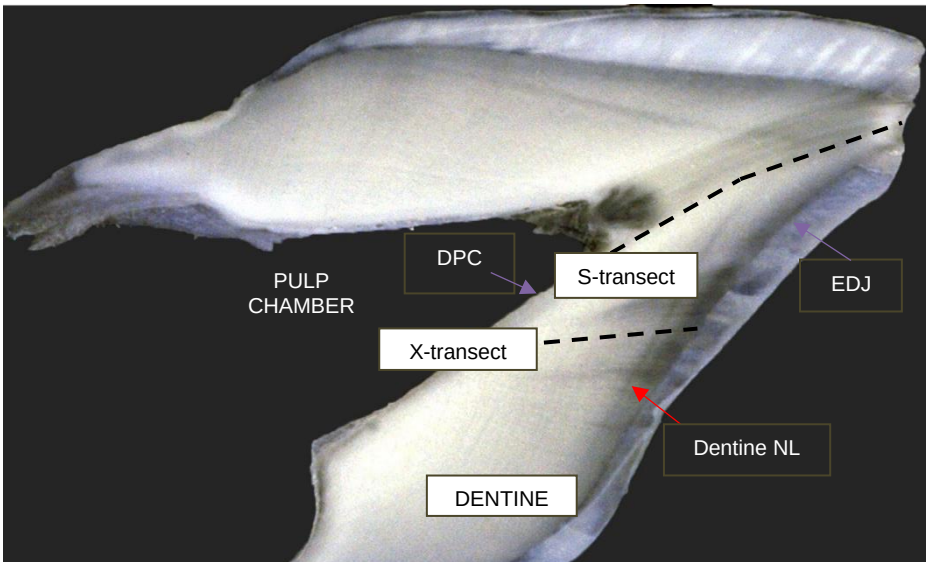

Figure 2.1 Representative photograph of a 200 µM ground section before LA-ICP-MS procedure. The ablation transect was marked from EDJ to DPC (including dentine NL) following the dentinal tubules (dashed black lines).

2.3.2 *Trace element analysis using Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS)*

Analysis of trace elements was carried out based on a protocol that has been developed by Shepherd et al, (2012). Briefly, a Geolas 193 nm ArF excimer laser coupled to an Agilent 7500c ICP-mass spectrometer was used to evaporate dentine tissue in 100 µm diameter (constant energy density of 10 J/cm^2; pulse rate of 5 Hz). This was produced a series of ablation pits along the dentinal tubules from the EDJ toward dentine-pulp chamber (DPC), known as a transect, and was guided by the histological landmarks, such as neonatal line and dentinal tubules (Figure 2.2). This was carried out at the School of Earth and Environment facility, Leeds University. The distance between pits was approximately 100-150 µm. The laser ablation process was captured using a video camera with visible light sources integrated into the optical array to be displayed and monitored continuously on a computer screen. The specification and operating conditions of LA-ICP-MS device is summarised in Table 2.1.

A reference material NIST SRM Glass 610, which was analysed before and after each analytical session, was used for instrument calibration and cross reference to replicate analyses of NIST SRM Glasses 612 and 614 to determine the instrument performance and within-run standard errors. SRM Bone Meal 1486 was employed as an external unknown since it has a matrix similar in chemical and mineral composition to dentine. The ion intensities of ^{66}Zn, ^{44}Ca, ^{40}Ca, and ^{88}Sr obtained from LA-ICP-MS were processed offline using SILLS; a software programme specifically written for the signal integration of laboratory laser systems by Murray Allan (University of Leeds) and later modified by Dimitri Meier and Marcel Guillong (Die Eidgenossische Technische, Zurich). The data was then presented as elemental ion (Zn or Sr) intensity relative to Ca ion intensity (cps).

Laser model	GeoLas ArF excimer
Laser wavelength	193 nm
Laser energy	10 J/cm2
Laser pulse rate	5 Hz
Pulses per ablation analysis	200
Beam diameter	100 µm
ICP-MS model	Agilent 7500c quadrupole MS
Scanning mode	Peak jumping
Background acquisition time	~20 s
Signal acquisition time	~40 s
Dwell times	10 ms ^{44}Ca, ^{40}Ca, ^{66}Zn, ^{88}Sr

Table 2.1 Laser and ICP-MS specifications and operating conditions

2.3.3 *Age assignment using dental histological analysis*

After the LA-ICP-MS procedure, all ground sections were observed under a polarized light microscope (Olympus BX51). The images were captured (x4 objective) and processed using Q-Imaging Micropublisher 3.3 RTV camera and Imporvision Openlab 5.0.2 image software. The images were sent back to Leeds University and the ablation pits were labelled for sample identification. The tooth sections were then further prepared by polishing down the section into 80-100 um thickness, and individually mounting on a microscope slide. The histological analysis was then carried out to determine the age time point for each ablation pit.

Firstly, a photomontage was generated for each ground section by merging captured images (x4 objective) using Adobe Photoshop CS6 software (Figure 2.2). The centre of ablation pit was then marked in photomontage and lines were drawn at the top and bottom border of ablation pits and perpendicular with dentinal tubules as shown in Figure 2.3.

The image of ablation pit was then captured individually (x40 objective). The dentine DSR was determined for each ablation pits and gaps between ablation pits by measuring the daily (Von Ebner's) line as presented in Figure 2.4. Using photomontage, the width of ablation pits and gaps between ablation pits were measured (Figure 2.3). All measurements were carried out using ImageJ software.

By dividing the width of ablation pits and gaps between ablation pits with DSR, the times covered by each pit and gap were could be determined. Using the NL as the zero point, the age range (days) for each ablation pit were established and the median value was considered as the age point (days) of the ablation pit. The age points for ablation pits laid in dentine from NL to EDJ were started with minus (-) days.

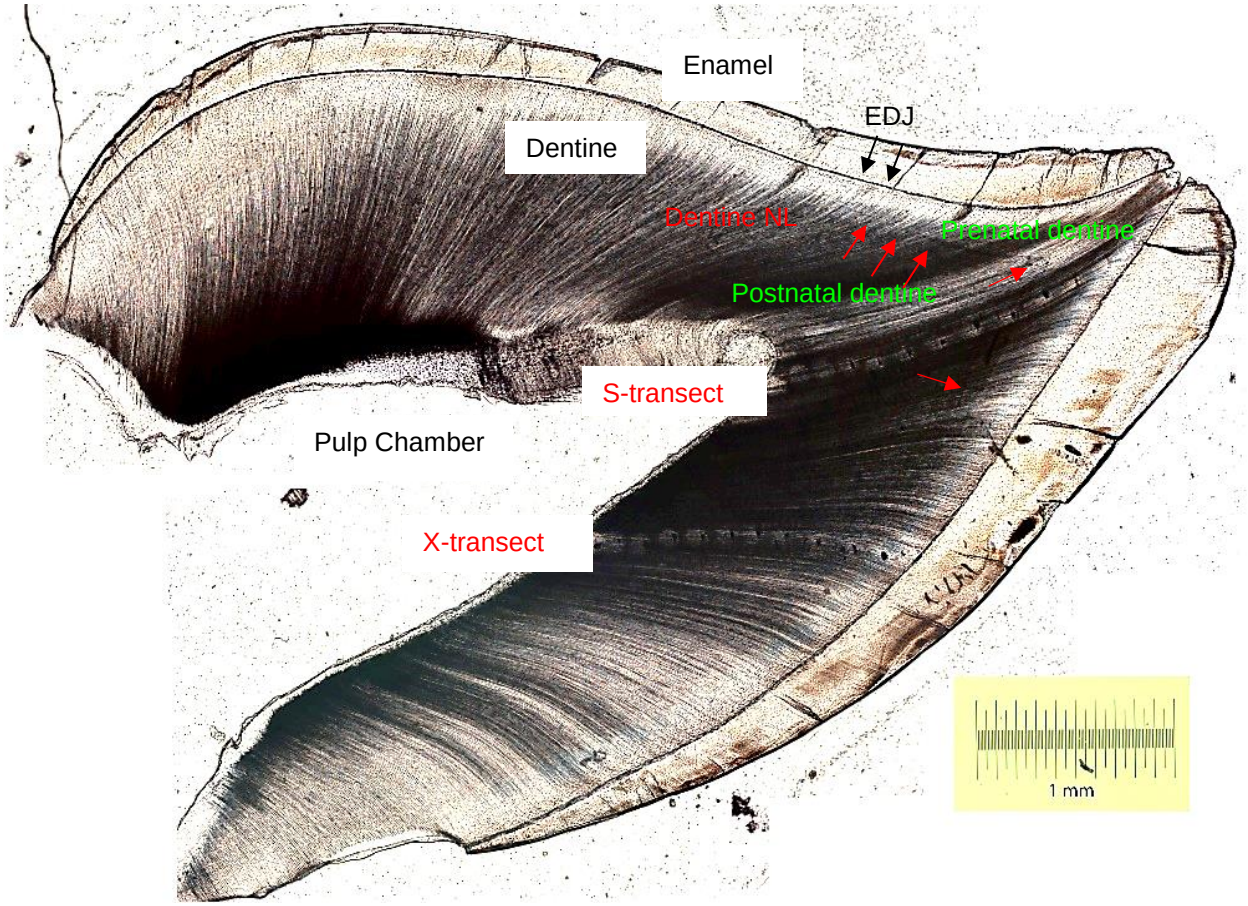

Figure 2.2 A representative image for photomontage of a ground section tooth section (x 4 objective) shows the series of ablation pits in S- and X-transects. The dentine NL was used as the zero point, which divides dentine into prenatal and postnatal dentine is shown by red arrows.

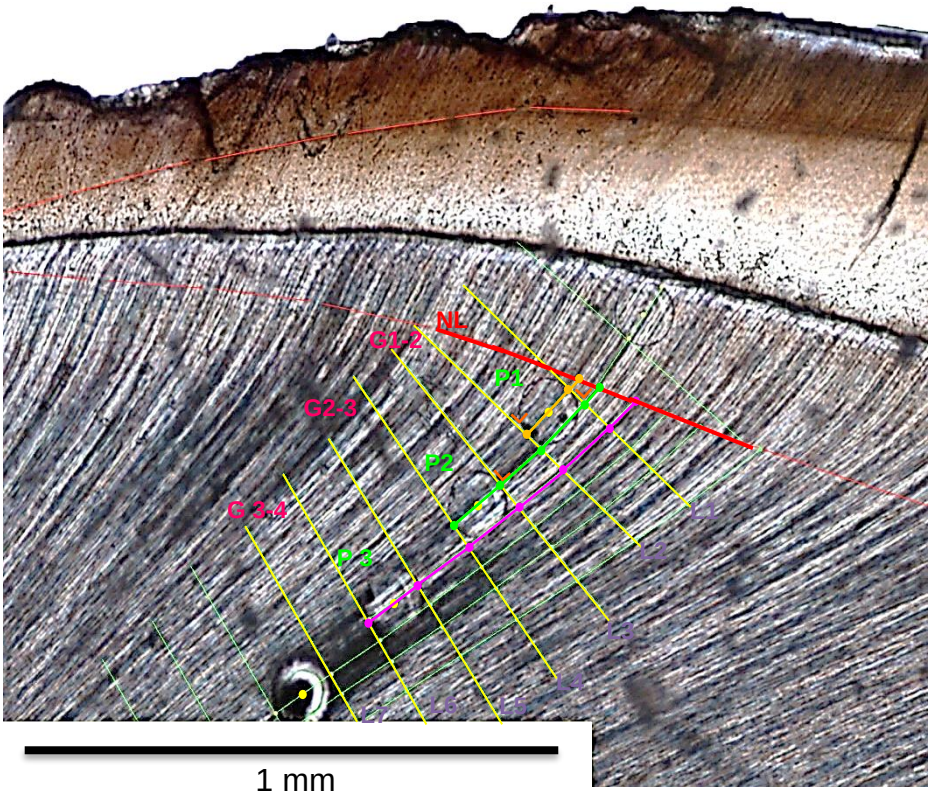

Figure 2.3 A representative image for a series of ablation pits in a transect (x40). The border of ablation pits (L1, L2, L3, L4, L5, L6, L7) were drawn perpendicular to dentinal tubules and NL was marked (in red). The distance from NL to ablation pit was measured from NL to the top border of ablation pit (L1, L3, L5). The width of ablation pit was measured from the top to bottom border of pit across the central point of ablation pit. For example, the width of P1 was measured from L1 to L2. The gap between two pits measured from the bottom border of one ablation pit to the top border of the following pit. For instance, the gap between P1 and P2 was measured from L2 to L3.

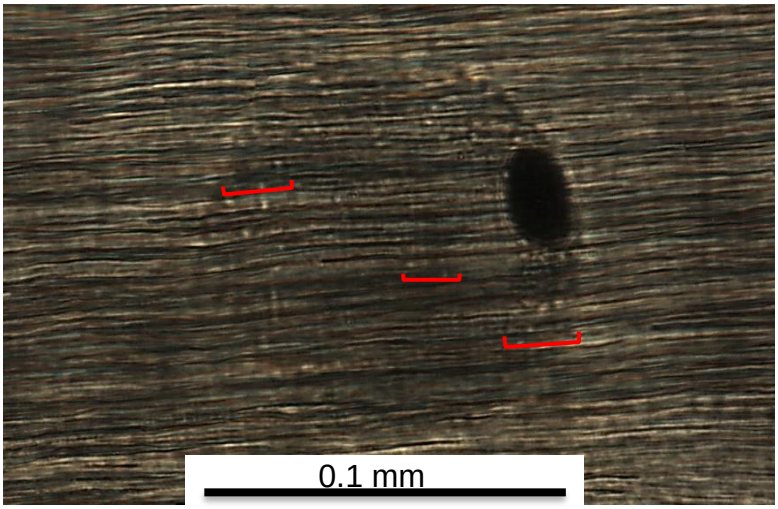

Figure 2.4 A representative image of an ablation pit (x 40). The dentine secretion rate was obtained by measuring daily Von Ebner's line. At each measurement, five daily Von Ebner's line were included as shown in red curves.

2.4 Cell line

2.4.1 *Cell culture technique.*

Cell culture was carried out at the Cell Culture Laboratory, Oral Biology, School of Dental Sciences, Newcastle University. An aseptic technique was applied routinely in handling cultured cells. All procedures involving cultured cells were performed in a class II laminar flow hood. The equipment was sterilised before use, also all the reagents were sterile and suitable for cell culture conditions.

2.4.2 *Growing cells*

In this study, Caco-2 cells (human intestinal), SH-SY5Y (human neuroblastoma), and HDP-hTERT (human dental pulp) were used. The HDP-hTERT cells, have been previously developed by Kitagawa, et al, from Hiroshima University, Japan, (Kitagawa et al., 2007) and are not available commercially. Minimum Essential Medium Eagle Alpha Modification (α-MEM) supplemented with 10% (v/v) foetal bovine serum (FBS, Sigma Aldrich) was used to grow and maintain HDP-hTERT cells in T175 flask (Bio-greiner). Every two days, the growth medium was replaced with fresh medium. Caco-2 and SH-SY5Y were cultured in Dulbecco-Modified Eagles Medium (DMEM) with 4.5 g/L glucose, 0.11 g/L sodium pyruvate and 0.11 g/L L-glutamine supplemented with 10% (v/v) FBS in T75 flask (Bio-greiner). The cells were evaluated for confluency using light microscopy and passaged at 70-80% confluency in an appropriate ratio (HDP-hTERT; 1:2 ratio, Caco-2 cells; 1:4 ratio, and SH-SY5Y; 1:9 ratio). All cell lines were incubated at 37˚C humidified chamber with 95% air and 5% CO_2.

In the passaging process, the medium was removed and the cells were washed using phosphate buffer saline (PBS) with $MgCl_2$ and $CaCl_2$ (Sigma Aldrich). The cells were then detached using trypsin-EDTA as required (6 mL for T75 flask or 9 mL for T175 flask) and incubated for 2-5 minutes. Once cells were detached, equal volumes of growth medium were added to the flask to neutralise trypsin-EDTA (6 for T75 flask and 9mL for T175 flask). Cells were seeded in appropriate numbers into new sterile flasks.

2.4.3 *Cells quantification*

Cells were quantified using a haemocytometer. Just after the medium was poured to neutralise trypsin-EDTA, the cells were transferred into a 50 mL sterile tube and centrifuged at 400 x g at 18˚C for 10 minutes. The supernatant was then removed and

the cells pellet was re-suspended using fresh medium as required. A volume of 20 µL of cell suspension was diluted with 20 µL 0.4% (V/V) Trypan Blue, and 20 µL of the mixture was applied into haemocytometer. The viable (living) cells, which were not stained by the Trypan blue, were counted from 4 outer squares and 1 middle square from each main square. In total, the cells were counted from 10 squares. The viable cells per mL were calculated using the equation:

average cell number x 10^4 x 2 (as dilution factor) = number of cells/ml

2.4.4 *HDP-hTERT cell differentiation into secretory odontoblast-like cells*

HDP-hTERT cells were differentiated by seeding 750,000 cells into a T25 flask in α-MEM supplemented with 10% (v/v) FBS (Sigma Aldrich), with the addition of 50 µg/mL ascorbic acid (Sigma Aldrich), 10 mmol/L β-Glycero-phosphate (Cayman), and 0.1 µmol/L dexamethasone (Sigma Aldrich), defined as mineralisation-induced medium, as described in a previous study (Wei et al., 2007). The medium was replaced every 2 days and the cells appeared fully differentiated (as confirmed by immunofluorescence (Section 2.4.5) and Alizarin red staining (Section 2.4.6)) into odontoblast cells after 7 days.

2.4.5 *Immunofluorescence*

HDP-hTERT cells were seeded at 5x10^4 cells/well on round glass coverslip in 24-well plate with α-MEM supplemented with 10% (v/v) FBS (Sigma Aldrich). Three plates were prepared for 3, 7, and 14-day culturing period. The cells were incubated at 37°C humidified chamber with 95% air and 5% CO_2 for overnight. Next day, the medium of 12 wells were replaced with α-MEM supplemented with 10% (v/v) FBS (Sigma Aldrich), plus the addition of differentiation supplements; 50 µg/mL ascorbic acid (Sigma Aldrich), 10 mmol/L β-Glycero-phosphate (Cayman), and 0.1 µmol/L dexamethasone (Sigma Aldrich), while the other 12 wells replaced using α-MEM supplemented with 10% (v/v) FBS (Sigma Aldrich) only (the undifferentiated group). The medium was replaced every two days using the appropriate medium according to the group type.

At the end of culturing period (3, 7 and 14 days), the medium was removed and the cells were immediately fixed using 100% (V/V) methanol for 5 minutes at room temperature. Fixed cells were washed in five stage cycles using PBS for 2 minutes and

stained using 1 mL primary dentine sialophosphoprotein (DSPP) antibody (Santa Cruz; 1:50 dilution) at 4˚C overnight. On the following day, the primary antibody was removed, cells were washed in three stage cycles using PBS for 2 minutes and stained using secondary anti-IgG –FITC conjugate antibody (Santa Cruz; 1:100 dilution) at 4˚C for 2 hours. The plate was covered with tinfoil as the secondary antibody is light sensitive. After 2 hours, the secondary antibody was discarded and the cells were washed in three stage cycles using PBS for 2 minutes. Cover slips containing stained cells were removed from plate well using tweezer and mounted cell side down using Vectashield (Vector laboratories) onto glass slides.

The stained cells were examined using Olympus BX61 microscope (Olympus Corporation in 10 X magnification with Alexa Fluor 488 and 594. Representative images were captured using a microscope-mounted Olympus XM10 monochrome camera and analysed using ImageJ software (Java-based image processing program—National Institute of Health (USA)).

2.4.6 *Alizarin Red Staining*

HDP-hTERT cells were seeded at $5x10^4$ cells/well on round glass coverslip in 24-well plate with α-MEM supplemented with 10% (v/v) FBS (Sigma Aldrich) overnight. On the following day, the 24 wells were randomly divided into differentiated and undifferentiated group, so each group of 12 wells maintained in the undifferentiated and differentiated medium as described above in Section 2.4.5. The cells were cultured for 14 days and incubated at 37˚C humidified chamber with 95% air and 5% CO_2. The medium was replaced every two days using an appropriate medium for each group.

At the end of culturing period, the medium was removed and cells were washed using PBS. An immediate fixation was carried out using cold 70% (V/V) ethanol for 1 hour at room temperature. Fixed cells were washed one time using sterile distilled water and stained with Alizarin Red dye (40 mmol/L at pH 4,2; Sigma Aldrich) for 20 minutes. The dye was then discarded, and stained cells were washed twice using sterile distilled water to remove excess stain. Representative photographs were taken for each group using digital camera.

2.4.7 *Cell viability assay*

HDP-hTERT cells were seeded at 5000 cells/well into a 96-well plate with α-MEM supplemented with 10% (v/v) FBS (Sigma Aldrich) overnight. On the following day, cells were treated with different concentrations of extracellular $ZnCl_2$ by adding 10, 20, 40, 60, 80, 100, and 200 µM into the growth medium and incubated for either 4 or 24 hours. Extracellular $ZnCl_2$ solution was prepared using a 100 mM stock $ZnCl_2$ dissolved in sterile distilled water. Cells without Zn treatment were used as control. Following incubation, AlamarBlue® (Invitrogen) was added to the cell medium at a volume of 10% (V/V) of the total well medium. Absorbance values were measured using a BioTek Synergy HT plate reader at 570 and 600 nm. Cell viability was calculated following manufacture's protocol as:

Oxidized Alamar blue in Zn treated cells: untreated control cells.

2.5 Total RNA extraction

2.5.1 *Total RNA extraction from dental pulp*

The RNAlater was discharged and the tooth was rinsed using nuclease-free water just before pulp removal and RNA extraction. The pulp was removed using barber broached needle from cervical site and transferred into a sterile RNAse free tube contained 0.5 mL of Trizol (Invitrogen). The tissue was disrupted and homogenised using a sterile plastic pestle operated by cordless motor (Kimble Kontes) and incubated overnight at -80˚C. The separation phase was carried out the day after by adding 0.1 mL chloroform into homogenate tube. The tube was shaken vigorously by hand for 15 seconds, and incubated 3 minutes at room temperature. Then, the homogenate-trizol-chloroform mixture was centrifuged at 12,000 x g for 15 minutes at 4˚C. The colourless phase was discharged carefully and transferred into a new RNAse free tube. An addition of 7.5 µg glycogen (Glycoblue, Invitrogen) was poured into the tube, followed by 0.25 mL 100% (V/V) isopropanol, gently flicked, and incubated at room temperature for 25 minutes. After the tube was centrifuged at 12,000 x g for 10 minutes at 4˚C, the supernatant was removed and a pellet was visible at the bottom of the tube. The addition of 0.5 mL 75% (V/V) ethanol to wash the RNA, and centrifuged at 7,500 x g for 5 minutes at 4^0 C. The supernatant was discharged and the visible pellet air dried for 7.5 minutes. Total RNA resuspension was achieved by adding 50 µL nuclease-free water. Then, NanoDrop™ 1000 Spectrophotometer (Thermo fisher scientific) was used to measure the concentration and quality of total RNA extracted.

2.5.2 *Total RNA extraction from cell line models*

HDP-hTERT cells were seeded at $7.5x10^5$ cells into a T25 flask with α-MEM supplemented with 10% (v/v) FBS (Sigma Aldrich) as normal growth medium overnight. On the next day, the cells were cultured using α-MEM supplemented with 10% (v/v) FBS (Sigma Aldrich), 50 µg/mL ascorbic acid (Sigma Aldrich), 10 mmol/L β-Glycero-phosphate (Cayman) as the mineralisation-induced growth medium for 7 days. The medium was replaced every 2 days.

On the seventh day, the medium was removed and cells were washed using a Dulbecco's PBS with $MgCl_2$ and $CaCl_2$ (Sigma Aldrich). The cells were then treated with extracellular Zn by adding $ZnCl_2$ into α-MEM medium at a concentration of 10 µM and 60 µM for 4 and 24-hour incubation period. The cells, which were cultured using α-MEM medium only, were used as control.

After the incubation period, the medium was discharged, and cells were washed using Dulbecco's PBS without $MgCl_2$ and $CaCl_2$ (Sigma Aldrich). Total RNA was immediately isolated using PureLink® RNA Mini Kit (Invitrogen). A fresh amount of β-mercaptoethanol were added into lysis buffer just before RNA isolation. The 0.3 mL of lysis buffer were poured into each flask and incubated at room temperature for 5 minutes. Cells were then thoroughly scrapped using disposable small scrapper (Bio-Greiner). Cells and lysis buffer mixture were transferred into new RNA-se free tube, homogenized using Tissue Lyser LT (Qiagen) for 5 minutes at 50 Hz, and centrifuged at 12,000x g for 15 seconds. Then, the binding, washing and eluting RNA steps were following protocols for PureLink® RNA Mini Kit (Invitrogen). NanoDrop™ 1000 Spectrophotometer (Thermo fisher scientific) was used to measure the concentration and quality of total RNA. Only samples with RNA 260/280nm ratio >1.8 were used for further analysis.

2.5.3 *DNAse clean up*

All cDNA samples were analysed by PCR (T100™ Thermal Cycler, Biorad) using GAPDH as housekeeping gene to examine if there was any genomic DNA contamination at RNA level. If the contamination was defined, a DNAse clean up protocol was applied using Turbo™ DNAse (Ambion). Contaminated RNA was diluted into 1.25 µg per 50 µL using nuclease free water into a RNA/DNAse free PCR tube,

added with 5 µL (0.1 v/v) 10x Turbo DNAse buffer and 1 µL Turbo DNAse, mixed gently. The tube was then incubated at 37˚C for 30 minutes using T100™ Thermal Cycler (Biorad) and immediately placed in the ice. A 5 µL (0.1v/v) DNAse inactivation were applied and mixed by flicking the tube 2-3 times during 5-minute incubation period at room temperature, and centrifuged at 10,000x g for 1.5 minutes. A pellet was shown at the bottom of tube. The clear supernatant was carefully removed without drawing the RNA pellet .

2.5.4 *cDNA synthesis*

cDNA was synthesized using High Capacity cDNA Reverse Transcription Kits (Applied Biosystem). The manufacture's protocol was followed to synthesize 1 µg RNA for each reaction. A mixture of 2 µL 10X RT Buffer, 2 µL 10X RT Random Primers, 0.8 µL dNTP Mix (100mM), 1 µL MultiScribe™ Reverse Transcriptase, and 4.2 µL nuclease-free water, were incorporated with 10 µL of RNA in 1.5 mL RNA/DNAse free tube. A negative reaction was synthesized for each sample, containing all the above with the subtraction of the RNA, was prepared to ensure no genomic DNA contamination in the RNA. The thermal cycle conditions were applied using a T100™ Thermal Cycler (Biorad, which were divided into step 1; 25˚C for 10 minutes, step 2;37˚C for 120 minutes, step 3;85˚C for 5 minutes. cDNA samples were stored at -20˚C until used.

2.5.5 *Real time q-PCR for detection of Zn transporters in human pulp tissue*

The Real Time-Polymerase Chain Reaction (RT-qPCR) amplification was used to detect Zn transporter genes expression at mRNA level. The list of Zn transporter genes and two housekeeping/reference genes completed with product length, annealing temperature, and cycling condition are shown in the relevant sections of Chapter 6. A standard curve for each primer set was carried out to measure the efficiency of working primers using RNA prepared from human RNA (Ambion RNA tissue panel), Caco-2 and SH-SY5Y cells, as these cells are known to express the genes of interest (GOI) (*see Appendix D*). The efficiency of the primers was calculated using the equation:

$$E = (10(-1/slope) - 1) \times 100$$

Working primer pairs, which have efficiency value within the range of 80-120%, were chosen.

cDNAs were amplified using SensiFAST SYBR mix No ROX (Bioline). In each RT-qPCR reaction, 6.25 µL SensiFAST SYBR mix was added to0.5 µL (10 µM) Forward primer, 0.5 µL (10 µM) Reverse primer, 5.25 µL nuclease free water and cDNA template. The amplification was performed with polymerase activation at 95˚C for 2 minutes, and continued with denaturation at 95˚C for 5 seconds and annealing/extension step at annealing temperature for 15 seconds. The plate was read after each cycle, while melting curve was obtained at 55˚C to 90˚C after all cycles completed and read every 1˚C increments for 5 seconds. Each sample was measured in duplicate.

The Zn transporter genes expression were measured using the relative quantification method and the cell type and tissue known to express the genes of interest (GOI) were chosen as the calibrator. The Ct value for ZnT gene expression was normalised using GAPDH and 18S as the reference genes according to Pfaffl method (Pfaffl, 2001). As the positive control, the calibrator was analysed for Gene of Interest (GOI) and reference gene in each qPCR plate.

2.5.6 *End-point PCR*

The Polymerase Chain Reaction (PCR) amplification was used to detect the zinc transporter genes expression within HDP-hTERT cell lines. The list of Zn transporter genes and two housekeeping/reference genes completed with product length, annealing temperature, and cycling condition are shown in the relevant sections of Chapter 6.

In each reaction, a mixture of 12.5 µL BioMix™ Red (Bioline), 2.5 µL 10 µM forward primer, 2.5 uL 10 µM reverse primer, 2 µL cDNA, and 5 µL nuclease free water. The PCR was performed using T100TM Thermal Cycler (Biorad) and initiated by melting at 94˚C for 5 minutes, followed by 30 cycles of melting at annealing temperature for 1 minute, extension at 72˚C for 1 minute, and melting at 94˚C at 1 minute. At the end of the PCR cycle, the extra extension at 72˚C for 10 minutes. No template PCR reaction, which was contained nuclease free water only, was running for each working primer to detect any contamination in PCR preparation process.

Gel electrophoresis was carried out to analyse the PCR products. A 2% (w/v) agarose gel (Sigma Aldrich) was made up by boiling a mixture of agarose gel powder and

1xTAE buffer. The gel mixture was then incorporated with Gel Red dye (Bioline) to visualize the gel under ultraviolet light using gel doc system. Once the gel was set in a tray, it was placed into the gel tank filled with 1xTAE buffer. In each well, 5 µL PCR product was loaded and compared with 5 µL Hyperladder 100bp (Bioline) as the DNA marker. The gel tank was connected into a gel electrophoresis apparatus (Biorad) and running at 80 Volt for 90 minutes. A gel image then was produced using gel doc system device (Syngene) and GeneSys software.

Chapter 3 Dietary intake of Zn during pregnancy, infancy and current periods

3.1 Introduction

Zn is a trace element that has an essential function in human metabolism. However, Zn is not produced by and/or stored in the human body, and therefore is continuously acquired from foods. Animal-based foods, such as organs and/or flesh of beef, pork, poultry, fish and shellfish, are known as Zn-rich foods, while eggs and dairy products have lower Zn content (International Zinc Nutrition Consultative Group (IZiNCG) et al., 2004). These high protein foods are also known to enhance Zn absorption. Legumes are Zn-rich foods, but these are also high in phytate, which has been recognised as an inhibitor of Zn absorption. The availability of Zn for absorption by the intestine is often associated with the presence of phytate or protein (Gibson, 2012).

Furthermore, the individual variety of diets should be considered to determine the Zn requirement on a daily basis. IZiNCG suggested an estimated average requirement (EAR) value for daily Zn intake based on the individual type of diets, such as mixed/refined vegetarian diets and or unrefined, cereal-based diets. The EAR considers the physiological requirement of Zn as well as Zn absorption level from both groups in different populations, where the unrefined/cereal-based diet group indicates a higher Zn requirement compared with the mixed/refined vegetarian diet group (International Zinc Nutrition Consultative Group (IZiNCG) et al., 2004). Therefore, a robust dietary assessment method was required in this study to obtain adequate information on the participant diets during pregnancy and infancy periods in relation to Zn requirement and availability.

Several dietary assessment methods have been studied previously, such as 24-hr dietary recall, diet record, diet history, food frequency questionnaire (FFQ), and brief dietary assessment method (Thompson and Subar, 2008). Each method has its own strengths and weaknesses. Thus, the selection of dietary assessment should be based on the aims and type of study being undertaken; such as the information of food type or nutrient intake type, the accuracy of information, targeted sample, time periods, human resources and cost.

Diet record is a tool to measure the absolute intake of respondent on a daily basis by weighing, measuring, or estimating the portion size. It is appropriate to be used in a prospective study, but the information accuracy could be reduced when there are more days to record. This could also affect the dietary habits of the respondent. Previous studies have shown level of under-reporting intake when using this method, therefore adequate training for both investigator and respondent is required (Shim et al., 2014; Thompson and Subar, 2008).

24-hr dietary recall provides a quantified intake for the last 24 hours or a day before. The respondent should report any foods consumed and estimate portion sizes. The accuracy is highly related to respondent's memory, but does not modifying the respondent dietary habits. However, an under-reported intake is common. Thus, it should be repeated more than once, to get information on individual habitual intake (Gibson et al., 2003). Moreover, the investigator needs to acknowledge that there will be unreported consumed foods by respondent (Thompson and Subar, 2008).

FFQs have been defined as questionnaires that contain a list of foods or meals alongside a consumption rate within a month, a week or a day. They often contain portion size information of the consumed foods or meals (Cade et al., 2002). Using the consumption rate multiplied by the portion size, it is possible to estimate the daily nutrient intake. This method allows the investigator to estimate a relative nutrient intake in a specific time period and does not tend to affect dietary habit of the respondent (Thompson and Subar, 2008). FFQs can be self-administered or completed through an interview with the investigator. However, there are difficulties in gathering accurate information on portion size, thus, several methods have been developed and studied, such as using special plates and bowls, weighed food units, two- or three- dimension food portion models, as well as food photographs (Toobert et al., 2011).

A previous study reported the food photographs enhanced the level of agreement between weighed records and FFQ. Food photographs can be used in a single food portion, and the respondents may estimate their portion as fraction or multiply the portion displayed on the photo, or in a series of food photographs with different portion shown in each photo. A series of food photograph is more effective if it is shown in even number of photos rather than odd number as the respondent may tend to choose the middle portion (Nelson and Haraldsdóttir, 1998). Using coloured photographs, the

weight of portion chosen in food photographs series was significantly associated with the weight of eaten portion, and it was independent to age, gender and BMI (Turconi et al., 2005). Thus, food photographs can be useful to help respondent estimating their food portion through a complex process of perception, conceptualization, and memory (Nelson et al., 1994).

Diet history is a quite similar method to a FFQ, where the information on frequency and portion of foods or meals are collected. It also allows the investigator to explore more information on how foods were cooked and served. Burke and colleagues, who developed this method, included an interview to obtain information on the habitual eating patterns, questionnaire with a food list to capture frequency and portion of consumed foods or meals, and a 3-day diet record (reviewed in Thompson and Subar, 2008). This method is applicable in a retrospective study, but it is not cost effective and is time consuming for investigator and respondent (Shim et al., 2014).

A meta-analysis study investigated the correlation coefficient of dietary mineral intakes based on the study's quality index to compare FFQ and other reference methods, such as diet record versus 24-hr dietary recall, as a part of EURopean micronutrient RECommendations Aligned (EURRECA) Network of Excellence study, and it found that FFQ is an acceptable instrument to assess long-term intake of Zn within 1 year retrospectively compared with diet record (Serra-Majem et al., 2009). A study in the Middle East analysed the validity and repeatability of FFQ in Saudi adults (20-30 years and 60-70 years participants) and suggested that FFQ was adequate to assess the differences of Zn intake between age and gender groups (Alsufiani et al., 2015). In addition to this, the sufficiency of FFQ to assess Zn intake in infants and pre-school children has been previously reported (Roman-Viñas et al., 2010). A more recent study showed FFQ was adequate to assess key micronutrient intake during pregnancy, such as Vitamin A, B6, B12, and folate, 4-6 years post pregnancy (Mejía-Rodríguez et al., 2012)

For this study, children aged 6-11 year, who were attending the Child Integration Clinic, Dental Education Hospital of University Indonesia, Central Jakarta, Indonesia, for deciduous teeth extraction, were recruited. We also recruited their primary carer, where all cases in this study are the mothers. Retrospective information on Zn dietary intake during pregnancy and infancy periods were gathered by interviewing the

mothers. Thus, FFQ was considered as a suitable dietary assessment method due to the ease of administration and time with respondent, also as this was a retrospective food analysis. As there is a lack of information regarding the use of FFQ in Indonesia populations and also a lack of data using FFQs to assess Zn intake, a dietary assessment was tailored for this specific population, taking into the differences in demographic conditions, ethnicities, religion, cultural, and social economic levels among this (Larson and Story, 2009; Thompson and Subar, 2008). The initial study was carried out to develop and validate the FFQ prior to use in the main study.

3.2 Aims

This chapter aims:

1) to develop and validate a food frequency questionnaire to assess relative daily Zn intake in Indonesian populations during pregnancy, infancy, and at present time periods.
2) to analyse daily Zn intake in children and their mothers, who participated in the main study, using the developed and validated FFQ.

3.3 Materials and Methods

3.3.1 Development of the FFQ

Firstly, a comprehensive search of electronic databases for publication from 2000-2013 was carried out to gain information on FFQ that has been developed and validated for an Indonesian population as presented in Table 3.1. There was a very limited information about this. The search was then focused on literature relating to FFQ used to assess dietary intake in pregnant mothers and children during their infancy periods.

Study	Utilization of FFQ
Wakai et al., 2000	Estimation of fat intake
Humphrey et al., 2000	Determination Vitamin A status
Lipoeto et al., 2004	Evaluation of dietary pattern related to the risk of coronary heart
Amtha et al., 2009	Evaluation of dietary pattern related to the risk of oral cancer

Table 3.1 Journal articles in which dietary analysis has been estimated by use of FFQ for Indonesian populations.

A FFQ used for recording dietary intake during pregnancy and the infancy period, was previously validated in Mexico (Mejía-Rodríguez et al., 2012, 2014), and was adapted

to contain commonly consumed Indonesian foods to assess dietary Zn intake for the Indonesian population. To adapt the FFQ with appropriate foods, an online, 1-day diet record was carried out to compile foods and meals that are commonly consumed by Indonesian people. Twenty-five females were recruited through word of mouth in this preliminary study. All participants in this initial study were treated based on the guidelines assigned in the Declaration of Helsinki and given informed consent.

A list of 82-food items was then incorporated into this FFQ (Table 3.2). In addition, portion size options were included and displayed as food photos. An additional main questionnaire was generated and included to gain personal information about the children and their parents. Other information related to specific conditions was also requested in the main questionnaire, such as the mothers' medical conditions or supplements taken during pregnancy, feeding practices, and weaning history during infancy. The long FFQ can be found in Appendix A.

Rice and cereals	**Fish and Seafood**	**Fruits**	Pak choy
Rice	Mussels	Avocado	Eggplant
Brown rice	Prawn	Apple	Pumpkin
White bread	Crab	Guava	Bitter gourd
Wholegrain bread	Squid	Orange	Green beans
Pasta	Fresh-water fish	Mango	Beansprout
Breakfast cereal	Salt-water fish	Pineapple	Long beans
Oatmeal		Banana	Peas
Noodle	**Eggs**	Melon	Jackfruit
Red meat	Eggs	Watermelon	Radish
Beef		Papaya	Beetroot
Beef sausage/meatballs	**Dairy**	Strawberry	Lettuce
Cow's offal	Yogurt	Grapes	Watercress
Veal	Cheese	Mangosteen	Kale
Veal sausage/meatballs	Milk	Kiwi	Asparagus
Lamb		Apricot	Zucchini
Lamb sausage	**Plant-based protein**	Peaches	Bell pepper
Lamb's offal	Tofu	Snake fruit	Cauliflower
White meat	Tempeh	**Vegetables**	Kangkong
Chicken	Kidney beans	Broccoli	Cassava leaves
Chicken sausage/nugget	Mung beans	Spinach	Cucumber
Chicken's offal	Soy beans	Mushroom	Potato
Pork	Peanuts	Cabbage	
Pork sausage/ham	Cashew	Chinese leaf	

Table 3.2 A 82-food-items FFQ. Commonly consumed foods by Indonesian people were gathered using a one-day diet record completed by 25 female volunteers.

Based on previous studies, a shortened FFQ has been used to gain adequate information on dietary Zn intake (Cade et al., 2002; Samman et al., 2010). The use of the shortened FFQ was preferable, saving time in completing the questionnaires was considered important, as our study was hoping to gain information from three different periods (pregnancy, infancy, and at present). Therefore, a shortened FFQ (28-food items) was developed from the full FFQ by focusing on Zn-rich foods, such as red meat, offal, avocado, broccoli, spinach, and by grouping foods into an 'other vegetables' category which contain less Zn in one item, like cabbage, carrot and lettuce.

Protein source	**Rice and cereals**	**Vegetables & fruits**
Beef /lamb	Rice	Corn
Beef sausage/meatball	White bread	Potato
Chicken	Pasta/noodle	Spinach
Chicken nugget	Breakfast cereal	Mushroom
Eggs		Broccoli
Cow'/Chicken's offal		Other vegetables
Salt-water fish	**Beans and nuts**	(carrot, cabbage, etc)
Fresh water fish	Long beans	Avocado
Seafood	Mung beans	**Dairy**
Tofu	Peanuts	Milk
Tempeh		Cheese
		Yogurt

Table 3.3 Food items included in the shortened FFQ.

Each photograph was displayed carrots, cauliflower, cabbage into other vegetables (Table 3.3). Some items that were discovered to be rarely or never consumed by this population, such as brown rice, veal, pork, were eliminated (for Shortened FFQ see Appendix A).

In this study, the food photographs were generated to help the respondent in estimating their usual food portion. Portion sizes were presented using food photos developed according to recommendation from previous study (Nelson and Haraldsdóttir, 1998). Each food was divided into four different portion sizes (Figure 3.1), i.e. 25%, 50%, 100%, and 125% from commonly consumed portion or available portion from commercial product (*see Appendix B*). The common consumed portions were obtained from 1-day diet record carried out previously. An electrical scale was utilised in measuring the weight of each portion in grams. Thus, the option might capture adult and child food portions. The amount of Zn for each food was obtained from USDA

Nutrient Database for Standard Reference (United State Department of Agriculture, 2013) and Nutrisurvey 2007 for Indonesian food database (http://www.nutrisurvey.de), and previous studies (Chan et al., 2007; Norhaizan and Nor Faizadatul Ain, 2009)a plate or bowl containing the food, with spoon and fork at each side. The photo size was 4x8 cm in size with white background and taken using Nikon 3100D camera with macro lens.

The main questionnaire and both versions of FFQ (long and short) (*see Appendix A*) were then tested by interviewing thirteen women at Al-Hidayah kindergarten, Bekasi, West Java, Indonesia. On the first day, the volunteers were interviewed to complete the main questionnaire and shortened FFQ to gain the daily Zn intake of their children in the previous month. A follow-up interview was carried out three days later to complete the longer version of FFQ. The estimation of daily Zn intake of their children during the previous month was acquired from both FFQ by multiplying the frequency of foods consumed in a month with the portion size. The frequency or how often the respondent consumed chosen foods ranged from never to the highest frequency of the respondent have for a food per day. Results were compared to analyse whether the shortened FFQ was in a good agreement with the longer version to estimate the Zn intake. Statistical analysis was carried out using Mann-Whitney test and Bland-Altman plot.

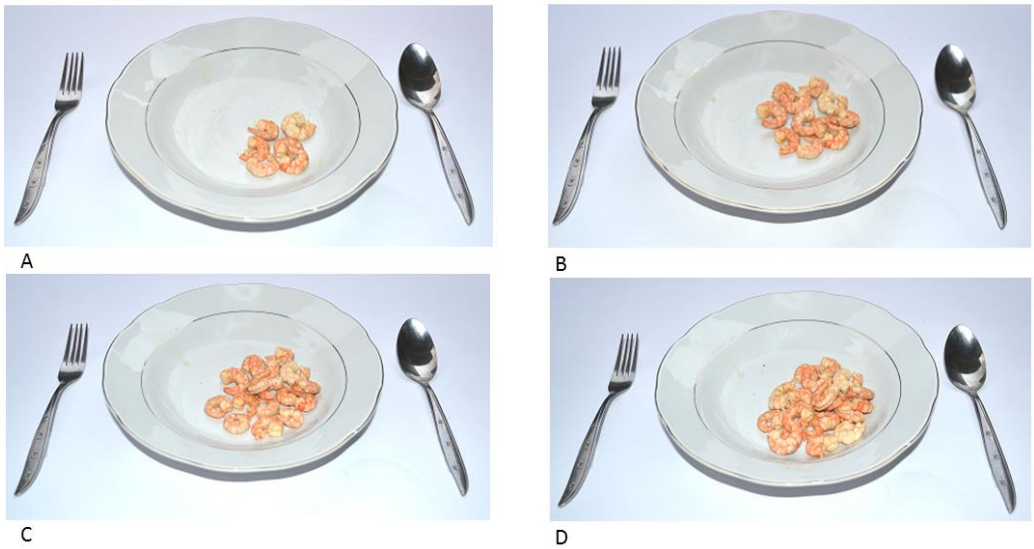

Figure 3.1 A set of food photos for one type of food.

3.3.2 *Validation of FFQ*

A 3-day diet record was used to validate both FFQs and carried out with a further 6 female volunteers. A week later, the participants were interviewed using the long FFQ

(L-FFQ), while the shortened FFQ (S-FFQ) was completed three days later. All the dietary assessments were used to estimate only the daily Zn intake of the participants. In summary, the process of FFQ development and validation is shown in a flow diagram (Figure 3.2).

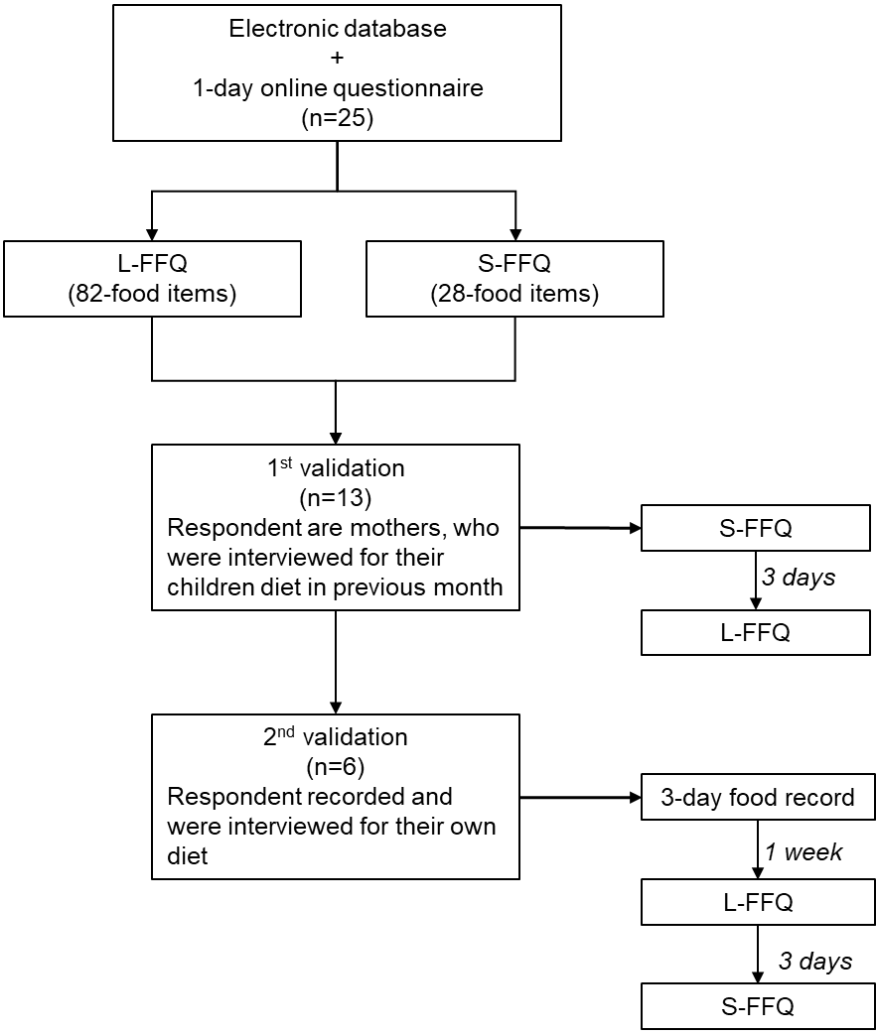

Figure 3.2 A diagram shows the flow of S-FFQ development and validation used in this study.

3.3.2 Estimated Zn dietary intake during pregnancy, infancy, and at-present periods in main study

An ethical clearance was obtained from the Committee of The Medical Research Ethics of the Faculty of Medicine University of Indonesia (526/UN2.F1/ETIK/2014). Twenty-one mother-child pairs were recruited for this study through attendance at Dental Hospital, Jakarta. The mothers completed the main questionnaire and the three sets of S-FFQs relating to retrospective food consumption regarding time periods, i.e. pregnancy, infancy, and child current consumption. All participants in this initial study

were treated based on the guidelines assigned in the Declaration of Helsinki and given informed consent (for Consent form see Appendix A).

The main questionnaire was completed before the FFQs to build a good rapport with the mothers. Furthermore, before beginning the FFQ for any periods, the investigator would remind the mother of certain conditions related to those times. For example, before completing the FFQ during pregnancy, the mothers were asked about morning sickness, their cravings for foods, any illness, etc. They were also requested to describe what kind of foods they consume on a daily basis from breakfast to dinner. Any information given by the participants was treated as confidential.

3.4 Results

3.4.1 *Analysis of the 1-day diet record*

The 1-day diet record identified 82 food items that were reported by the 25 volunteers (Table 3.2). All food items were included in the longer version of FFQ. The shortened FFQ presented 28 food items based upon Zn-rich or phytate-rich foods was also compiled (Table 3.3). Both FFQ were completed with the portion size option for 27-food items displayed by food photos (Figure 3.1).

3.4.2 *The Long and Shortened FFQ analysis*

To validate the long FFQ and short FFQ, 13 different volunteers were recruited. The assessment using the long FFQ (L-FFQ) revealed no differences between daily Zn dietary intake for the children (mean ± SD=7.73 ± 3.57 mg/day) compared with shortened FFQ (S-FFQ) (mean ± SD=8.05±2.83 mg/day; p=0.45) as shown in Figure 3.3. There was also a strong significant correlation between L-FFQ and S-FFQ (r=0997, p=0.01). A Bland-Altman analysis was carried out to demonstrate if the use of L-FFQ compared with S-FFQ resulted in any bias in measuring daily Zn intake across the range of intake values and to gain intake values for limits of agreement between both FFQs. Thus, the differences in estimated daily Zn intake between both FFQs (L-FFQ and S-FFQ) were plotted against the mean daily Zn intake between both FFQs (L-FFQ+S-FFQ/2) (Figure 3.4). The result showed the daily Zn intake obtained using S-FFQ was in a good agreement with L-FFQ because the differences of daily Zn intake between L-FFQ and S-FFQ were still in the range of differences standard deviation, which was 2.6 for the upper limit and -3.26 for the lower limit.

Data analysis was carried out to reveal if there were any significant differences in daily Zn intake among children according to the educational background of their mothers. The results show no difference was recorded in daily Zn intake across maternal educational background when using the S-FFQ as shown in Figure 3.5.

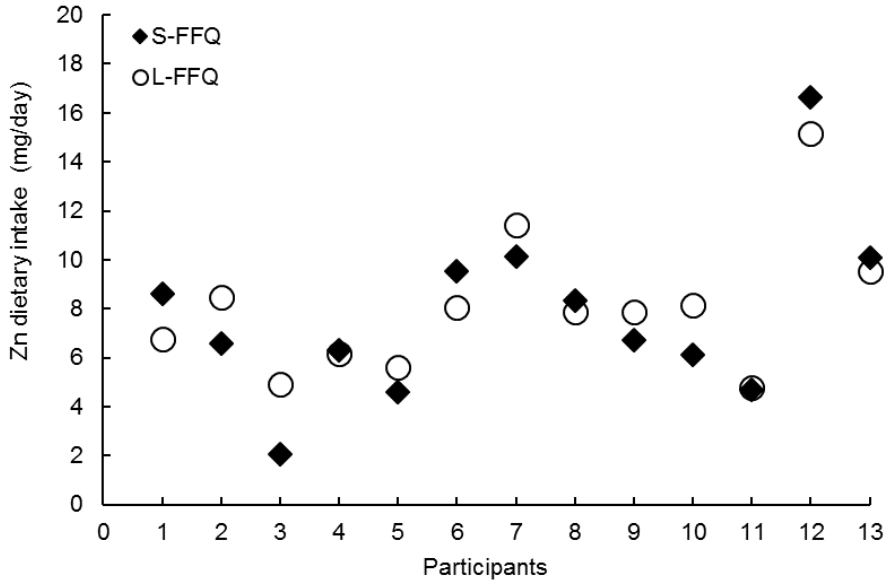

Figure 3.3 A graph represent estimated daily Zn dietary intake using L-FFQ and S-FFQ. The differences were not significant between both FFQs (Mann-Whitney, p=0.871), while there was a significant correlation between L-FFQ and S-FFQ (Spearman correlation, r=0.806, p<0.01) (n=13).

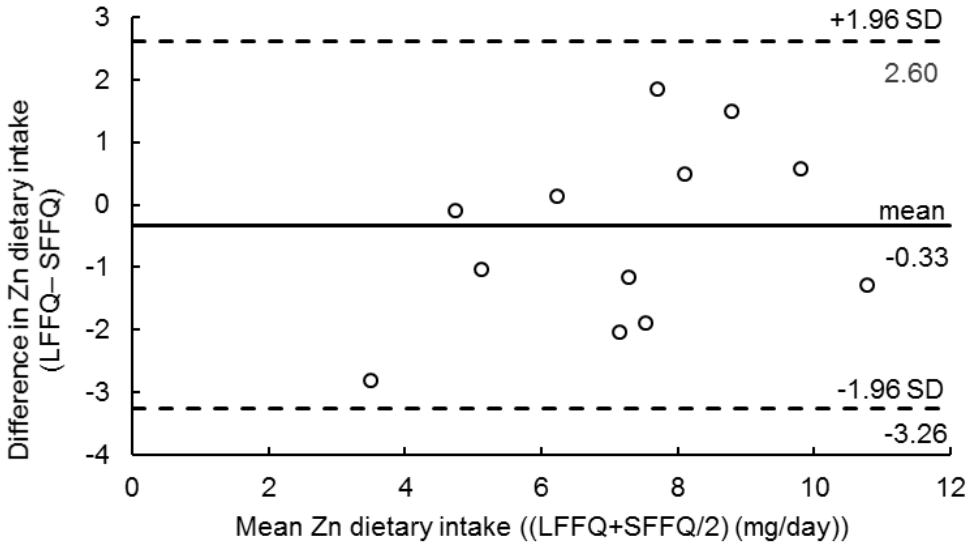

Figure 3.4 A graph represent Bland-Altman plot to analyse the level of agreement between L-FFQ and S-FFQ. The graph is showing limits of agreement and mean differences of daily Zn dietary intake from L-FFQ and S-FFQ, that was -i.e. -3.26 to +2.6, mean difference –0.33 mg/d (n=13).

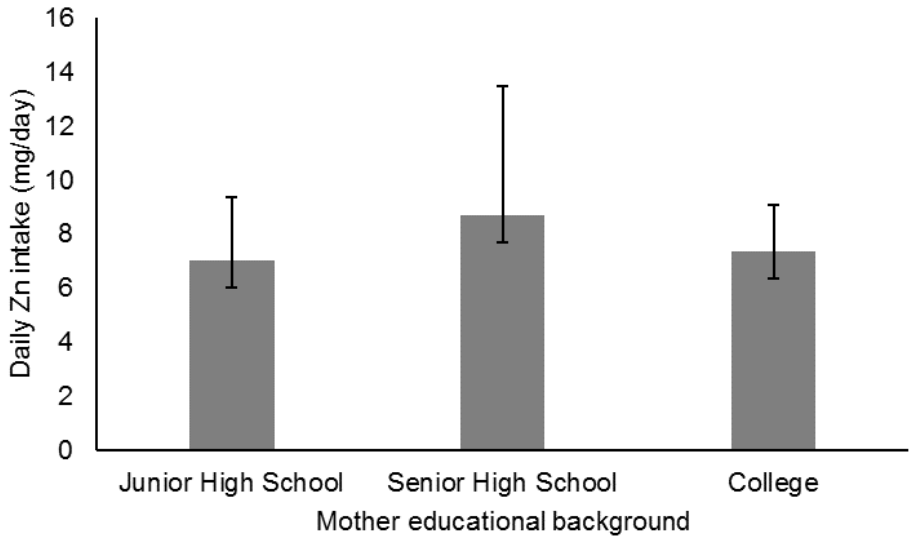

Figure 3.5 The mean value of daily Zn intake according the mother education background. The differences were not significant between each group (Kruskal-Wallis test, p=0.737) (n=13).

3.4.3 Validation of FFQ

A relative validation was carried out by comparing daily Zn intake obtained from S-FFQ, L-FFQ and the 3-day diet records (DR) in 6 individuals. There was no significant difference in daily Zn intake gathered using DR (mean ± SD=6.38 ± 1.48 mg/day), S-FFQ (mean ± SD=6.19 ± 1.36 mg/day), and L-FFQ (mean ± SD=7.50 ± 1.29 mg/day) using Kruskal-Wallis test (p=0.24), and S-FFQ revealed a significant correlation with L-FFQ (Spearman's correlation; r=0.943, p=0.005) (Figure 3.6).

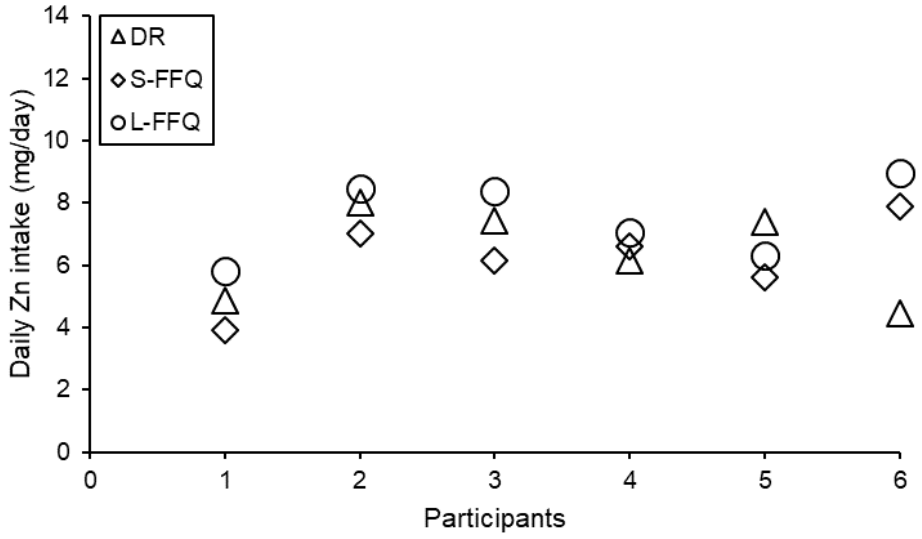

Figure 3.6 Estimated daily Zn intake assessed using DR, S-FFQ and L-FFQ (Kruskal-Wallis, p=0.24). There was a significant correlation between S-FFQ and L-FFQ (Spearman's correlation; r=0.943, p=0.005) (n=6).

A Bland-Altman plot was performed to evaluate the limits of agreement between S-FFQ compared with DR. Thus, the differences in estimated daily Zn intake between DR and S-FFQ were plotted against the mean daily Zn intake between DR and S-FFQ. The differences between the two assessments were within the limit of agreement as revealed in Figure 3.7.

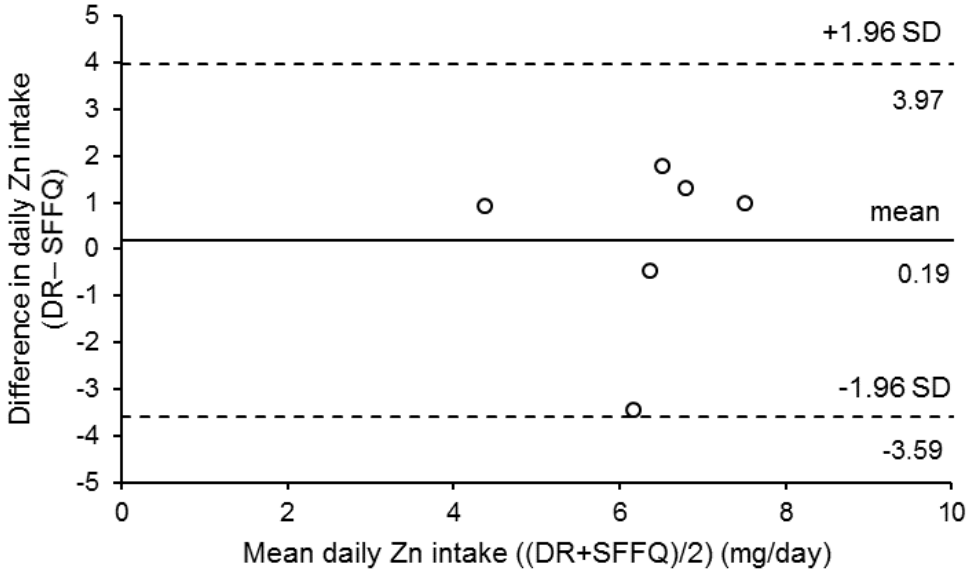

Figure 3.7 Bland-Altman plot demonstrates limits of agreement and mean differences of daily Zn intake from DR and S-FFQ. The limits of agreement were -3.59 to +3.97 and the mean differences is –0.19 mg/d (n=6).

3.4.4 *Estimated daily Zn intake of mothers during pregnancy and of children at infancy and current time, in larger study cohort*

Twenty-one children paired with their mothers were eligible and recruited in this study, but one participant was excluded due to inadequate information on child's intake during infancy and at present provided by the mother. Thus, 20 participants, consisted of 8 boys and 12 girls, were included in this study. The characteristic of participants, such as age, medical history, socioeconomic level, prenatal and postnatal history, is shown in Table 3.4. As the dental hospital was located at central Jakarta, majority of the participants were originated from the local area.

The daily Zn and phytate intake of the mother during pregnancy, the child during infancy and child at current time were estimated using previously developed and validated S-FFQ contained 28 food items (Table 3.3) completed with 26 food photo series *(Appendix B).* The portion for offal was presented using the same picture for the red meat (for cow's offal) and or obtained from the respondent's description for the

number of chicken liver and or gizzard consumed, and for eggs were based on the respondent description (half, one, two, or three eggs). On average, daily intake of Zn, phytate, and Zn:phytate molar ratio of the mothers during pregnancy, and the children during infancy and at present are presented in Table 3.5.

General Information		n	(%)
Child's Gender	Male	8	(40)
	Female	12	(60)
Mean child age (years)		8.15 ± 1.35	
Special needs		0	(0)
Medical history	Illness	0	(0)
	Allergies	5	(25)
Socioeconomic level			
Father's education	Less than high school	1	(5)
	High School	14	(70)
	Higher education	5	(25)
Mother's education	Less than high school	3	(15)
	High School	12	(60)
	Higher education	5	(25)
Monthly family expenses (Indonesian Rupiah)	< 1,000,000	3	(15)
	1,000,000-<3,000,000	9	(45)
	3,000,000<5,000,000	5	(25)
	≤5,000,000	3	(15)
Pre-natal and post-natal history			
Mean mother pregnancy age (year)		27.32±5.43	
Hyperemesis		7	(35)
Hospitalized		1	(5)
Drug/Food Supplement/Vitamin		20	(100)
Delivery time	Pre-term	2	(10)
	At-term	18	(90)
Delivery method	Vaginal birth	16	(80)
	Caesarean section	4	(20)
Birth weight (kg)		3.15±0.55	

Table 3.4 Characteristic of participants (n=20)

	Estimated daily intake (mg/day)		
	Zn (mean±SD)	Phytate (mean±SD)	Zn : Phytate ratio (mean±SD)
Pregnancy	11.99 ± 6.99	583.61 ± 575.64	4.41 ± 2.48
Infancy	6.12 ± 2.82	117.12 ± 62.66	2.16 ± 1.87
Childhood			
(4-8 years old)	8.08 ± 3.93	193.95 ± 173.60	2.18 ± 1.51
(9-13 years old)	6.52 ± 3.26	191.52 ± 91.03	3.19 ± 1.25

Table 3.5 The mean daily Zn and phytate intake (mg/day), also viable Zn (Zn:phytate ratio) during pregnancy (n=20), infancy (n=20), at-present (n=20).

Based on the estimated average requirement (EAR) for Zn in mixed or refined vegetarian group, the mean daily Zn intake among three groups were above the EAR value suggested by IZiNCG (International Zinc Nutrition Consultative Group (IZiNCG) et al., 2004), which were 8 mg/day for pregnant women, 3 mg/day for children aged 1-3-year old and 4-8-year old, and 5 mg/day for children aged 9-13-year old. Using EAR value for Zn, the individual participants were classified into low and high Zn intake groups. There were 9 mothers who classified into low Zn intake (LZ) group, while 11 other mothers belonged to high Zn intake (HZ) group. Whereas, during infancy, there were 2 children classified into LZ group and the other 18 were belonged to HZ group. From analysis of child dietary Zn intake at the present time, three children were included into LZ group, while 17 others were classified into HZ group as shown in Table 3.6.

Category	Pregnant Mother[a] (n=20)	Infancy[b] (n=20)	At-Present[c] (n=20)
Low	9	2	3
High	11	18	17

Table 3.6 Frequency of individuals categorised for Zn intake based on EAR of Zn for different age group. According Estimated Average Requirement (EAR) for Zn for mixed or refined vegetarian diets in pregnancy[a] (EAR=8 mg/day), 1-3 year old during infancy[b] (EAR=2 mg/day), current intake for 4-8 year old children[c] (EAR=3mg/day) and 9-13 years old children (EAR=5mg/day) (International Zinc Nutrition Consultative Group (IZiNCG) et al., 2004).

In the questionnaire, information on baby feeding just after birth was also included. There were 13 children who were exclusively breastfed, including 10 children who were breastfed until 6 months, and 3 children who were breastfed for less than 6 months and continued by having formula or no milk but solid food. Instead of breastfed, there was 1 child fed by formula milk, while 6 children were given mixed of breastmilk and formula milk.

Mother's education	Weaning age ≤ 6 months	Weaning age ≥ 6 months	p (n=20)
Less than high school	2	3	1.00
High school and above	5	10	

Table 3.7 The weaning age in relation with mother's education levels. The differences of weaning age were not significant between mother's education levels (Fisher's exact test; significance level at p≤0.05).

The weaning ages varied across individuals in this study. According to WHO, weaning should not be started before 6 months of age (WHO, 2003). In the current study, 7 children started the weaning process before 6 months, whilst 13 others were weaned after 6 months. The reason for early weaning, however, was not related to the educational level of the mother as presented in Table 3.7. The information of weaning was also obtained, where less children were having their first solid food earlier than 6 months old and banana was a common first food (Table 3.8).

	< 6 months (n=7)	≥ 6 months (n=13)
Banana	4	5
Baby rusk	2	3
Rice porridge	1	5

Table 3.8 The introduction of solid foods or weaning.

3.5 Discussion

FFQ format was selected for use in this study as it is an appropriate tool to estimate relative nutrient intake and has been used successfully in retrospective studies. A previous study demonstrated a FFQ was sufficient to assess mineral intake, such as calcium, iodine, and Zn in the European Community's 'EURopean micronutrient RECommendations Aligned (EURRECA)' Network of Excellence study (Serra-Majem et al., 2009). However, it is important to exclusively develop FFQ for different populations (Shim et al., 2014). This preliminary study aimed to develop and validate a suitable FFQ to gather information on the Indonesian population's daily Zn intake, as previous studies were limited. Current, validated FFQ were deemed inappropriate for this study as food choices included are highly associated with demographics, cultures, ethnicity, and religions of a population and food availability in the local area. This was another reason why the development of bespoke FFQ was essential. For example, anecdotally, tempeh, a fermented soy bean is consumed by Indonesian people on daily basis. Thus, the first stage of the preliminary study was to establish foods and meals that are commonly consumed by Indonesian people.

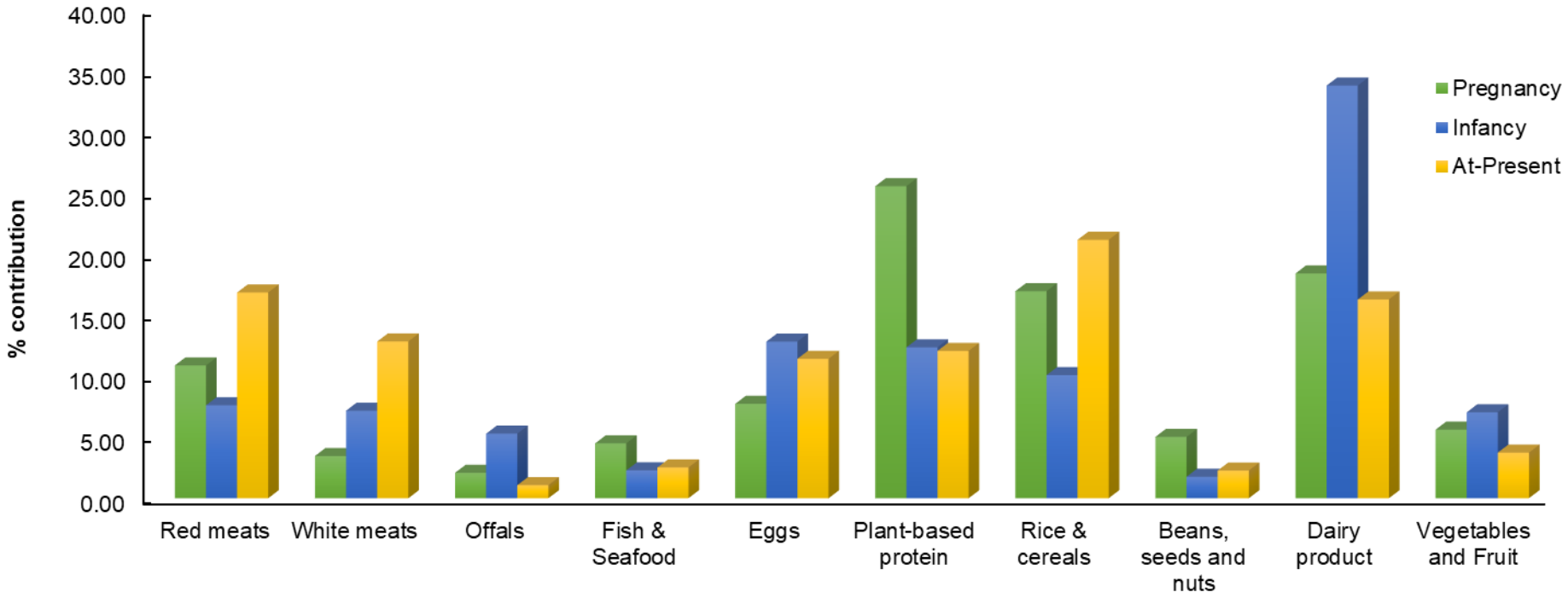

Figure 3.8 Percent contribution of food groups contributing to estimate daily Zn intake of pregnancy, infancy, and children at present group.

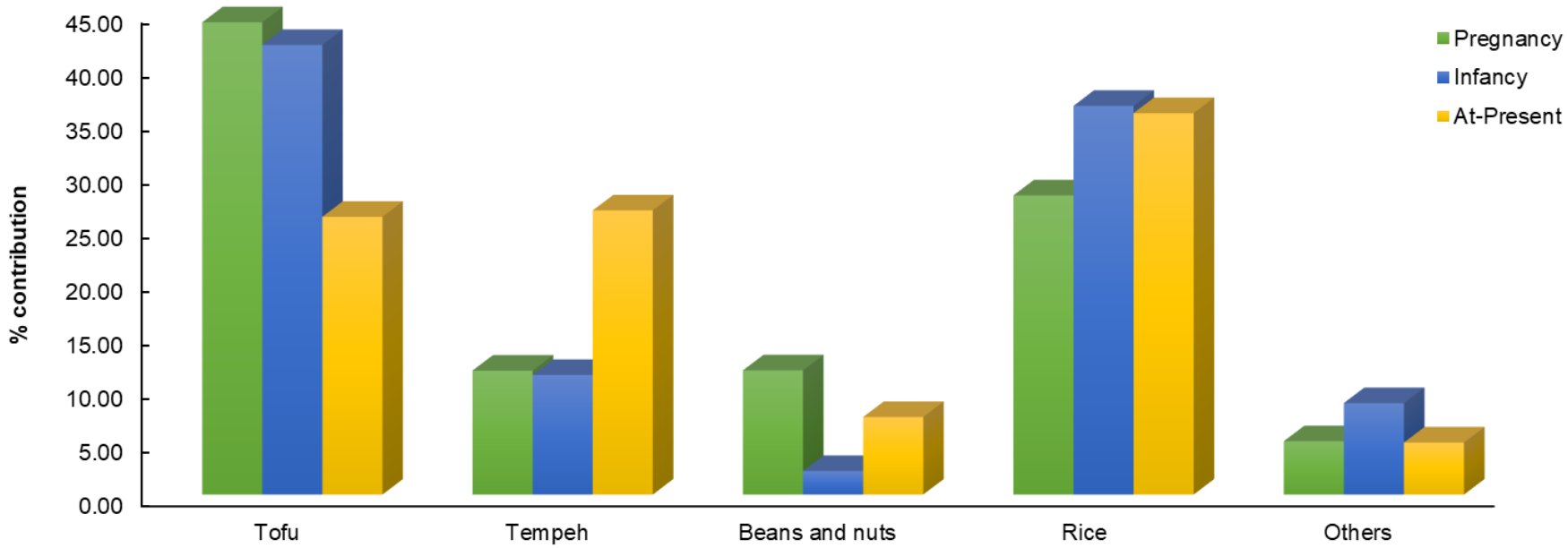

Figure 3.9 Percent contribution of food group contributing to estimate daily phytate intake of pregnancy, infancy, and children at present group.

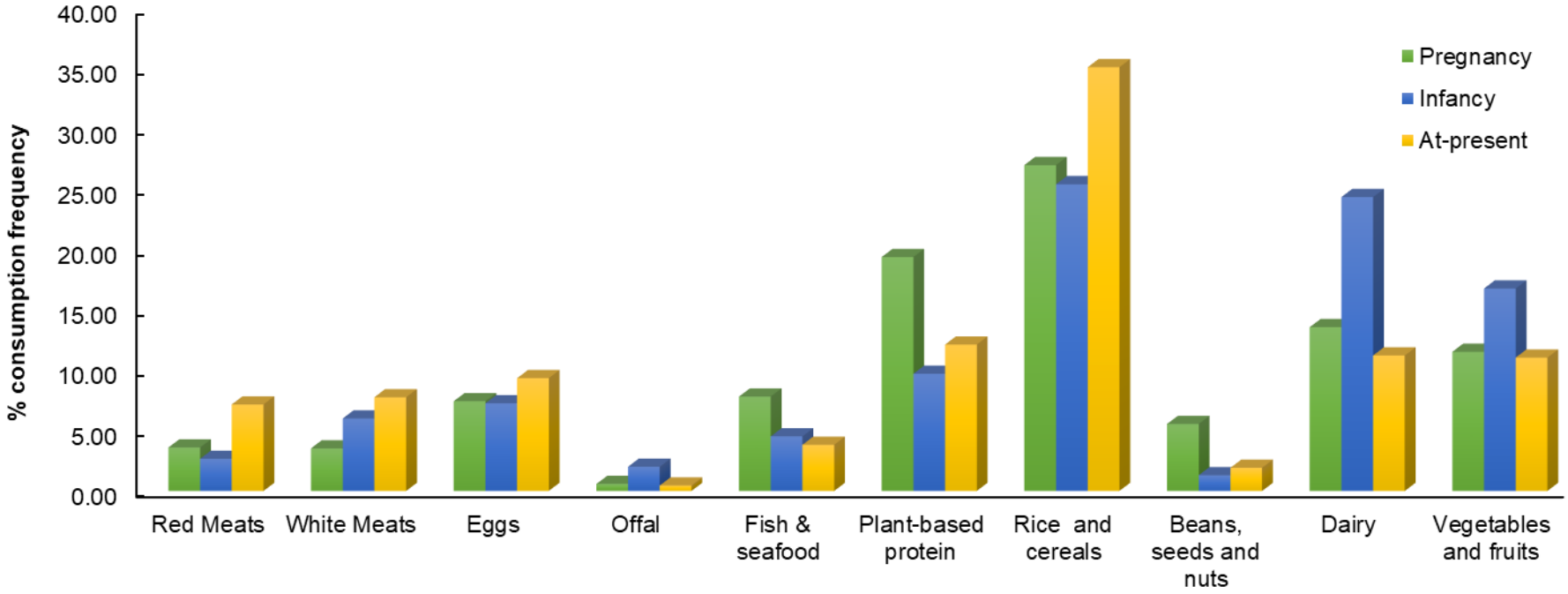

Figure 3.10 Percent frequency of food group consumed in pregnancy, infancy, and children at present group.

The compilation of foods and meals were reviewed to create a regularly consumed food list that would be incorporated in the FFQ. There was a large variety of meals prepared from only one type of food. This variety was taken into consideration to develop a food-based FFQ rather than meal-based. As, the main study would be performed at the dental hospital located in central Jakarta, a capital city occupied by more than 10 million people with different socioeconomic, ethnicities, cultures, and religions background, anyone could be recruited as long they were eligible for the study. Therefore, if the meal-based FFQ was applied, it might not accommodate meals consumed by all people, thus, increased the possibility of unreported intake (Shim et al., 2014).

After the first stage 1-day food record, 82 food items were included in the food list in L-FFQ. The average time used to complete the FFQ was approximately 15-20 minutes. Hence, the participants in the main study would spend about 60 minutes to complete three sets of long FFQ. The main concern was the participants might feel exhausted which would affect the quality of their answers. In addition, the interviewer could lose focus because they need to repeat questions several times. Therefore, a S-FFQ containing 28-food items was developed and compared with L-FFQ in the preliminary study. The food items, which were not consumed frequently, such as pork, veal, cow's organs, were eliminated from S-FFQ food items list. The results indicate that there was no difference in daily Zn intake between S-FFQ and L-FFQ. The S-FFQ in the second stage of the preliminary study was then evaluated and validated using a 3-day diet record in the third stage of preliminary study. The differences of daily Zn intake between S-FFQ, L-FFQ, and 3-day diet record were not statistically significant. As the S-FFQ was comparable to the L-FFQ and has been validated using 3-day diet record as a 'gold standard', it was decided to be applied in main study to reduce pressure on the interviewers and participants (Samman et al., 2010).

The second stage of the preliminary study revealed that average daily Zn intake in children measured in both FFQs met the EAR for children 4-8 years old in the mixed/refined vegetarian group as suggested by IZiNCG (3 mg/day) and the RDA for 4-6 years old of Indonesian populations (5 mg/day). Interestingly, the assessment in adult females volunteered in third stage of the preliminary study showed daily Zn intake using L-FFQ, S-FFQ, and 3-day diet record were lower compared with EAR suggested

by IZiNCG and the RDA for Indonesian populations. Thus, the maternal educational background was considered since the participants in the third stage had a degree in higher education compared with the second stage of the preliminary study. However, there were no significant differences in daily Zn intake between different maternal educational backgrounds (p=0.70) demonstrated in the second stage of preliminary study as well as in the main study (Table 3.7). Maternal education has previously been associated with the type of children's diet as the children from lower maternal education seems to choose less healthy foods (Cribb et al., 2011), therefore, sociodemographic background among participants should be carefully considered.

The FFQ can be interviewer-administered or self-administered. A previous study demonstrated a higher correlation coefficient between FFQ and the reference measurement when using the interviewer-administered FFQ compared to self-administered questionnaire (Cade et al., 2002). Therefore, it was decided that interviewer-based FFQ would be more accurate. However, the circumstances during interview session, such as privacy and confidentiality, should also be taken into consideration. In the second stage of preliminary study, the investigator was aware of the presence of other mothers in possibly provoking the participants to misreport their child's nutrient intake in an attempt to influence any negative opinions of other mothers towards them if they did not give their children certain foods, particularly in terms of "healthy" or "luxury" foods, such as sausage, chicken nuggets, or vegetables. Social desirability and approval may lead bias on nutrient intake report, where women are more affected by compared to men (Farajian et al., 2015; Hebert et al., 1995; Horner et al., 2002). Thus, the privacy and confidentiality must be guaranteed during interview in the current study. The cross-check questions are also important to avoid misreporting during the FFQ completion (Cade et al., 2002).

In this study, 26 food photo series were produced to display the food portion size incorporated in the FFQ. The portion size of each food was presented using four different food photos following a guideline by Nelson et al. (1998) in that even numbers were considered to avoid photo selection in the central position. Furthermore, the use of food photos to assist participants in estimating the food portion size has been documented previously (Bouchoucha et al., 2016; Naska et al., 2016; Nelson and Haraldsdóttir, 1998).

The previously developed and validated S-FFQ was applied in the current study to estimate daily Zn intake during pregnancy, infancy, and in child at present. A recent study showed the usefulness of FFQ to estimate nutrient intake 3-5 years after birth (Mejía-Rodríguez et al., 2014). Since this main study required the information in daily Zn intake of more than nine years prior, the interview process was added and optimised to gain valid information. Initially, the interview was begun with a conversation to bring participants back to the targeted time periods. The interview was started by asking participants' daily routines, such as time to get up from bed, kinds of breakfast, any morning sickness, type of activities they usually did during pregnancy, and they were also asked to give an illustration on commonly consumed foods within a week. So, if there were 7 days in a week, how many days they ate red meats, white meat, fish, seafood, egg, etc. The same methods were also applied in probing the information of their child's dietary intake history, especially during infancy. All answers were then recorded and used to indicate overreported or misreported intake when the interview was continued to complete the FFQ. However, a recent study reported women were capable of remembering exactly their diets and food supplements consumed during pregnancy (Mejía-Rodríguez et al., 2012).

The daily Zn intake of participants were estimated using a shortened FFQ containing 28 food items. FFQs are sufficient in assessing relative nutrient intake and allows us to rank individuals into groups, but are not suitable to measure the absolute value of nutrient intake (Cade et al., 2002; Ho et al., 2016). Thus, we divided the participants into low (LZ) and high Zn (HZ) intake groups based on EAR established by IZiNCG (International Zinc Nutrition Consultative Group (IZiNCG), 2004) as shown previously in Table 3.6.

Overall, rice was the most frequent food consumed by all three groups, but contributed less Zn per day compared with plant-based foods during pregnancy and milk during infancy. An earlier study using data from an Indonesian socioeconomic survey (2008) suggested rice was the most commonly consumed food by Indonesian people and contributed to the cause of micronutrient deficiency (Radix et al., 2012). It has also been observed that women who lived In Jakarta urban areas tend to serve low price foods which are high in carbohydrates, low protein and fibre level, even though they are able to buy foods containing better nutrition (Kolopaking et al., 2011). Our participants were all from Jakarta, an urban area, presumably high consumption of rice

during pregnancy could be a reason why almost half of the mothers in this study were classified in low Zn intakes group.

In the current study, plant-based protein foods (tempeh), and milk as part of dairy product, were the second and third most frequently consumed foods by mothers, during pregnancy, respectively. Only three mothers reported they did not consume milk during their pregnancy. The results were in line with a recent study reporting that plants foods were commonly consumed by pregnant women in Bogor district, Indonesia. They suggested pregnant women consumed more milk compared to pre-pregnant women. Apparently, the pregnant mothers considered milk as a good source of nutrition for a better pregnancy outcome (Madanijah et al., 2016).

Milk was the highest contributor in daily Zn intake among children during infancy period (after weaning). Most of the Indonesian mothers still provide breast milk until their children approximately 2 years old, and complemented with either formula milk, ultra-high temperature processing (UHT) milk, fresh milk, or nothing at all. The dietary slogan “4 healthy, 5 perfects” which refers to staple foods, protein sources, vegetables, and fruits (4 food items) that is completed with milk to be perfect (5 food items), has been established since 1955 by Indonesian government to improve the health level of Indonesian population on that time. Nowadays, the slogan is quite embedded in Indonesian people’s mind (Kolopaking et al., 2011). UHT milk, that is available in ready-to-drink package with a variety of flavours, is popular and well-liked by Indonesian children (Muslimatun and Wiradnyani, 2016). It might be the reason that milk was the second Zn-source food in older children.

The current study showed eggs were the second contributor of daily Zn intakes and second most frequent consumed foods during infancy and at-present. In a recent study, eggs were the most commonly consumed food among 1-5 years old children in Bandung, West Java. Likely, eggs are good candidates as a protein source that are readily available in the market with low prices and easily prepared into various meals (Muslimatun and Wiradnyani, 2016). In contrast, the consumption of plant-based foods as the protein sources were less frequent compared to vegetables during infancy, but not in older children, and their contribution to daily Zn intake was still considerably high. It might also be affected by the dietary slogan that indicated the importance of vegetables.

In general, children at present consumed more diverse foods since the consumption rates among food items in 'child at-present' group were distributed more evenly (Figure 3.10), particularly plant-based foods (tofu and tempeh), red meat products, vegetables, dairy products, and cereals. However, red meat products, such as meatballs (known as *bakso*) and sausages, were prepared by mixing a small amount of red meat with a lot of starch flour and added seasoning and flavouring enhancer. Unfortunately, this is a typical food that loved by urban children. The urban mothers also tend to serve easy-to-prepare and ready-to-eat foods, which might encourage the older children to prefer this typical food (Kolopaking et al., 2011).

Red meat was consumed extremely less frequent compared to plant-based food to fulfil the protein requirement in all groups, even though it contains high Zn. It might be led by the high price of red meats in the local market. A recent study suggested the low consumption of red meats in 12-23 months old children in West Java, Indonesia, was related to socioeconomic levels, where the children from medium socioeconomic group consumed meats more often compared to low socioeconomic group in urban areas (Fahmida and Santika, 2016). However, in general, Sari et al. (2010) suggested the consumption of animal source foods were considerably low in Indonesia, either in rural or urban poor area, which was about 12-33 gram/day. Nevertheless, the other Zn-rich foods with lower price compare to red meats, such as offal (chicken and/or cow liver), were the least consumed foods across three groups. The lack of knowledge on nutrition among mothers and false perception on liver as an unhealthy diet might responsible for this (Kolopaking et al., 2011; Radix et al., 2012).

On average, daily Zn intakes during pregnancy were more than fifty per cent supplied by plant foods, consisting of rice, cereals and noodles, plant-based protein (tofu and tempeh), seeds and nuts, also vegetables and fruits (Figure 3.8). Thus, the viable Zn was also concerning since plant foods containing high phytate acts as an inhibitor in Zn absorption. Daily phytate intake was mostly contributed by tofu and rice across three groups, while tempeh contributed less. Even though, soybean is the main ingredient for both foods, the process in making tofu is different compares to tempeh. In producing tempeh, the soybeans undergo fermentation by adding Rhizopus species (Astuti et al., 2000), which may decrease the phytate content (Chan et al., 2007).

Hence, the phytate intake and Zn:phytate molar ratio should be considered in analysing the adequacy of Zn intake in the current study.

Instead of dietary slogan socialized by the government, there is a culture to feed the baby with solid food in less than 6 months old, and even worse only few weeks after birth. The differences relating to child's weaning age and educational level of mothers, however, were not significant in this study. In contrary, a previous study in Hong Kong demonstrated that mothers, who have lower educational level, had longer breastfeeding period compared with the mothers from middle educational level, as most of them did not have a commitment to full time jobs (Tarrant et al., 2010).

Banana is a common baby first food and sometimes mixed with steamed rice. In current study, seven children were introduced solid food less than 6 months old, and most of them were given banana. This finding was consistent with a previous study which demonstrated approximately 40 per cent of mothers in Indonesia introduced solid foods to their babies within 1 week after birth (Muslimatun and Wiradnyani, 2016). Instead of banana, the mothers reported baby rusk and rice porridge as the first food for their babies (Table 3.8).

In future work, the use of the FFQ that is developed and completed with food portion photographs in this study to measure Zn intake during early life for an Indonesian population, needs to be validated using a longitudinal study. This can either be in prospective or retrospective cohorts, and/or measuring Zn in human tissue that is suitable in reflecting Zn dietary intake, like umbilical cord and/or serum which has been stored in tissue banks for research purposes. Thus, Zn content in this material may validate the level of Zn intake measured using FFQ carried out six or seven years later.

3.6 Conclusion

A shortened FFQ completed with food photos to display portion sizes, which has been developed and validated in this study, seems sufficient to estimate the relative daily Zn intake of the mother during pregnancy, also the children during infancy and at-present in the Indonesian population. Moreover, the use of the shortened FFQ may also be extended to assess other relative nutrient intakes.

Chapter 4 Developing a robust method to measure Zn distribution within the dentine of deciduous teeth at different points in time

4.1 Introduction

Early life events have been extensively studied in relation to health status of individuals in later life (Barker, 1995; Gluckman et al., 2008), such as nutritional status, illness, and/or stress. The usefulness of hair, nails, bone, and teeth have been explored for this purpose. Deciduous teeth, commonly referred to primary teeth or baby teeth, start to form approximately 8 weeks intra utero with mineralisation beginning around 14 weeks intra utero for incisors (Hillson, 2014). They continue to develop and emerge into the oral cavity after birth from about 6 months of age with permanent (adult) teeth replacing them around 6 years onwards. Thus, deciduous teeth span between the prenatal and childhood period, and present them as a good candidate to record any life events in early life (Dean, 2017). Moreover, the moment of birth is recorded within the deciduous tooth, shown as a neonatal line both in the enamel and dentine tissues. The neonatal line is an important landmark to distinguish enamel and dentine developed during prenatal (prior to birth) and postnatal (after birth) period, therefore the identification of neonatal line, which appears as a distinctive sharp dark band in a longitudinal tooth ground section, is useful in reconstruction of life history using histological techniques in deciduous teeth as described later in Figure 4.1 (Hurnanen et al., 2017).

The incremental pattern, as produced through the mineralisation process, is a feature of dental hard tissue that enables age measurement from the dentine or enamel structure. The usefulness of dentine structure is less explored in comparison to enamel, especially in deciduous teeth. Enamel is the outer layer of tooth of which 96% is composed of mineral, where the crystallite dimension of enamel is much larger compared to dentine. Nonetheless, enamel mineralisation continues during the tooth's life course even after the tooth emerges into the oral cavity as influenced by ion minerals contained in saliva (Dean, 2017).

On the other hand, dentine, as an abundant layer of the tooth structure, covered by enamel in the coronal part and cementum in root part. It is accepted that dentine is not expose to and affected by oral environment, unless there is an access due to caries and/or fracture in enamel. Furthermore, dentine is normally not remodelled. When the

level of certain minerals is not adequate in human body, bone acts as a reservoir and is involved in homeostatic mechanism, such as release of Ca and Zn, whereas dentine does not act in this way (Linde and Goldberg, 1993). Any external substances deposited during dentine mineralisation remain fully incorporated until the maturation stage. Evidence for this is observed when tetracycline is given during tooth development, where there are dense, bright and sharp lines in dentine compared with bands in enamel that are more diffuse with lower intensity when examined under ultraviolet light (Dean, 2017). Other study suggests that it is due to a lesser time lag between matrix secretion and mineralisation in dentine compared with enamel (Austin et al., 2016).

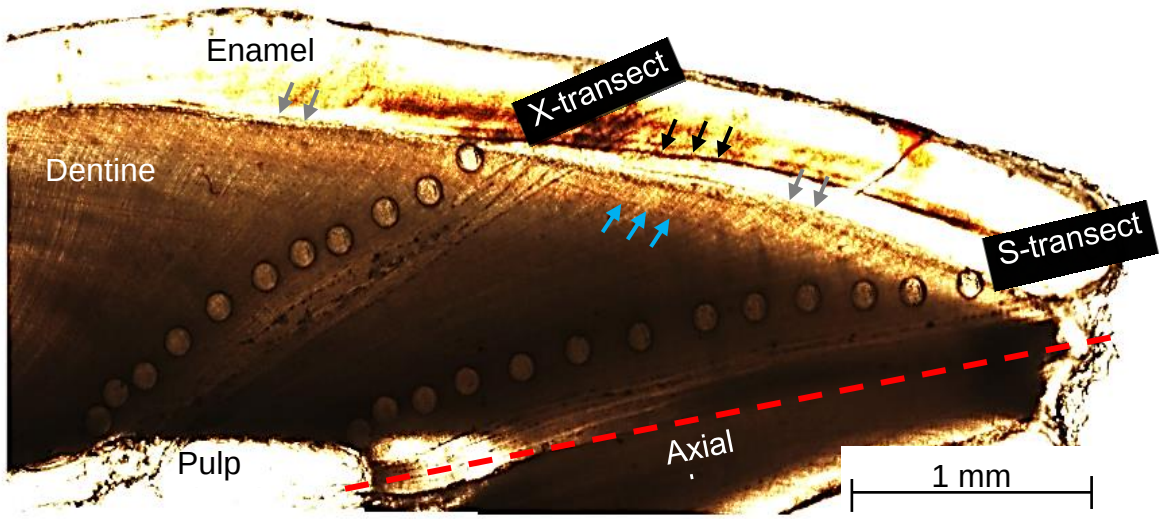

Figure 4.1 A representative image of a longitudinal ground tooth sections of an incisor. The section was labelled with enamel, enamel dentine junction (EDJ) (grey arrows), dentine, pulp, neonatal line in enamel (black arrows) and dentine (blue arrows), S and X-transect, labelled. The location of S-transect was closer to the axial plane (red dashed lined).

During the tooth's life course, dentine develops in three stages as primary, secondary, and tertiary dentine, as illustrated in Figure 4.2. The primary dentine comprises the bulk of the tooth structure under a tight-regulation of odontoblast cells. Once the shape and size of the tooth has been completely developed from the crown to the apical root, secondary dentine starts to form as a physiological process in response to age increments. The odontoblast cells regulate the formation of secondary dentine. Thus, the appearance of secondary dentine is similar to primary dentine, however is lower in proportion compared with primary dentine (Smith et al., 1995). After the development of primary and secondary dentine, tertiary dentine can develop as a response to

trauma (caries, tooth fracture and/or tooth wear) at the border of dentine-pulp junction and consequently reduces the space of pulp chamber (Klinge, 1999). In response to mild stimulus, such as mild caries, attrition, erosion and/or abrasion, the tertiary dentine is formed by primary odontoblast cells that develops the primary and secondary dentine. While, a new generation of 'odontoblast-like' cells develops the tertiary dentine when the primary odontoblast cells have died due to strong stimulus/trauma, such as severe caries (Smith et al., 1995).

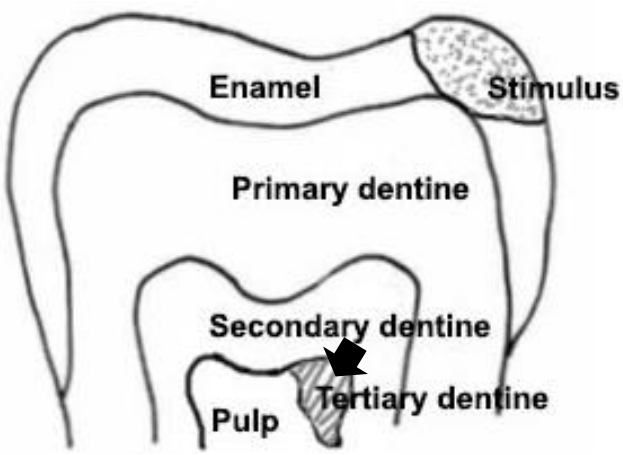

Figure 4.2 A schematic illustration of primary, secondary, and tertiary dentine in a human tooth (adapted from Smith et al, 1995).

The calculation of age in reconstructing life history in teeth is based on the incremental layers by obtaining the number of DSR in dentine. Studies have been conducted to measure DSR in dentine by either injecting external substances or dyes into living things, such as tetracyclines. This is followed by measuring the width of dye bands in a tooth ground section; or measuring the daily (von Ebner's line) and long period lines (Andresen line) of dentine using a tooth ground section or demineralised tooth section with light microscopy (Dean, 1998; Kawasaki et al., 1977; Kawasaki and Fearnhead, 1975; Massler, 1946).

The DSR of dentine in permanent teeth has been reported to be approximately 4-6μm with the highest rate located in the axial plane of cuspal area and the lowest rate shown closer to the pulp (Dean, 1998; Dean and Scandrett, 1995; Kodaka and Higashi, 1994; Reid et al., 1998). Longer distances between two lines can be 15-30 μm and represents dentine secretion within 6 to 10 days in permanent teeth (Dean and Scandrett, 1996).

In addition to the incremental nature of dentine, analysis of stable isotopes, such as nitrogen (N) and carbon (C), as well as trace elements, like lead (Pb), strontium (Sr), barium (Ba), manganese (Mn), and zinc (Zn), in collagen and mineral of dentine structure may retrieve important information related to dietary changes, migration, and or pollution. Development of micro-sampling techniques, which enable investigators to obtain small amounts of sample from specific locations (including laser ablation, serial or sequential sampling, and micro-drilling), has potential to explore elemental distribution at different time points based on the incremental nature of dental hard tissue (Humphrey, 2014), thus, reconstruction of human life history is now possible. For example, a previous study using stable isotope analysis in prehistoric human demonstrated different profiles of nitrogen and carbon in correspondence with the consumption of different food types, weaning and breastfeeding, also any stress event, including malnutrition and or disease during their lifetime (Beaumont et al., 2015). Certain trace elements in dentine, such as Pb and Mn, were related to environmental exposure in human, while Sr, Ba and Pb were associated with external stressor in primate (Arora et al., 2011, 2017; Austin et al., 2016a; Shepherd et al., 2012).

Shepherd et al (2012) reported a high variation in the Zn profile from different individual at different age interval using laser ablation - inductively coupled plasma - mass spectrometry (LA-ICP-MS) combined with histological analysis in dentine in a small number of samples (n=14). This study used longitudinal tooth sections to investigate Pb exposure in children from the north-east England. The findings revealed for the first time, a profile of Zn in humans that may represent valuable information related to human life history.

LA-ICP-MS has been used in geological samples, such as rocks, to obtain information on chemical and physical process during formation. This technique is rapid and sensitive, it also allows multi-element information from small samples at one time (Cox et al., 1996; Lee et al., 1999). Considering the aim of the present study, the LA-ICP-MS and histological analysis technique developed by Shepherd et al (2012) was adapted and optimised to investigate Zn distribution in dentine at different age intervals. Sr distribution was also analysed to confirm the diet type of respondents across different age time points as Sr has been previously studied and shown to be a good marker in dietary changes (Austin et al., 2016b; Dean, 2017; Humphrey, 2014).

4.2 Aims

This chapter aims to describe:

1) the development and optimisation of the LA-ICP-MS method and histological analysis technique to analyse Zn and Sr distribution across age points spanning over prenatal and postnatal dentine of deciduous teeth.
2) to analyse Zn and Sr distribution in dentine of deciduous teeth using the previously developed and optimised LA-ICP-MS method and histological analysis technique.

4.3 Materials and methods

4.3.1 *Developing and optimising LA-ICP-MS method and histological analysis technique*

Teeth were collected just after extraction at the Child Integration Clinic, Educational Dental Hospital of Indonesia University (RSGM-P Universitas Indonesia), and stored in RNA-later solution until the pulp was removed for molecular analysis. The 5% (v/v) Chloramine-T was used to disinfect the tooth for a week after pulp removal followed by 1% (v/v) Chloramine-T storage until they were sectioned into 200 µm thickness using low-speed saw blade with water coolant at Dental Hard Tissue Laboratory, School of Dental Science, Newcastle University. For ground section, the 1/3 middle in mesial distal direction was used for incisors, while in molars at least 2 cusp tips were included in buccal lingual direction as shown in Figure 4.3. Only intact tissues were used for sections. A group of sections was then embedded on one glass slide (up to 8 tooth sections) as presented in Figure 4.4, photographed and labelled before being sent for LA-ICP-MS at University of Leeds (School of Earth and Environment). LA-ICP-MS protocol was carried out by Tom Shepherd and is described in Chapter 2. Zn and Sr distribution were presented as ion intensities (count per second/cps) relative to Ca ion intensity (cps) (Zn/Ca and Sr/Ca ratio).

Tooth sections were then further ground (lapped) and polished manually using finest sandpaper into 100 µm section thickness and checked using a digimatic micrometre (Mitutoyo, Manual No. 1038, Serie N^0 293), subsequently each tooth section was mounted onto a histological glass slide using xylene and self-cured glue resin. Finally, the tooth sections were ready for histological analysis to estimate the age time point of each ablation pit.

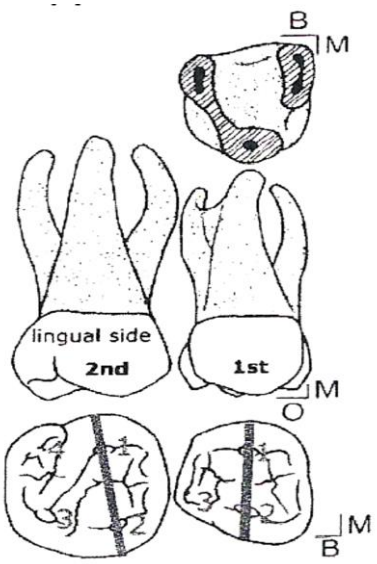

Figure 4.3 A diagrammatic representation of the area where a tooth was sectioned to produce 200 µm-thickness ground section. A molar tooth includes 2 cusps in the buccal lingual or buccal palatal direction (image courtesy of Pam Walton)

Firstly, each tooth section was observed to identify the ablation pits, neonatal line as well as any long-period and accentuated lines using a polarized light microscope (Olympus BX51). A series of images was captured (x4 objective) with an embedded-microscope camera (Q-Imaging Micropublisher 3.3 RTV camera) and processed by Imporvision Openlab 5.0.2 image analysis software. Then, a photomontage was produced using Adobe Photoshop CS6 that showed a full-shaped tooth as seen Figure 4.5, including the enamel, EDJ, neonatal line (NL), long-period and accentuated lines, as well as tertiary dentine. Next, each ablation pit and gap between pits were captured to produce an image as presented in Figure 4.6. Using ImageJ software, these images were used to measure the daily (Von Ebner's lines), which represent the DSR of dentine. At each time, five daily lines were measured in a different area of the pit and the average number was considered as the DSR of the pit. This method was also applied to measure DSR in the gap between pits. The diameter of pit and distance of gap between pits were then measured and divided with DSR to determine the time covered by each pit and gap. Thereafter, using the distance from neonatal line that was measured in the photomontage, the age of each pit was established as illustrated in Figure 4.7.

Figure 4.4 Ground tooth sections (200 µm thickness) mounted on a glass slide and labelled for sample identification.

4.3.2 *LA-ICP-MS method and histological analysis technique used in main study*

In the main study, the LA-ICP-MS method and histological analysis technique developed and optimised in the pilot study was applied with extra steps added into the protocol as follows (described in Chapter 2):

1) The tooth ground section was manually marked by a straight diamond dental eye bur under a light microscope before the LA-ICP-MS procedure.
2) After LA-ICP-MS was carried out, each ground section was observed using the polarized light microscope (x4 objective, Olympus BX51) to identify the laser ablation pits and images were captured (Q-Imaging Micropublisher 3.3 RTV camera) as shown previously in a representative image Figure 4.1 and archived.
3) The distance between each ablation pit to neonatal line was measured by following the dentinal tubules.

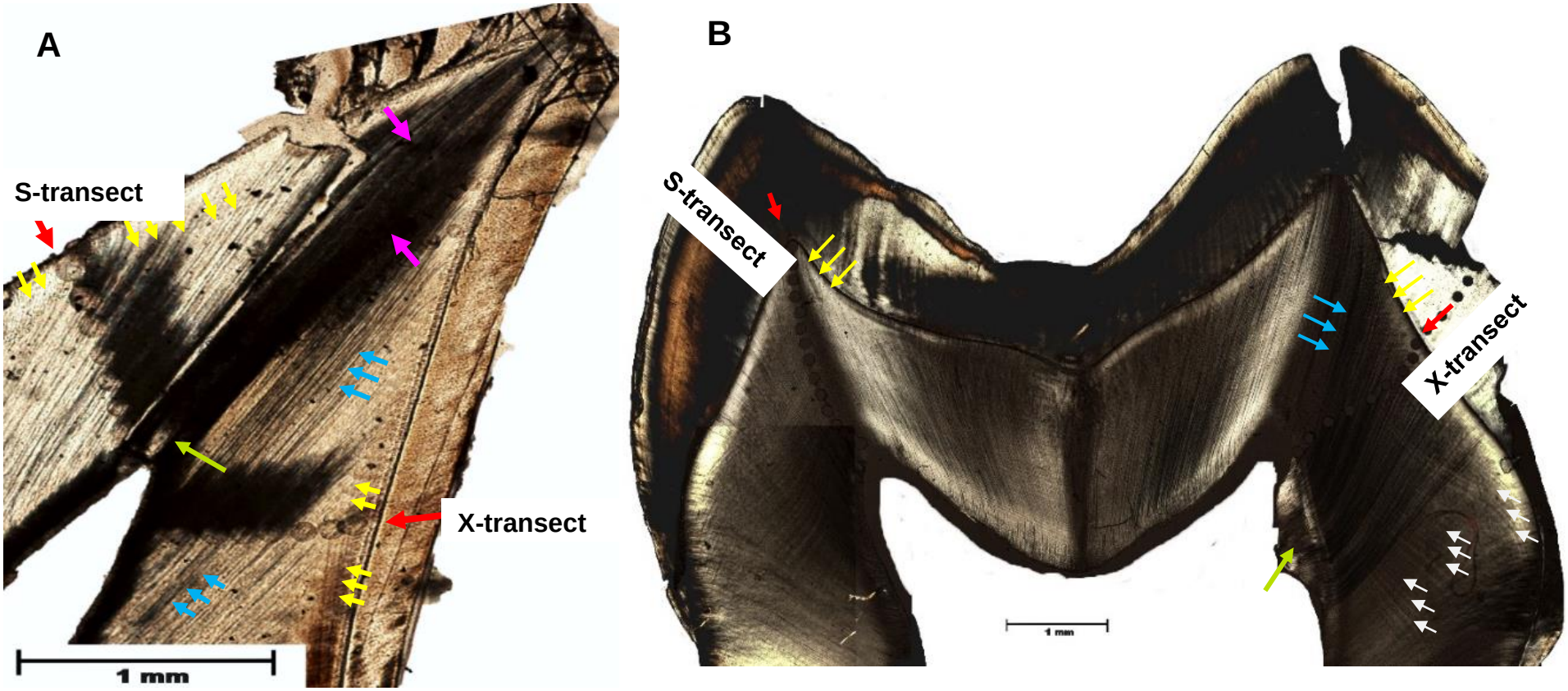

Figure 4.5 A representative photomontage of a longitudinal ground section of incisor (A) and molar (B). The identification of the ablation pit series (red arrows), neonatal line (yellow arrows), accentuated line (white arrows in B), dentinal tubules track (blue arrows), and tertiary dentine (green arrows) were carried out. Two further transects are not visible in parallel to axial plane due to their location in the dead tract, shown by purples arrows (in A).

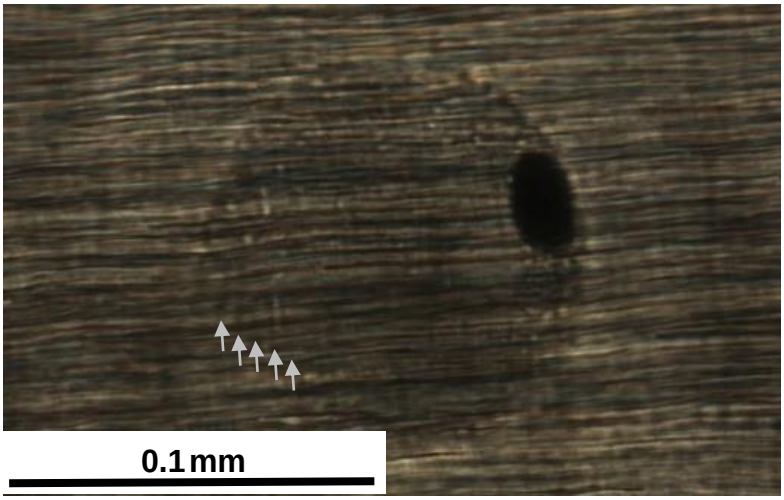

Figure 4.6 Daily Von Ebner's line within the ablation pit. It is shown as a dark and light line as following the circadian rhythms within 24 hours.

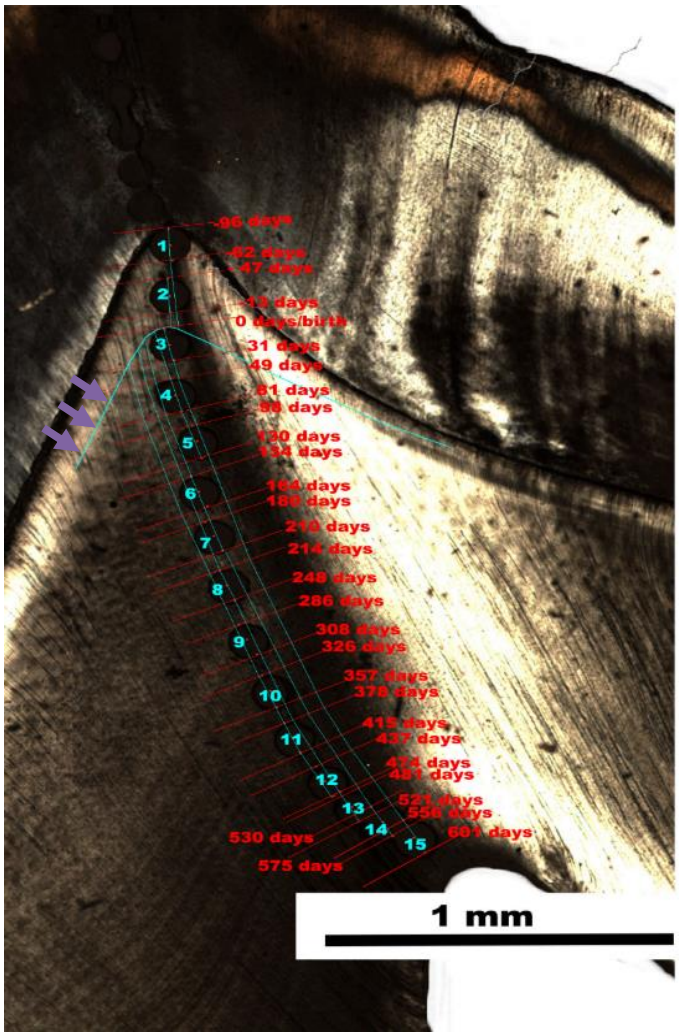

Figure 4.7 A representative image of age assignment. Using a montage, a transect was marked by dot points at the edge and centre of ablation pits (red dots), lines were drawn perpendicular with dentinal tubule at the edge of ablation pits (red lines) and each pit was measured for the distance to neonatal line (blue lines pointed by orange arrows) by following dentinal tubules (blue line).

4.4 Results

4.4.1 *A pilot study to validate the LA-ICP-MS method and dental histology approach for measuring Zn in dentine*

As a pilot study, two different teeth (incisor and molar), from different children (Z0172 and Z0255) were prepared for LA-ICP-MS and histological analysis to develop a robust technique that would be applied to the remaining study samples. Evaluation of the technique was carried out using the first two samples as shown previously in Figure 4.5. It was confirmed that 100 µm thickness of tooth section was adequate to identify tooth elements (enamel, dentine and pulp chamber), the ablation pits, long period lines, accentuated lines including neonatal line, as well as secondary and tertiary dentine using a microscope. Daily lines within ablation pits and gaps between pits were also visible and possible to measure.

Using the photomontage, certain points were identified and marked using Adobe Photoshop CS6 and ImageJ before the measurement, such as: 1) neonatal line in dentine; 2) the edge and central point of pits; 3) any ablation pits laid on tertiary dentine; and perpendicular lines to dentinal tubules were then drawn at each edge of pit along the transect as presented in Figure 4.7.

Four transects were performed in Z0172, where two transects laid parallel with the axial plane. These transects (parallel to the axial plane) were not visible for histological measurement due to a wide area of dead tract. The other two visible transects, located in more cervical area, were not oriented in a track of dentinal tubules as shown previously in Figure 4.5B. Two transects were applied in Z0255 and visible for histological measurement, but the orientation of X-transect did not follow the dentinal tubule (Figure 4.5B). The different location of transects were carried out to identify the sensitivity of LA-ICP-MS technique in analysing Zn distribution within dentine.

The dentine DSR in Z0172 (incisor tooth) was measured as 2.8 µm/day on average prior to birth (prenatal) and at an average of 2.7µm/day after birth (postnatal) at S-transect, while X-transect showed an average DSR of 2.9 µm/day in prenatal dentine and an average of 2.8 µm/day in postnatal dentine. The DSR in Z0255 (molar tooth) was slightly higher, showing an average of 3.6 µm/day in prenatal dentine and an average of 3.2 µm/day in postnatal dentine at S-transect, whereas an average of 3.5 µm/day for prenatal dentine and 3.6 µm/day for postnatal dentine were measured in X-

transect. The age range of each ablation pit measurement was not always identical between different transects in both teeth (age ranges from each transect are shown in Table 4.1). Based on the diameter of ablation pits (100 µm) and an average of DSR from all transects (3.1 µm), a 30-day age interval was determined.

	Z0172		**Z0255**	
	S-transect	X-transect	S-transect	X-transect
Prenatal (before birth)	56	38	96	12
Postnatal (after birth)	567	538	562	583

Table 4.1The age range (days) in prenatal and postnatal dentine for transects in Z0172 and Z0255.

After the age points were assigned for each ablation pit in the transect, Zn and Ca ion intensity (cps) at each transect were plotted against the age points as shown in Figure 4.8. The Ca was constantly distributed within dentine from EDJ toward the pulp. Thus, Ca was used as a reference value and the variation in Zn distribution was presented relative to Ca as Zn/Ca ratio. As seen later in Figure 4.9, although the age points of ablation pits were not identical between each transect, the profile of Zn/Ca ratio between two transects in each tooth showed an excellent agreement and a similar trend at different age points.

Zn/Ca ratio increased in last couple of pits at all transects from both samples. It was noted that the raise may not be related to age, but more related to the distance of the last ablation pit to the pulp chamber since the secondary and tertiary dentine exist closer to the pulp chamber. Zn/Ca ratio was then re-plotted against the distance of the ablation pit to pulp chamber as shown in Figure 4.10. The plot indicated the raise in Zn/Ca ratio closer to the pulp was more relevant to the distance from the ablation pit to pulp rather than age of the pit, as ages did not correlate, however distance correlated well. Considering these factors, a cut-off was established by statistical analysis (Friedman test; $p<0.05$) using data from all samples ($n = 18$). The cut-off was deemed to avoid misinterpretation in data analysis.

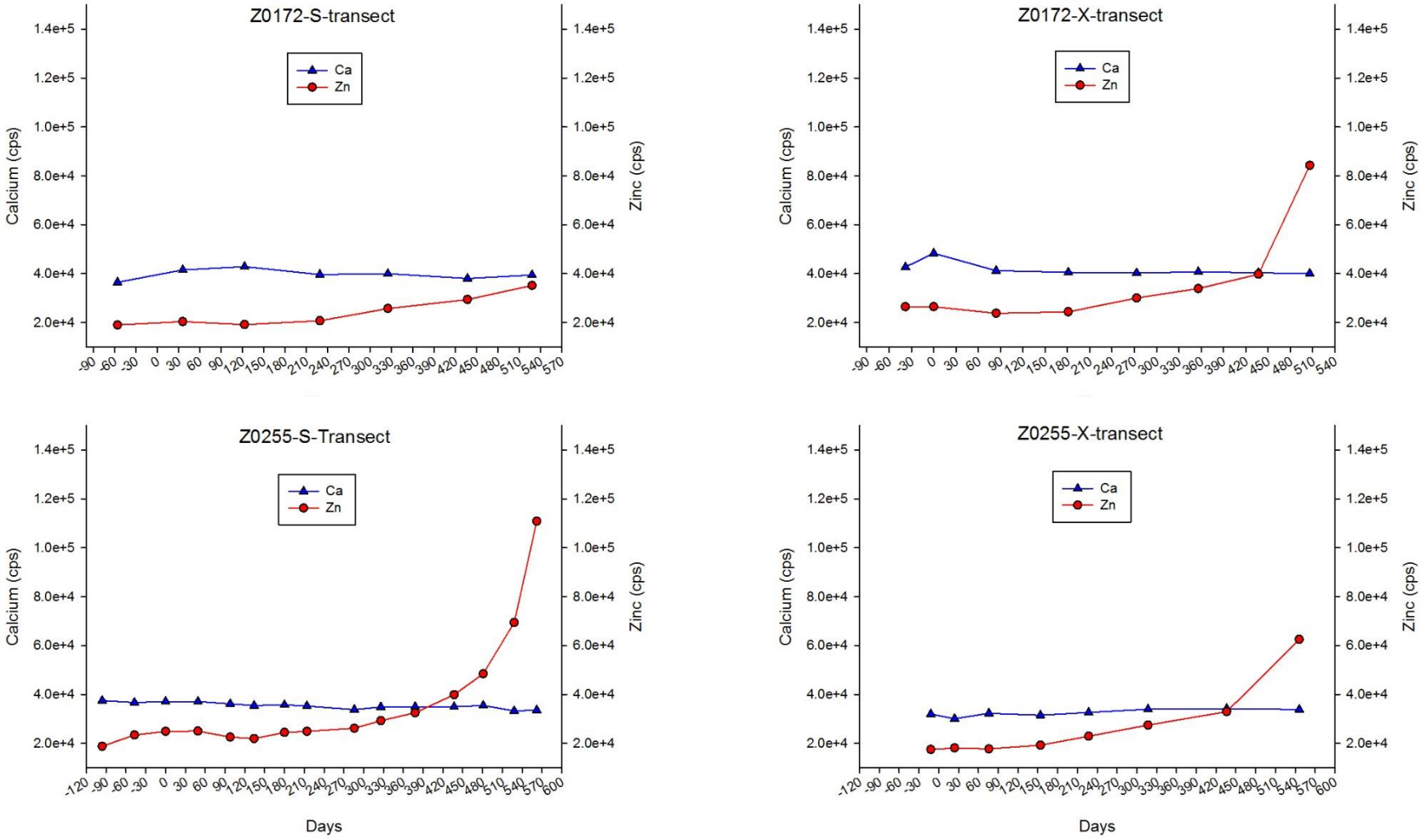

Figure 4.8 Graphs show Zn and Ca distribution that present as ion intensities (cps) in S-transect and X-transect in both tooth samples (Z0172 and Z0255).

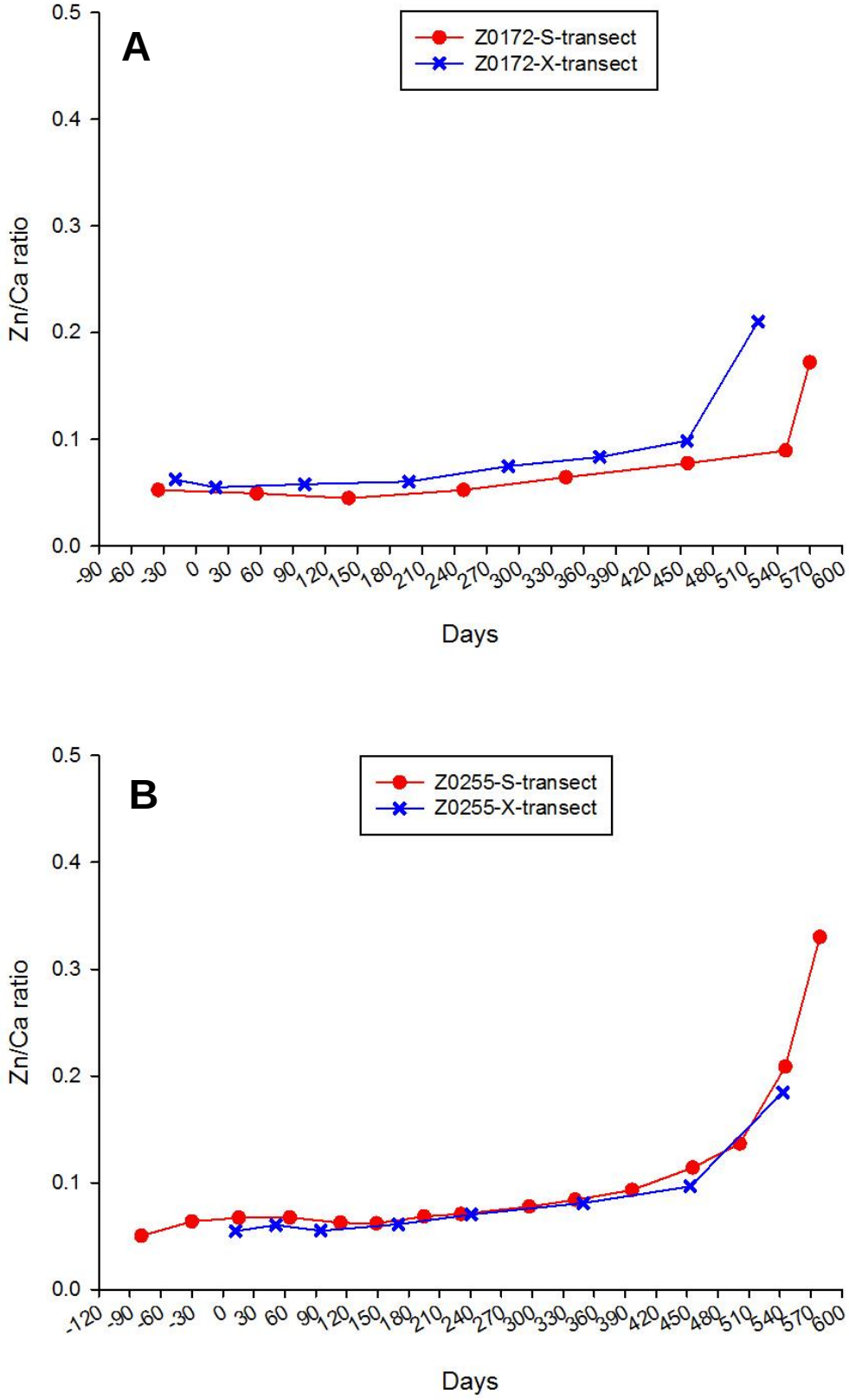

Figure 4.9 Zn distribution in tooth Z0172 (A) and Z0255 (B) at different age interval (days). Zero (0) days represents the time when the child was born (birth point). The plots from 2 samples show markedly increase at last couple of ablation pits.

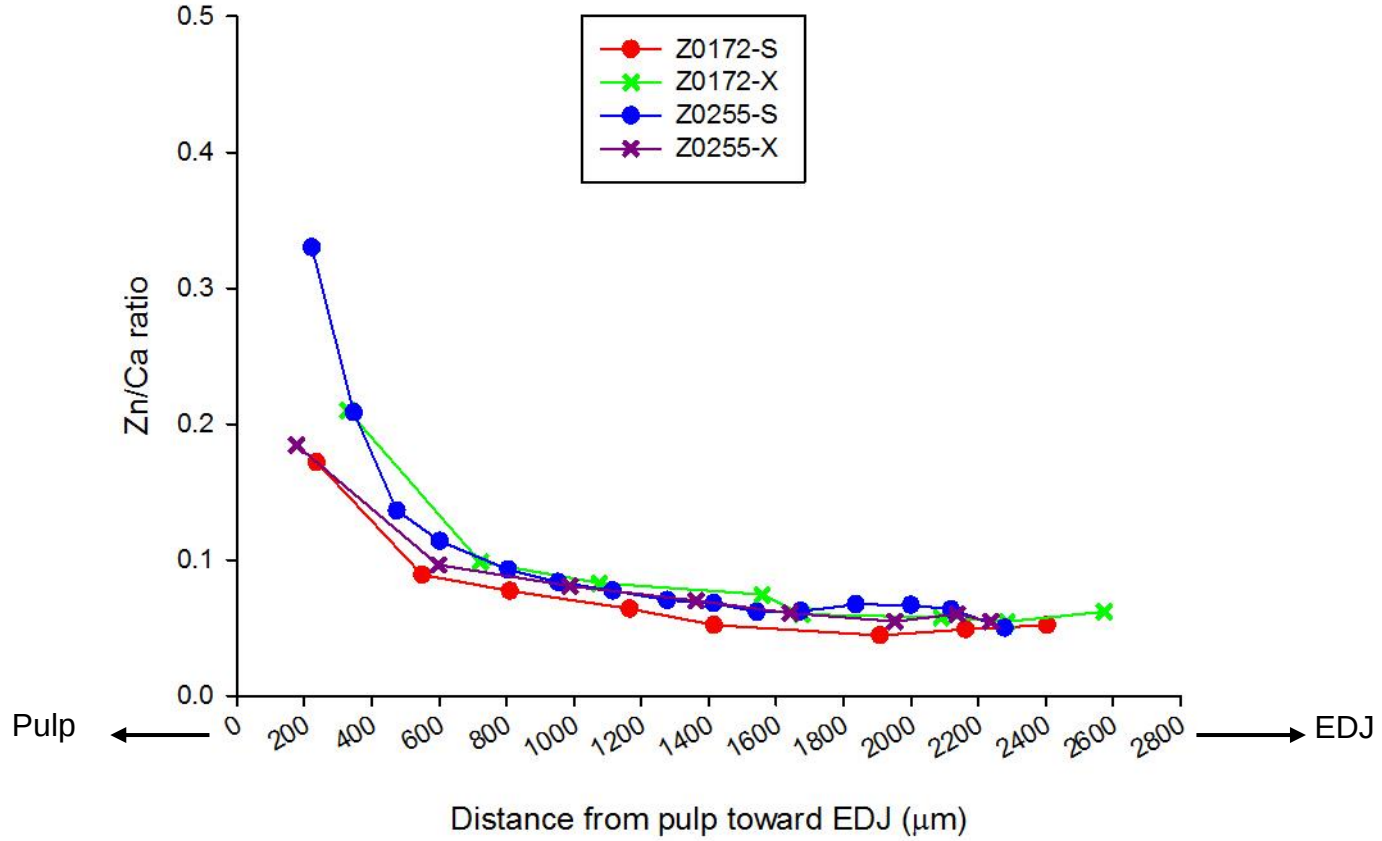

Figure 4.10 Zn distribution at different location from pulp to EDJ. A dramatically increase was addressed at less than 400 µm distance to the pulp in both transects from two tooth samples.

4.4.2 *Zn distribution in deciduous tooth dentine at different ages by LA-ICP-MS in combination with histological analysis technique*

Twenty-one children were recruited, but only 18 tooth sections were included for the element analysis within dentine since one participant has been excluded due inadequate information provided by the mother on dietary intake during infancy and at present, and two ground sections from two participants were not eligible because either the remaining hard tissue was not sufficient to produce an appropriate longitudinal section, or the dental histology analysis could not be performed as a large area of dead tract was located in the regions where the transects were created. Thus, the element analysis (Zn and Sr) at different age points were carried out using or LA-ICP-MS and histological analysis developed and optimised in the pilot study in 14 incisors and 4 molar teeth (n=18). Although a good agreement was already observed, this had only been demonstrated in two teeth and so two transects were carried out in each tooth, where S-transect was closer to the axial plane and X-transect was more cervical (as previously shown in Figure 4.1).

The thickness of prenatal and postnatal dentine of each tooth was measured in both transects and it was significantly different between dentine in each type of tooth as shown in Table 4.2. The prenatal dentine in incisors was thicker than molars, but the thickness of postnatal dentine was lower in incisors compared with molars, although this was not statistically significant. Interestingly, the DSR was measured in all tooth sections and demonstrated a significant different between prenatal and postnatal dentine as shown in Table 4.3.

Tooth	Prenatal dentine (mm)				Postnatal dentine (mm)				p
	min	med	Max	mean±SD	min	med	max	mean±SD	
Incisor	0.06	0.29	0.99	0.43±0.31**	1.26	1.6	2.16	1.63±0.21	<0.01*
Molar	0.01	0.24	0.46	0.24±0.14**	1.86	2.1	2.15	2.07±0.13	

Table 4.2 The thickness of prenatal and postnatal dentine combined from two transects in each tooth. *Kruskal-Wallis test, p<0.001; **Dunn's post-hoc test, p<0.001 comparing pre-natal with post-natal in incisors.

In line with the thickness and DSR of prenatal and postnatal dentine, this study also observed, based on the position of transect toward neonatal line as previously illustrated in Figure 4.1, the coverage period of S-transect in the prenatal age, up to 125 (± 61 days), was significantly longer than in the X-transect (38 ± 24 days) (paired

t-test; p<0.001). The age range from postnatal dentine was significantly shorter in S-transect 477 (± 92 days) compared with X-transect (511 ± 71 days) (paired *t-test*; p=0.044) (Figure 4.11). Similar with the pilot study, on average, an ablation pit represented 31 days (SD ± 5 days). Based on this observation, and the level of finer detailed information, that we can deduce from the prenatal period, we have used S-transects for the results shown in Chapter 5.

	Prenatal dentine (µm/day)				Postnatal dentine (µm/day)				
	min	Median	max	mean	min	median	max	mean	p
Tooth									
Incisor	3.0	3.8	4.5	3.8**	2.6	3.2	4	3.3	
Molar	3.2	3.6	3.8	3.6	3.2	3.4	3.6	3.4	<0.001*

Table 4.3 Dentine DSR combined from two transects in each tooth of prenatal and postnatal dentine (n=18). *Kruskal-Wallis test, p<0.001; **Dunn's post-hoc test, p<0.001 comparing pre-natal with post-natal in incisors.

Based on the pilot study, no visible difference in Zn/Ca ratio value were observed between two transects, therefore a Bland-Altman plot and correlation coefficient analysis were carried out to evaluate the agreement between two transects in this larger scale analysis (Figure 4.12). Using a Bland-Altman plot, the differences in Zn/Ca ratio between S-transect and X-transect were plotted against the mean of Zn/Ca ratio between both transects (S and X-transect). The plot suggests that Zn/Ca ratio in S-transect is in a good agreement with Zn/Ca ratio in X-transect. To carry out this analysis, Zn/Ca ratio was measured in randomly chosen ablation pits at similar time points (less than 7-day differences) from S-transects and X-transects . It was not possible to compare Zn/Ca ratio between all pits in the S-transects and X-transects because the time points from each pit between both transects were not all the same, as there were some missing time points in both transects. Zn/Ca ratio demonstrated a significant strong correlation between S-transect and X-transect (r=0.667; p=0.00183) (Figure 4.13).

An atypical increase of Zn distribution in the last couple of pits closest to the pulp, identified in this pilot study and also previous studies (Shepherd et al., 2012), was observed in all teeth analysed and a cut-off was determined using data from the S transects where Zn measurement began to differ statistically. The distance of each pit from the pulp toward EDJ was measured and grouped into 200 µm distance intervals to minimise missing values as shown in Figure 4.14. Zn/Ca ratio of ablation pits were

significantly different in different locations from pulp to EDJ (Friedman test, p=0.002). Zn/Ca ratio from pits located at position 0-200 µm and at position 200-400 µm were significantly different to the other pits (Student-Newman-Keuls; p<0.05). In contrary, Sr distribution was not affected by the location of pits as the Sr/Ca ratio differences between ablation pits were not significant from pulp to EDJ (Friedman test, p=0.207).

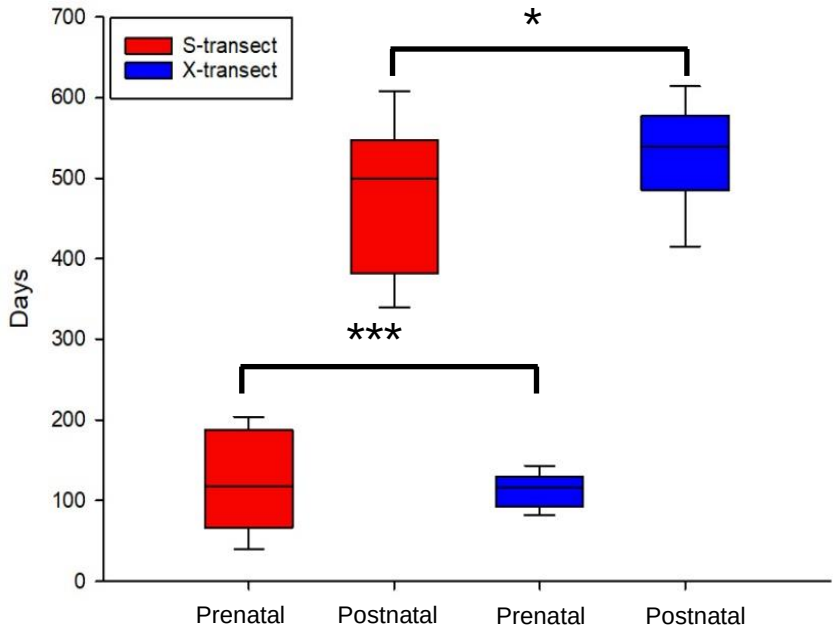

Figure 4.11 The number of days covered in S- and X-transect. S-transect showed a significantly longer prenatal period than X-transect (n=18) (paired T-test, p<0.001***), while there was a significantly shorter postnatal period in S-transect compared to X-transect (paired t-test, p=0.044*).

4.5 Discussion

A previous study demonstrated that the neonatal line in enamel is visible using a 100 µm thickness of tooth ground section (Eli et al., 1989), which was also applied by Reid, et al (1998) to reconstruct dental development using enamel from medieval era. Dean (1998) used 80 µm to identify the daily lines in dentine. In this study, 100 µm (±20 µm) thickness was performed in all samples and was adequate to observe some landmarks in dentine of deciduous tooth, such as EDJ, neonatal line, long-period lines, accentuated lines, and tertiary dentine.

The neonatal line observed in deciduous teeth, and sometimes in first permanent molars, is useful in age assignment. The previous studies demonstrated the neonatal line as a disruption in mineralisation process due to the environmental and nutritional changes that happen after birth as well as the stress-related process of birth (Eli et al.,

1989; Hurnanen et al., 2017). In this study, the neonatal line in dentine was used as point ‘zero’, where the Zn distribution in ablation pits located from neonatal line to the EDJ indicates Zn incorporation in prenatal dentine while ablation pits located from neonatal line to the pulp present Zn incorporation in postnatal dentine. Thus, the age points for ablation pits from neonatal line to EDJ were assigned as a minus (-) days, means the Zn was incorporated at certain days before birth.

The distance from each ablation pit to neonatal line was measured by tracking the corresponding dentinal tubules to determine the age points. A procedure in following dentinal tubules was necessary, as dentinal tubules spans from EDJ to the mineralisation front in dentinogenesis, and the configuration of dentinal tubules reflects the mineralisation path of dentine over tooth life course (Nanci, 2013). Thus, the tooth ground section was manually marked before the LA-ICP-MS procedure to create a transect as parallel as possible to dentinal tubules.

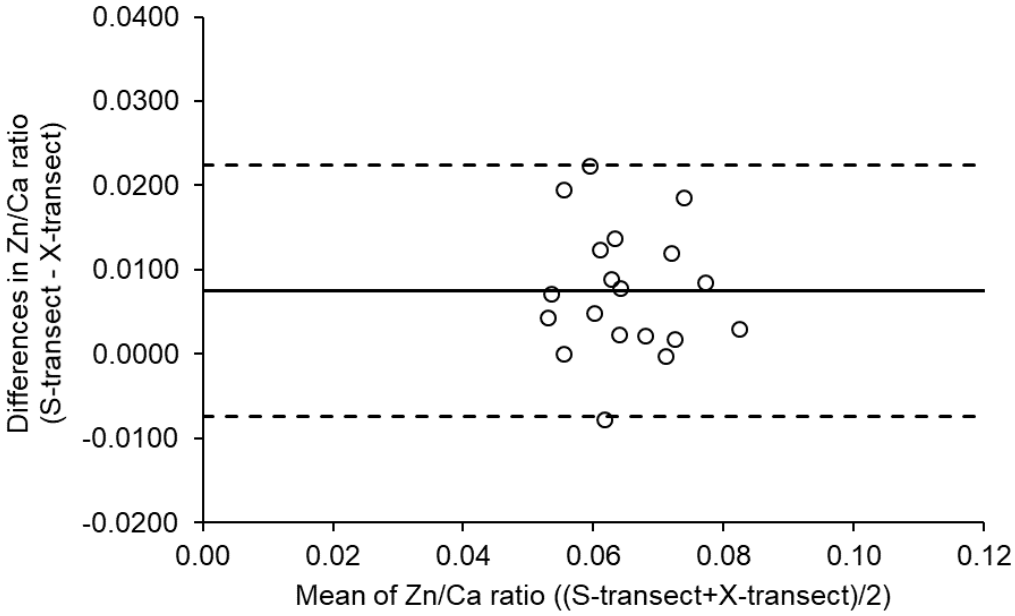

Figure 4.12 Bland-Altman plot showing a good agreement between the S-transect and X-transects in 18 deciduous teeth.

The initial mineralisation differs between tooth types, where it starts 12-16 weeks intra utero for incisors and 24-26 weeks intra utero for molars (Birch and Dean, 2014). Furthermore, incisors erupt earlier (begin at approximately 4.5 months after birth) than molars (start at approximately 10.5 months after birth) (AlQahtani et al., 2010). Incisors may develop faster than molars during prenatal period as shown here the prenatal

dentine DSR was significantly higher compared with postnatal dentine in incisors (Table 4.3).

In all tooth sections, the tertiary dentine was identified, which appears in the roof of pulp chamber as a regular dentine, similar to primary dentine, and or an irregular dentine (Smith et al., 1995). Tertiary dentine develops due to an external stimulus, such as attrition, caries, and or trauma. Attrition is common in deciduous teeth because the enamel layer is thinner than permanent teeth, thus, tertiary dentine is found quite often in deciduous teeth. (Klinge, 1999). In a previous study using dentine, the tertiary dentine was emitted from analysis since the development mechanism differs to primary dentine (Beaumont et al., 2015). Thus, the ablation pits located in tertiary dentine were excluded from this study because Zn incorporation in dentine may be regulated differently compared with primary dentine.

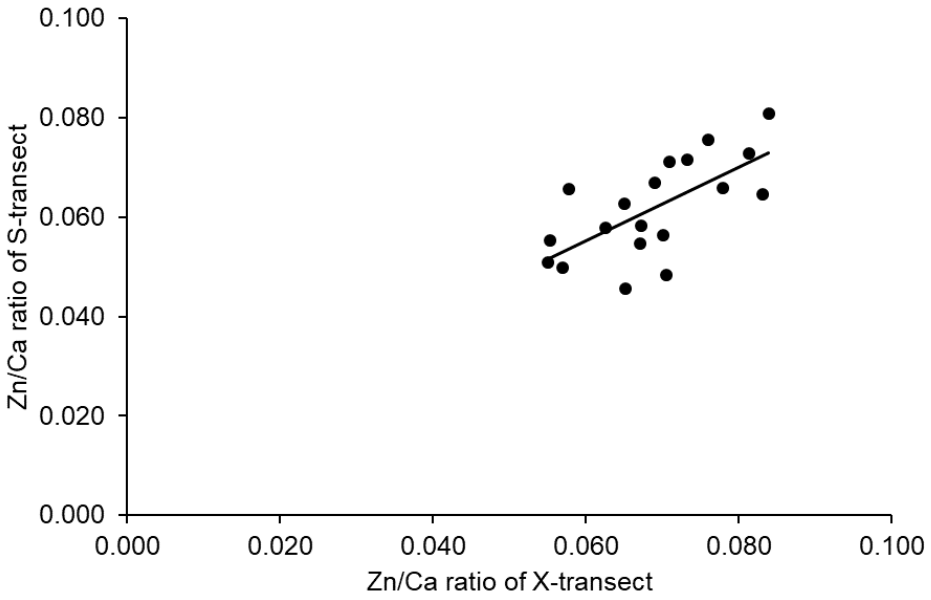

Figure 4.13 Zn distribution in S-transect and X-transect demonstrated a significant positive correlation (Spearman correlation, r=0.667; p=0.00183) in samples from 18 deciduous teeth.

Using LA-ICP-MS, information on several elements may be obtained from a small quantity of sample in one 'run'. Instead of Zn, Sr distribution was also identified in this study. The Sr distribution in teeth is recognised to relate to diet transition in individuals. A previous study in primate enamel indicated Sr distribution was increased when non-milk food was introduced (Humphrey et al., 2008b). In human, the lower Sr level was related to higher protein intake and higher Sr level was correspondent to lower protein intake (Dolphin et al., 2005).

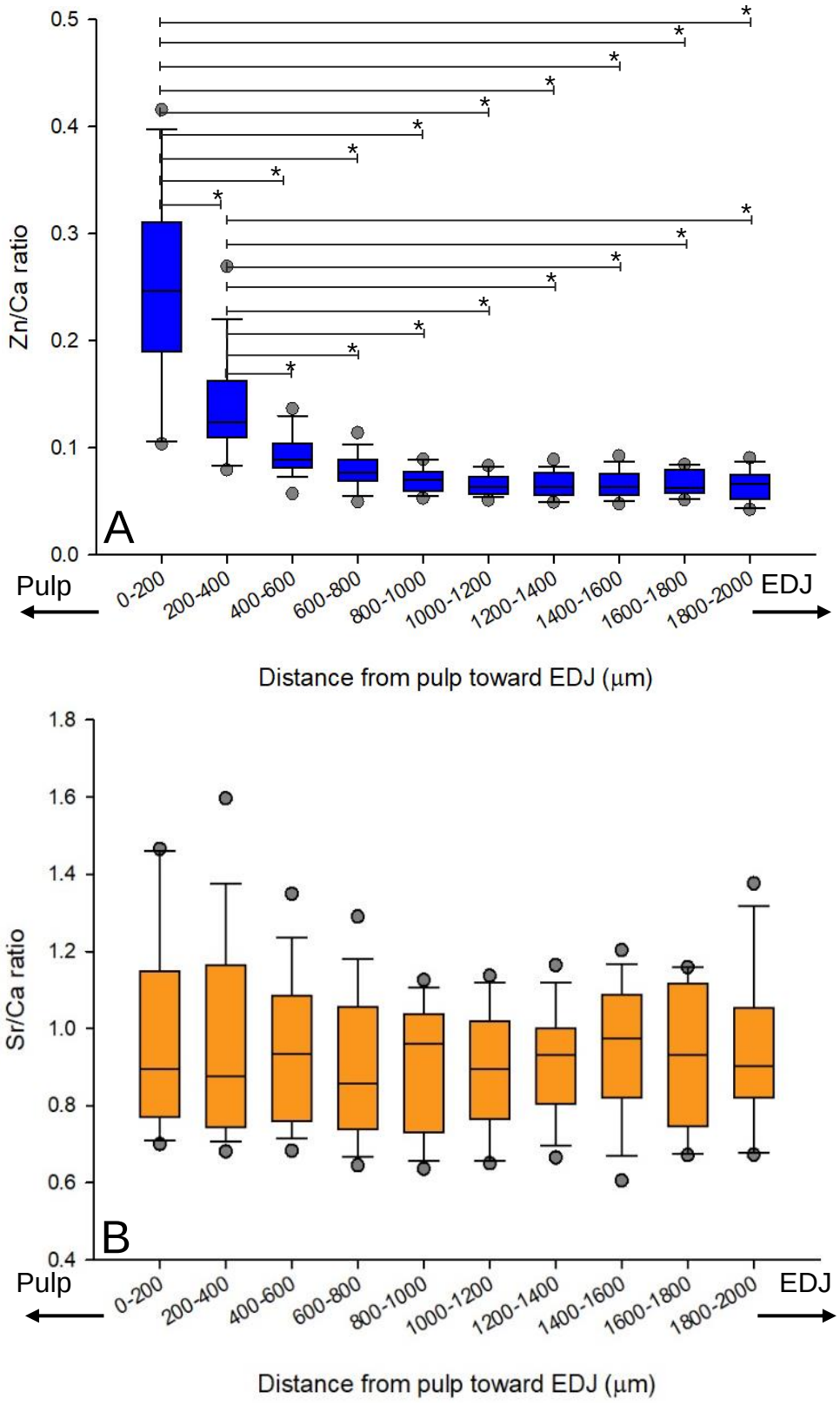

Figure 4.14 Graphs represent different trends of Zn distribution compared with Sr distribution from pulp to EDJ (n=18). A) a significant difference on Zn/Ca ratio at different locations of ablation pits from pulp to EDJ (Friedman test, p=0.002), where the ablation pits closer to the pulp, i.e. 0-200µm and 200-400µm, were significantly different to others (Student-Newman-Keuls as a post-hoc test; p<0.05*); B) the Sr/Ca ratio differences were not significant between ablation pits from pulp to EDJ (Friedman test, p=0.207).

Kang et al., 2004; Shepherd et al., 2012). Reference materials used in this study, was glass (NIST SRM Glass 610, 612, 614), which was used to calibrate the instrument, and also determine the instrument performance and within-run standard error, while SRM Bone meal 1485 was employed as an external unknown because it has a similarity in chemical and mineral composition to dentine. Thus, results are presented relative to Ca ion intensity from the same ablation pit for both Zn and Sr, since Ca did not fluctuate significantly across dentine in our study and a previous study (Shepherd et al., 2012). The relative standard deviation (RSD) between all ablation pits in dentine, however, was calculated for Ca, Zn, and Sr, which were 13%, 10%, and 12%, respectively.

According to Shepherd (2012), Pb was distributed similarly at different locations in dentine of paired-deciduous teeth from the same child after the age assignment was carried out. Therefore, one transect analysed from each tooth section was sufficient and reliable to reconstruct Pb distribution as well as for Zn, Mg, and Sr. The transect location, however, affects the amount of detailed information on Zn distribution from prenatal (prior to birth) and postnatal (after birth). Considering the location of the neonatal line, the number of age measurements from the prenatal period is wider when the transect was located at the axial plane of tooth (S-transect) than in the cervical area (X-transect), especially in incisors. Furthermore, the axial plane of the tooth is easier to identify by looking at the cusp tip, so the transect location can be located uniformly for all tooth samples, thus, the possibilities of measurement error due to variation of sampling area are reduced. Two transects were then performed, where one transect was located closer to axial plane of the tooth by using cusp tip as a guidance, and another transect was located more cervical to the previous transect in the same surface of tooth. Zn distribution showed good agreement, in our study, between the two transects at different age points, thus, any location up to 400µm from the pulp region, within dentine would provide the same information on Zn distribution across age points. The distance between incremental lines in different locations, such as in S- and X-transects, should be taken into consideration where it is narrower in the tooth cervical area (X-transect) rather than in the axial plane area (S-transect) as shown in a previous study using tetracycline deposition technique (Kawasaki et al., 1977), thus, the Zn content may be different between S- and X-transects measured by LA-ICP-MS. Overall, the S-transect (closer to the axial plane) allowed for more area to generate laser ablations pits for analysis of Zn distribution during the prenatal period

compared with the X-transect (more cervical) (Figure 4.11). Therefore, the S-transect was chosen for all further analyses of Zn content. (Chapter 5).

Based on this pilot study, an atypical increase in Zn was measured in the age points closest to the pulp (within 400 µm) with pulp set at 0 µm, even after ablation pits laid in tertiary dentine have been excluded. Similar findings have been reported with other elements distributed within dentine, such as Zn in human and F in horse teeth (Kierdorf et al., 2016; Shepherd et al., 2012). Several factors may be related to these findings, such as 1) secondary dentine, which is developed just after the root completion and known as a physiological reaction and related to ageing. Even though the secondary dentine is similar to primary dentine in terms of chemical composition and physical structure, the secretion rate of secondary dentine is much slower, i.e. 0.4 µm/day (Bleicher et al., 2015) and has time to potentially incorporate more Zn. Therefore, a higher Zn/Ca ratio is possibly measured by the ICP-MS from the ablation pits located at secondary dentine rather than primary dentine. Furthermore, dentinal tubules are more dense in older teeth closer to the pulp because peritubular dentine of secondary dentine are developed in primary dentine (Dean, 2017); 2) immature crystal apatite still has 'non-apatitic environment' that allows the ions exchange easily, but then along the crystal maturation and growth, these ions would be permanently incorporated into the crystal (Kierdorf et al., 2016). Another formal possibility is the level of protein in this area of the tooth. Since immature dentine would contain more dentine matrix protein, more Zn may be observed in this site, as Zn binds to protein (Kambe et al., 2015). Taking these reasons into consideration, a cut-off was determined to exclude the Zn/Ca ratio value from the ablation pits located at 0-200µm and 200-400µm from pulp since it may be not related to the Zn deposition during primary dentine development and may not represent the definite age measured. The Sr distribution, however, did not show the same trend from pulp to EDJ. Presumably, the incorporation of Sr into the crystal apatite differs from Zn, where Sr acts as a substitution for Ca and develops a stable compound within the crystal apatite. On the contrary, Zn atoms lay on the surface of crystal apatite (Shepherd et al., 2012). This result suggests the variation in Zn/Ca ratio within dentine may be related to Zn regulation in the human body.

4.6 Conclusion

The LA-ICP-MS method performed in this study is a robust technique for analysing Zn distribution within dentine and the age assignment using histological technique

enhances the ability of this method to analyse Zn distribution at different age points spanning between prenatal and postnatal dentine.

Chapter 5 Relationship between dietary Zn intake and Zn distribution within dentine of deciduous teeth in early life

5.1 Introduction

Zn, a trace element essential for human metabolism, is tightly regulated by homeostatic mechanisms in the human body. Most of the Zn in the human body (as percentage of total body Zn) is stored in skeletal muscle (60%), followed by bones (30%), and other organs (10%) (Hara et al., 2017). Generally, Zn in human tissue ranges from 10-30 ppm, but high concentrations of Zn can be found in hair (160 ppm), bone (100 ppm), liver (55 ppm), kidney (25 ppm), and teeth (130-280 ppm) (Brudevold et al., 1963). Zn in human teeth has been studied for decades and used to identify the environmental exposure to Zn, from pollution, disease, and dietary intake.

Using the same material, a recent study in Norway showed the Zn concentration in deciduous teeth was slightly increased compared with a 1970's study, although Zn exposure from the metallurgical and mining industries has been eliminated in this country since 1990. In addition, the Zn content in the Norwegian diet was slightly increased due to a higher consumption of meat, protein-rich cheese, and whole-meal flour (Tvinnereim et al., 1999). A different study showed differences in Zn concentration between UK and Ugandan deciduous teeth and associated these differences with dietary Zn intake (Brown et al., 2004).

Several studies have since been carried out to identify the association between Zn in human teeth and certain diseases, such as autism (Adams et al., 2007; Arora et al., 2017). Using the whole tooth, Zn concentration within deciduous teeth between autistic and non-autistic children was not significantly different (Adams et al., 2007). Whereas, a later study suggested Zn has an important role in regulating the absorption of other heavy metals, such as lead (Pb) and manganese (Mn), involved in pathophysiology of autism (Arora et al., 2017).

According to dietary intake, Zn distribution within enamel had been observed in women of the Solis Valley population, Mexico. Using LA-ICP-MS method, the study showed a significant difference in Zn levels between prenatal and postnatal enamel, but this did not correlate with levels of dietary Zn intake of the mother during pregnancy.

Surprisingly, the highest Zn level was found in prenatal enamel from an individual who consumed low-bioavailable-Zn diet during pregnancy. Thus, the study suggested that Zn distribution in enamel of children's deciduous teeth was not representative of Zn status of their mother's during pregnancy and lactation (Dolphin and Goodman, 2009).

At present the gold standard marker for Zn exposure is plasma/serum Zn levels, however, due to tight homeostatic control mechanisms (Zn transport proteins), marginal and subclinical Zn deficiencies are often un-detected. Moreover, a mild to moderate (marginal) Zn deficiency is difficult to detect because of unobvious clinical symptoms but it is thought to be prevalent worldwide with17.3 % of the population estimated to have inadequate Zn in their diet (Wessells and Brown, 2012). Pregnant and lactating women as well as infants are high-risk groups for suffering marginal Zn deficiency due to circumstances of high Zn requirement for metabolism, constant and/ or insufficient dietary Zn intake, disruption in Zn absorption and lack of Zn body storage (Ackland and Michalczyk, 2016; Donangelo and King, 2012). Furthermore, the availability of other nutrients in these groups, such as iron, cadmium, and or other food components such as phytate, can interfere with the absorption of Zn (Ackland and Michalczyk, 2016). At this stage, Zn deficiency may adversely affect the conditions of pregnancy and health status outcome of children in later life (King, 2000). In addition, growth stunting, alteration in taste and smell, irritability, bad appetite, and less resistance to infections are related to persistent marginal Zn deficiency in infants (Ackland and Michalczyk, 2016; Millward, 2017).

Generally, pregnant women need 18-36% more Zn intake from their diet compared with non-pregnant women, where more than half of the intake is being transferred to the foetus (Donangelo and King, 2012). Absorption during pregnancy and lactation of Zn fomr the diet is increased to meet these additional needs. A previous study showed an upregulation in total absorption of Zn during late pregnancy and lactation regardless of Zn bioavailability from the mother's diet (Hambidge et al., 2017). It seems that the placenta has an active role, as part of Zn homeostasis, to ensure the adequate level of Zn to support foetal growth (Terrin et al., 2015). The adjustment of Zn homeostasis is also possible by increasing renal Zn retention, intestinal Zn absorption, and Zn flux from the pools to other tissue when dietary Zn intake is equal and less than 9 mg/day. Although at certain levels of Zn deficiency, the homeostatic mechanism is not effective to meet the daily Zn requirement during pregnancy (Donangelo and King, 2012).

A high level of Zn is transferred to the foetus at late pregnancy and primarily bound in hepatic metallothioneins, accordingly neonates preserve 25 per cent of total body Zn at birth. The Zn level reserved in liver is gradually diminished from birth to a constant level at about 4 months of age (Ackland and Michalczyk, 2016; Aggett, 2000). In addition to maternal dietary Zn intake, the homeostatic mechanism is primed to meet a high demand of Zn by mobilizing maternal Zn pools, such as uterus, maternal blood, and trabecular bone (Donangelo and King, 2012).

Maternal Zn requirements are higher during lactation than pregnancy, as breast milk is the major nutrient source for the baby (Ackland and Michalczyk, 2016; Donangelo and King, 2012). In the neonate and infant, breast milk can vary in concentration from 0.7-1.6 mg/L. The highest Zn concentration is contained in colostrum (8 mg/L) which is secreted within first few days after birth. The breast milk concentration, then rapidly reduces until 1 month after birth, and continuously decreases to the level of approximately 0.5 mg/L at 12 months after birth (Ackland and Michalczyk, 2016; Terrin et al., 2015). The reduction of Zn concentration over time has been studied in different populations worldwide (Donangelo and King, 2012). On the other hand, a recent study showed the absorption of Zn was increased in both low- and high-phytate consumers along with the increase of total dietary Zn intake during early lactation (Hambidge et al., 2017). Thus, the reduction of Zn concentration seems to be physiologic and independent to maternal Zn status. The variation of Zn concentration in breast milk between individuals, however, depends on maternal Zn status, where a lower Zn concentration is produced by the mothers who are deficient in Zn, compared with mothers who have sufficient Zn levels (Donangelo and King, 2012).

Overall, measuring Zn status in human tissues is not easy because there are strict homeostatic mechanisms which maintain Zn availability for metabolism in the human body (Hambidge, 2003) and as such large fluctuations do not tend to occur due to the well-regulated mechanism to maintain Zn homeostasis during pregnancy and lactation period, Zn measurement during early life, is even more challenging.

The deciduous tooth spans from the prenatal period into childhood and it has been shown as a good candidate to record dietary changes in humans (Dean, 2017; Humphrey, 2014). Using the enamel of deciduous teeth, a previous study suggested Sr/Ca ratio levels dropped at birth and continued to decrease until the end of exclusive

breast feeding. This was then increased during the weaning period due to higher Sr/Ca ratio of complementary foods, with a further decrease of the Sr/Ca ratio observed when the child was fully weaned onto 'adult foods' (Humphrey, 2014), a reflection of the requirement of Sr as discussed later. In women, the absorption of Sr is gradually increased during pregnancy and lactation (Cabrera et al., 1999). A previous study demonstrated the differences in Sr level was not significant between maternal serum and umbilical cord, but Sr level of maternal serum had a positive and strong correlation with the Sr level from umbilical cord (Krachler et al., 2002).

Sr is a non-essential element, but is abundantly available in soil and water (Coelho et al., 2017). Human diets, such as meat, poultry, vegetables and fruits are low in Sr (from 0.3 to 5.1 mg/kg), while cereal, grain, and fish contains higher Sr (up to 25 mg/kg). The absorption of Sr is proportional to the content in the diet and is age-dependent, where the highest amount of Sr from the human diet is absorbed during infancy (almost 90%) and decreased in adults (only about 10%). The ability of gastrointestinal tract to discriminate Sr is well-established in adults (Humphrey, 2014). Sr is mostly deposited in the bone and teeth, and known as a substitute for Ca (Cabrera et al., 1999). Sr distribution has been studied widely in relation with diet exchange and migration (Humphrey, 2014).

In regard to micro-sampling techniques using LA-ICP-MS, it is possible to measure the elemental distribution within human teeth, of Zn and Sr, and determine against age time points using the dental histological analysis. Therefore, this study aims to explore the usefulness of the dentine of deciduous tooth in recording Zn exposure from early life nutrition in comparison with Sr distribution.

5.2 Aims

This chapter aims:

1) To analyse the differences between Zn/Ca and Sr/Ca ratio across age points within dentine of deciduous teeth.
2) To analyse the differences of Zn/Ca and Sr/Ca ratio within dentine of deciduous teeth between high-Zn and low-Zn consumer during the last trimester of pregnancy and weaning period.
3) To analyse the variation of Zn/Ca and Sr/Ca ratio within dentine of deciduous teeth in relation to infant feeding regimes.

5.3 Materials and methods

Ethics was obtained from the Committee of The Medical Research Ethics of the Faculty of Medicine University of Indonesia (526/UN2.F1/ETIK/2014). Twenty-one mother-child pairs were recruited at the Child Integration Clinic, Educational Dental Hospital of Indonesia University (RSGM-P Universitas Indonesia). The mothers completed the main questionnaire and the three sets of S-FFQs (as described in Chapter 3) to estimate dietary Zn intake of the mother during pregnancy and the child during infancy and at-present. The daily dietary Zn intake during these periods was calculated and analysed as described in Chapter 2. Removed deciduous teeth were processed to analyse the element distribution (for Ca, Zn and Sr) using LA-ICP-MS and dental histology for age measurement (as described in chapter 2). The age point from each ablation pit was determined using the median age range. As a general trend of Zn/Ca and Sr/Ca ratio from this study population, the average of Zn/Ca and Sr/Ca ratio in 3-month age interval were calculated and plotted.

5.3.1 Analysing the differences of Zn/Ca and Sr/Ca ratio within dentine of deciduous teeth across different age points

Firstly, the 3-month age intervals were determined as; -90 until -1 day prior to birth (late pregnancy), 0-3 month, 3-6 month, and 6 to 9 months after birth (weaning) periods. The Zn/Ca and Sr/Ca ratios from each age point were allocated into 3-month age intervals and presented as averages of Zn/Ca and Sr/Ca ratios. The differences of Zn/Ca over time were then analysed using the Friedman test and Wilcoxon sign rank test as the post hoc test ($p<0.05$) and repeated measurement ANOVA followed by Bonferonni post hoc test ($p<0.05$) was applied to analyse differences in Sr/Ca ratios between age intervals.

5.3.2 Analysing the differences of Zn/Ca and Sr/Ca ratios within dentine of deciduous teeth between high-Zn and low-Zn consumers during the last trimester of pregnancy and weaning period

Based on EAR for pregnant women and infants (1-3 years old), the daily Zn intake information obtained from S-FFQ (as described in Chapter 3) was used to classify the mothers into either high Zn (HZM) and low Zn (LZM) consumers as well as the infants (HZI for high Zn consumer and LZI for low Zn consumer). The differences of Zn/Ca and Sr/Ca ratios in late pregnancy between HZM and LZM and between HZI and LZI at weaning period, were then analysed statistically using Mann-Whitney test ($p<0.05$).

5.3.3 Analysing the variation of Zn/Ca and Sr/Ca ratio within dentine of deciduous teeth across infant feeding practices in first 6 months of life

Using the information on feeding regimes from the S-FFQ, the children were classified into exclusively breast feeding, mix breast and formula feeding, and formula feeding only group. Exclusively breast fed (BF) referred to children who consumed solely breast milk from birth up to 6 months of age, the mixed breast and formula feeding (MF) group included the children who consumed both breast and formula milk during first 6 months, while the formula fed (FF) group contained every child who consumed only formula milk from birth until 6 months of age. Any children, who were introduced with complementary foods before 6 months of age with or without breast milk or formula milk, were classified as early weaning group (EW). This study recruited children who were early weaned but still consuming breastmilk (EW+BF) or breastmilk and formula milk (EW+MF) until 6 months.

5.4 Results

Twenty-one mother-child pairs were recruited for this study but only 18 teeth samples were eligible for further analysis using LA-ICP-MS and dental histology to obtain Zn and Sr distribution within dentine at different age points. The three teeth samples were excluded because either the remaining hard tissue was not sufficient to produce an appropriate longitudinal section, or the dental histology analysis could not be performed as a large area of dead tract was located in the regions where the transects were created.

5.4.1 Zn and Sr distribution within dentine of deciduous teeth at different age intervals

The Zn and Sr distribution (presented as Zn/Ca and Sr/Ca ratios) were decreased from late pregnancy until the first 3 months after birth. Thereafter, the Zn/Ca ratio gradually increased from 3-6 months of age until the weaning period, but the Sr/Ca ratio continued, as expected to decrease (Figure 5.1), the reasons for which are described previously in this Chapter. The average value of Zn/Ca and Sr/Ca ratio at the four age-intervals from all samples were then assigned as average trends in this study.

The overall Zn/Ca ratio differed significantly across all time points analysed (Friedman test, $p=0.002$). Post hoc analysis using Wilcoxon Sign Rank test showed differences

in Zn/Ca ratio were significant between late pregnancy and 0-3 months (0.069 + 0.016 vs 0.063 ± 0.012; p=0.031), between 0-3 months and weaning (0.063 ± 0.012 vs 0.071 ± 0.015; p=0.008), and between 3-6 months and weaning (0.062 ± 0.013 vs 0.071 ± 0.015; p<0.001). There were no significant differences between late pregnancy and weaning (0.069 ± 0.016 vs 0.071 ± 0.015; p=0.231), between 3-6 months and late pregnancy (0.062 ± 0.013 vs 0.069 ± 0.016; p=0.53), nor between 0-3 months and 3-6 months (0.063 ± 0.012 vs 0.062 ± 0.013; p=0.819) (Figure 5.1 A).

There was a significant decrease in Sr/Ca ratio between late pregnancy and weaning periods (repeated measures ANOVA, p<0.01). Post hoc analysis using Bonferonni demonstrated the differences in Sr/Ca ratio were significantly lower at 3-6 months and weaning compared with late pregnancy (0.898 ± 0.161 and 0.888 ± 0.157 vs 0.997 ± 0.197; p<0.05) and also compared with 0-3-month period (0.898 ± 0.161 and 0.888 ± 0.157 vs 0.956 ± 0.182; p<0.05).

5.4.2 The Zn/Ca and Sr/Ca ratio in high and low-Zn consumers during late pregnancy and weaning period

The eighteen mothers were classified into HZM (n=10) and LZM (n=8) during pregnancy, while there were only two children categorised as LZI (n=2) and sixteen children identified as HZI (n=16) during weaning period (Chapter 3, Table 3.5).
The average trend of Zn/Ca and Sr/Ca ratio in deciduous teeth for this study decreased from late pregnancy until birth. The HZM and LZM group showed a similar trend in Zn/Ca and Sr/Ca ratio in deciduous teeth at the same time points.

Interestingly, the Zn/Ca ratio was higher in HZM and lower in LZM compared with the average level at all time points, and vice versa for the Sr/Ca ratio (Figure 5.2A and B; the average trend represented by grey line). The statistical analysis, however, was not able to discriminate HZM and LZM groups within the small range of differences in Zn/Ca and Sr/Ca ratio in deciduous teeth probably because of the small sample number in this study (Mann-Whitney, p>0.05).

The average trend of Zn/Ca ratio for this study gradually increased from birth to weaning period, while Sr/Ca ratio decreased from birth to weaning period in deciduous teeth from children in HZI and LZI groups (Figure 5.2C and D). At all-time intervals, the Zn/Ca ratio was higher in HZI and lower in LZI compared with the average level. In

contrary to Zn/Ca ratio, the Sr/Ca ratio of HZI was at the average level, but LZI showed a higher Sr/Ca ratio than average level at all time points. The mean differences of Zn/Ca ratio and Sr/Ca ratio, however, were not significant between HZI and LZI group (Mann-Whitney, p>0.05).

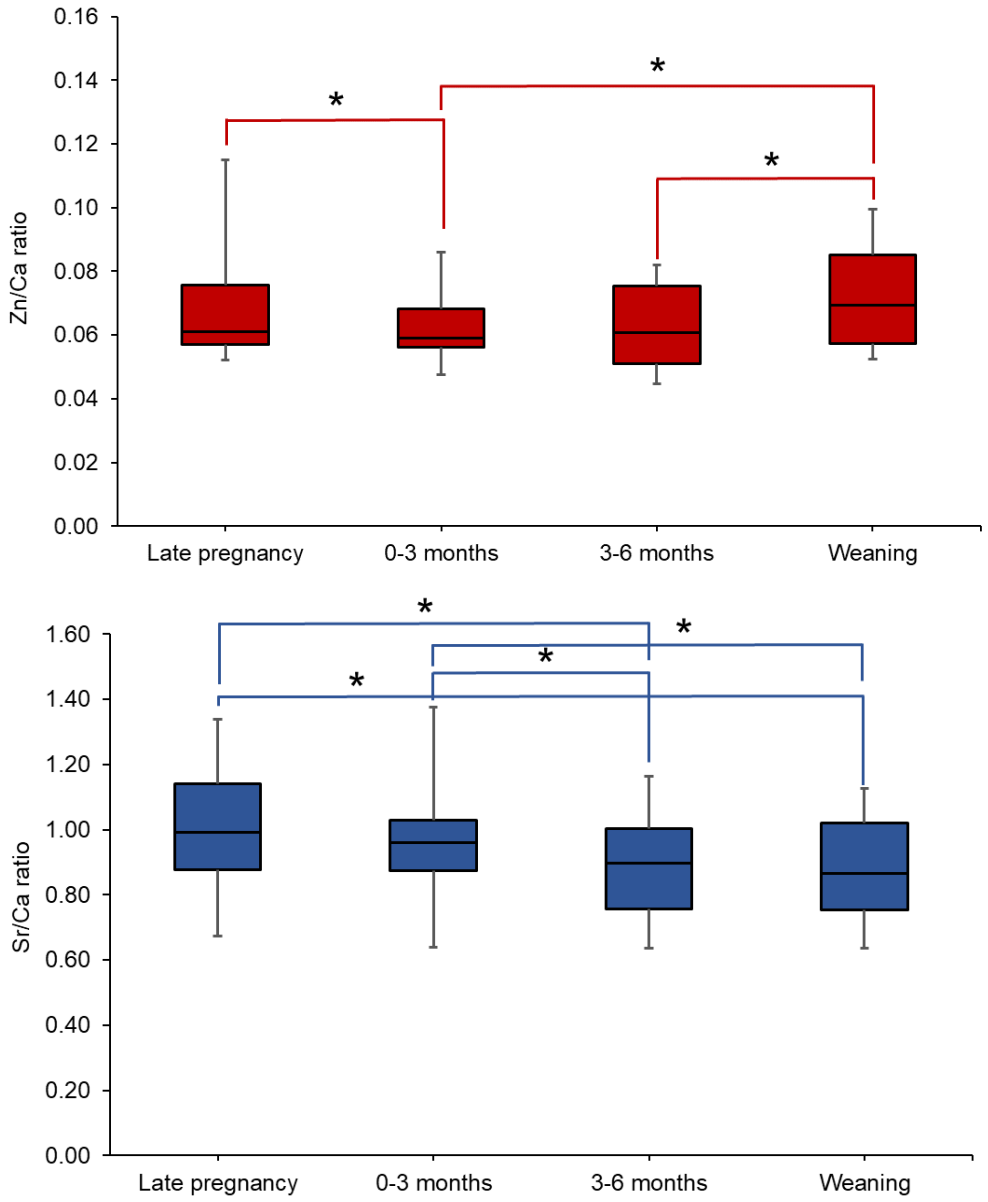

Figure 5.1 The box plot represents Zn and Sr distribution at different time points. The boxes show medians and extend from 25th to the 75th percentile with the whiskers extended from the smallest to the largest value (n=18). **A**) the differences of Zn/Ca ratio were significant at different time points (Friedman, p<0.002), and a post hoc test indicated a significant different between two groups (Wilcoxon sign rank post hoc test, p<0.05*); **B**) Sr/Ca ratio were significantly different across different time points (repeated measurement ANOVA, p<0.01), and the differences were significant between two groups (Bonferonni post hoc test; p<0.05*).

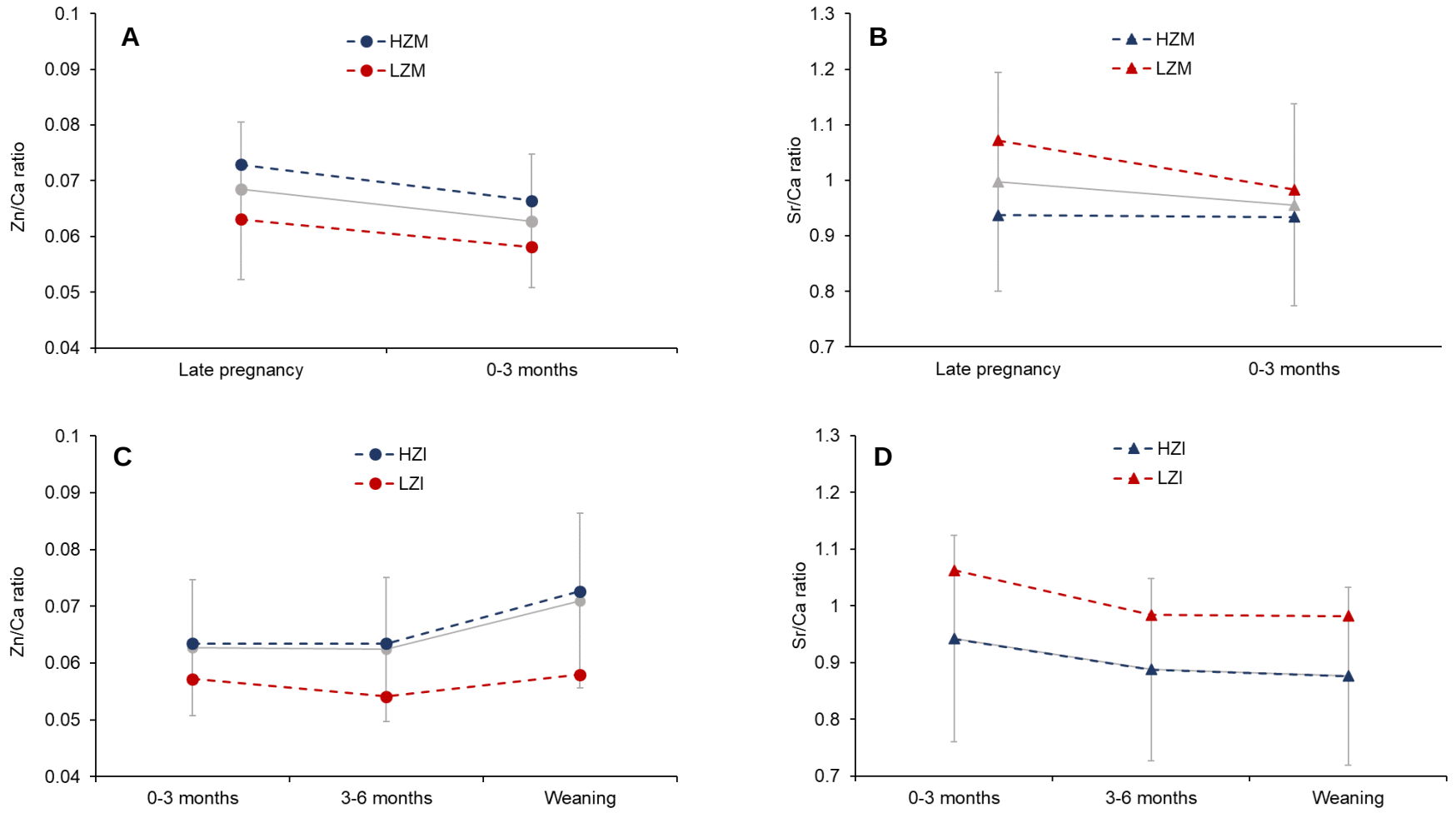

Figure 5.2 Graphs show the average value of Zn/Ca ratio and Sr/Ca ratio for this study population (line and symbol in grey) compared with high-and low-Zn-consumer at late pregnancy (A and B) and weaning period (C and D).

5.4.3 The Zn/Ca and Sr/Ca ratio distribution within dentine of deciduous teeth during the first six months of life between different feeding practices

There were five different feeding practices during the first six months after birth that were identified in this study as follows: BF (n=7), FF(n=1), MF(n=4), EW+BF (n=4), and EW+MF (n=2). The Zn/Ca and Sr/Ca ratio for each group were plotted against different age intervals as described in Figure 5.3.

The average trend of Zn/Ca and Sr/Ca in deciduous teeth for this cohort declined from late pregnancy until 6 months, the trend then increased at the weaning period for Zn/Ca ratio, but consistently decreased for Sr/Ca ratio. The different trend in Zn/Ca and Sr/Ca ratio emerged between different feeding practices (Figure 5.3A and B), although there were no significant differences between different feeding practices at the 0-3-month and 3-6-month period (Kruskal-Wallis test, $p>0.05$). This is probably due to the small numbers in each feeding group after stratification.

Interestingly, the trend of Zn/Ca ratio of deciduous teeth in BF and MF group decreased from late pregnancy until 3 months and marginally increased after 3 months, but EW+BF and EW+MF groups followed more closely the average trend of Zn/Ca ratio in this study. In addition, the Zn/Ca ratio was higher in BF and MF groups, but lower in EW+BF and EW+MF groups compared with the average level at all time points. A distinct trend was determined in the FF child; from the lowest level at late pregnancy, the Zn/Ca ratio of FF was increased dramatically by 3 months to almost as high as the BF group, with a sharp decline by 3-6-month period (Figure 5.3A). As there was only one child in this category, it is difficult to make any assumptions as to why we saw this pattern.

The average trend of Sr/Ca ratio for this cohort declined from 0-3- to 3-6-month period for all feeding practices as shown in Figure 5.3B. The Sr/Ca ratio in BF and MF groups was below the average level, but it was higher in the other groups compared with the average level at the same time points. Again, a distinct trend was determined in the FF child, where the Sr/Ca ratio increased by 3 months, and gradually decreased after 3 months

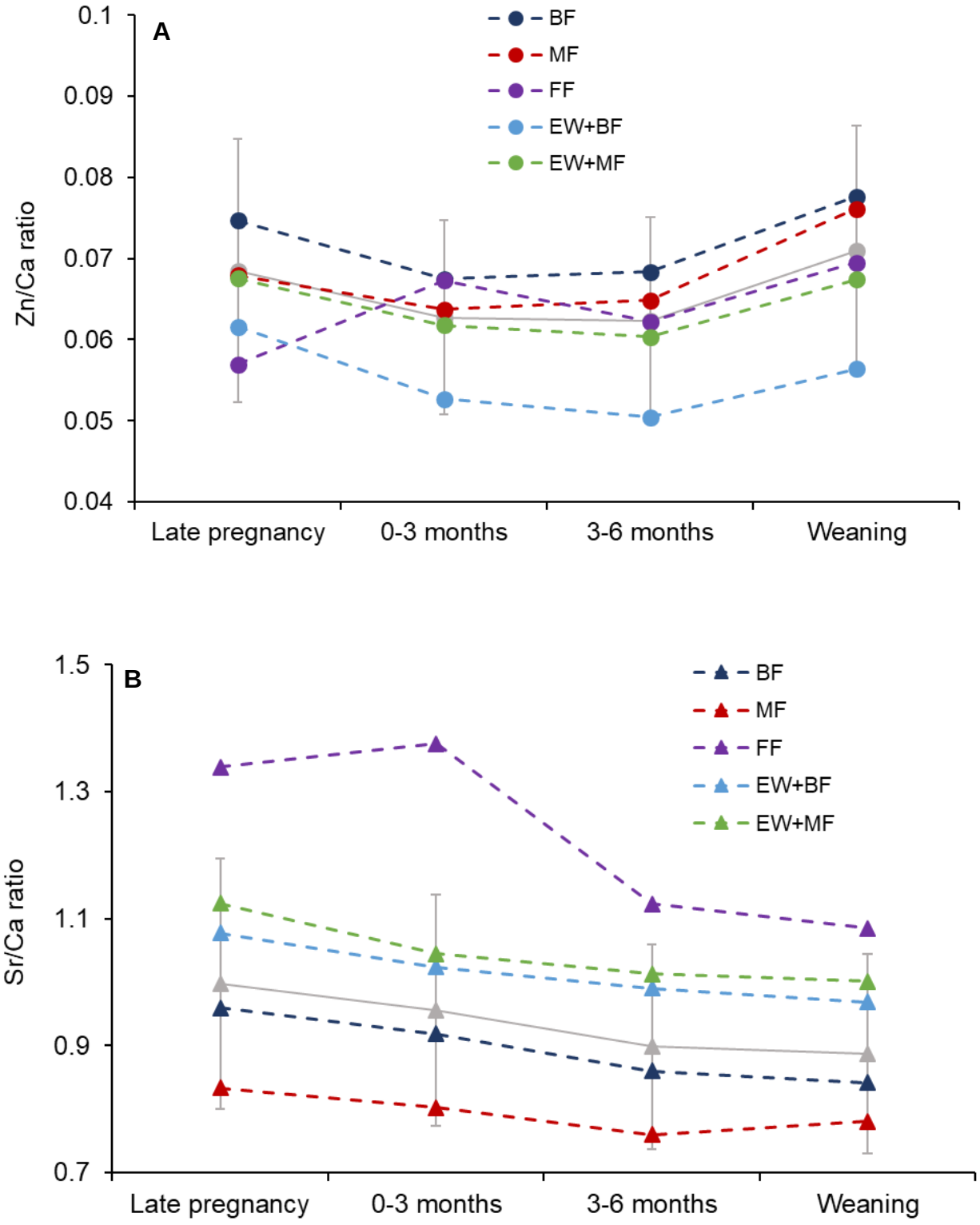

Figure 5.3 Graphs show the average value of Zn/Ca ratio (A) and Sr/Ca ratio (B) for this study population (line and symbol in grey) against different feeding practices at 0-3- and 3-6- month period.

5.4.4 Subject specific variations associated differences in Zn deposition within dentine of deciduous teeth

A child, who was born prematurely at week 24 of gestation and classified as low-birth weight (1.5 kg), participated in this study. This baby was fed breast milk and formula milk from birth. At late pregnancy, the Zn/Ca ratio was lower than average Zn/Ca level for this cohort in dentine determined by analysis of all the other samples in different feeding groups. The Zn/Ca ratio had increased within a month after birth and exceeded the average level, but declined dramatically to below the average level from day 56 until 112. Even though, the Zn/Ca ratio in dentine had increased again from day 168, it was consistently below the average level from this point until day 330. The trend of Sr/Ca ratio in dentine indicated a slight decrease after birth to below the average level, but had increased beyond the average level from day 168 until after weaning at day 224, then decreased from that time point until the observed day (Figure 5.4A).

A mixed-fed child, who started formula feeding at birth, showed an increase in Zn/Ca ratio compared with the late pregnancy, but the Zn/Ca ratio in dentine decreased at day 117. The trend of Sr/Ca ratio in dentine showed a similar trend with Zn/Ca ratio in dentine for this child. The trend for Sr/Ca ratio in dentine had increased gradually within 3 months after birth, and then decreased from day 117 until the final time point observed in this study. Interestingly, the dentine Zn/Ca ratio for this child exceeded the average level at all time points, and vice versa for Sr/Ca ratio. Based on the questionnaire, the mother noted the formula milk was stopped at 4-month old and she administered iron supplementation to her child at the same time as suggested by the paediatrician (Figure 5.4B)

There was one breast-fed baby who was introduced to solid food since birth and the mother stopped breast-feeding the child at day 90. The Zn/Ca ratio in the child's dentine decreased from late pregnancy to birth, with an increase for the next two months, but there was a marked decrease at day 105. The Zn/Ca ratio in dentine for this child was below the average level at all time points, however the Sr/Ca ratio seems to be consistent with the average trend of Sr/Ca ratio for this study, as seen in Figure 5.4C.

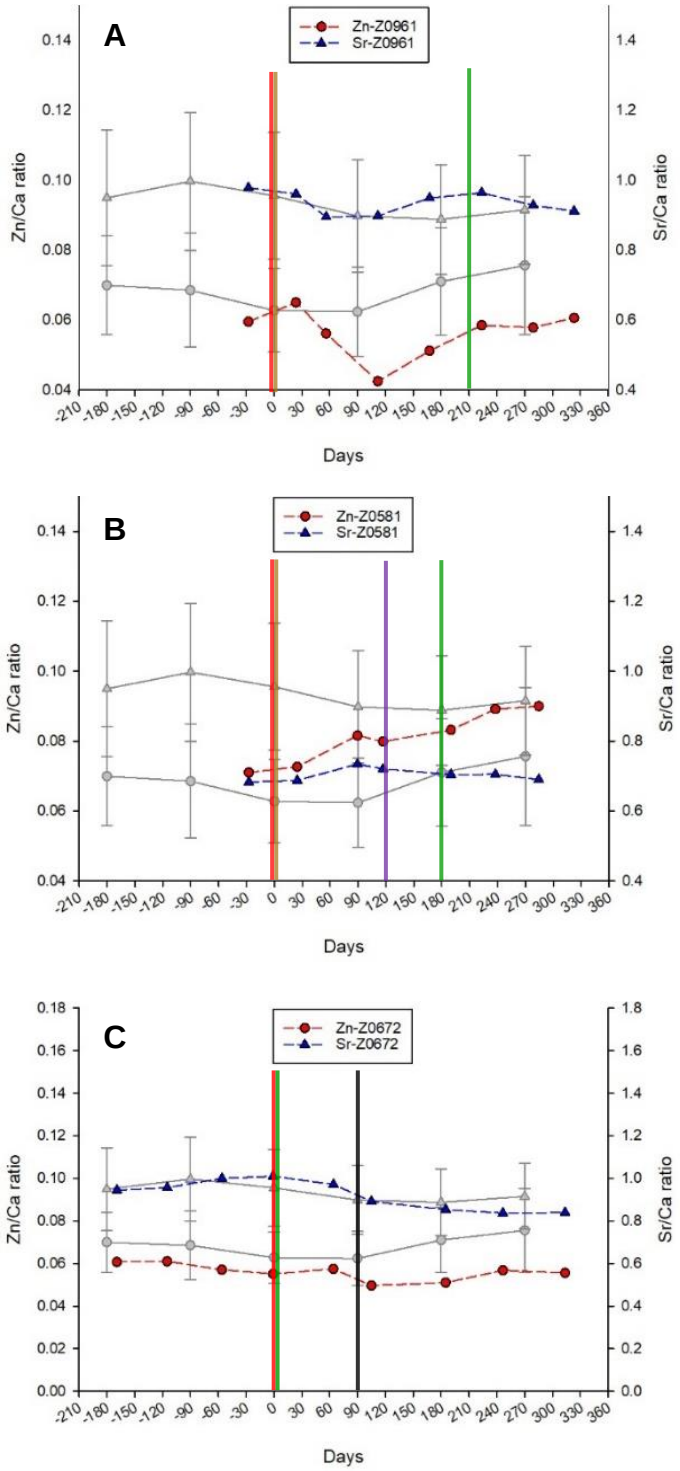

Figure 5.4 Graphs represent different trends of Zn and Sr distribution in children, who did not follow the observed trends, due to subject specific variation associated with birth weight and or unusual feeding regimes, from birth until 1-year old. Image: A) Z0961, who was born prematurely at week 24 of gestation with low birth weight (1.5 kg); B) Z0581, who was mixed-fed from birth and had iron supplementation at 4-month old; C) Z0672, a breast-fed child who had solid food immediately after birth and the mother stopped breastfeeding at 3-month old. Vertical lines are drawn as red line for birth as day 0, brown line for the beginning of formula milk, green line for the introduction of solid foods, purple line for end of formula milk, and black line as end of breast feeding.

5.5 Discussion

It has been reported that the highest Zn accretion of the foetus occurs after week 24 (last trimester) of pregnancy, as the Zn reservation in liver is developed (Terrin et al., 2015). In comparison to non-pregnant women, a higher Zn absorption was observed in pregnant women at late pregnancy and this was independent to dietary Zn intake (Hambidge et al., 2017). Zn levels in plasma or serum at late pregnancy have been reported to be 15-35% lower than pre-pregnancy (Donangelo and King, 2012). The accretion of Zn transferred to foetus may be reflected in the higher Zn levels stored within dentine of deciduous teeth at late pregnancy, observed in this study, which were significantly higher than 0-3-month and 3-6-month periods as presented in Figure 5.1. Consistent with these results, a previous *in vitro* study using osteoblast cells (MC3T3-E1 cell subclones), showed Zn deficiency decreased extracellular matrix protein synthesis and activities of both collagen type I and ALP, as well as Ca and P deposition, and reduced the calcification of extracellular matrix protein (Alcantara et al., 2011). A disruption in the formation of bone extracellular matrix was found in neonates from rats that had suffered from Zn deficiency. Also Zn content in their carcass and femur was significantly lower compared with the neonates from rats with sufficient Zn (Nagata et al., 2010). The technique of LA-ICP-MA and dental histology used in this study, however, was not able to separately analyse inorganic and organic forms of Zn in dentine.

This study demonstrated that the highest Sr/Ca ratio measured in teeth was found during late pregnancy and that this gradually decreased until the weaning period. A recent study showed the Sr concentration of maternal serum was perfectly correlated with Sr concentration of umbilical cord (Krachler et al., 2002), and the amount of Sr transferred to placenta is driven by serum concentration gradients (Rossipal et al., 2007). Furthermore, a previous study reported an increase in Sr absorption during pregnancy (Cabrera et al., 1999), which may be being reflected in the dentine of the teeth used in this study.

The results presented in this Chapter show the average trend of Zn/Ca ratio in dentine decreased significantly from late pregnancy until 6 months after birth, with a significant increase observed at weaning period (6-9 months). A previous study revealed Zn levels in infant hair significantly decreased from 2 months until 12 months of age, but

the Zn plasma level was constant over time (Özden et al., 2015). Opposed to these observations, Dolphin (2009) reported the Zn levels of prenatal tooth enamel were significantly lower than postnatal enamel, but the age assignment was not finitely determined. Using dental histology for age assignment, a more recent study demonstrated Sr distribution within enamel demonstrated a similar result with Sr distribution in dentine from prenatal until postnatal shown in our study (Humphrey et al., 2007).

Most of the children, recruited in this study, were breast-fed for durations longer than 6 months. Therefore, the reduction in Zn/Ca ratio in dentine within the first three months was expected, possibly due to the reduction of Zn concentration in breast milk and depletion of Zn reserves in the liver by 4 months postnatal. Moreover, the Zn concentration of breast milk is diminished with age (Ackland and Michalczyk, 2016; Krebs et al., 2014), while the fractional absorption of Zn was significantly increased during lactation regardless of the dietary intake of the mother (Hambidge et al., 2017).

The Zn/Ca ratio in dentine was notably increased at weaning, when taking the grouped results as a whole, as presented in Figure 5.1A. The introduction of complementary food at 6-month old, along with breast milk, as recommended by WHO, may increase Zn intake in infants, and appears to be recorded in the tooth with subsequent increases in Zn/Ca ratio in dentine. Zn/Ca ratio at weaning, however, was not significantly different compared with late pregnancy. In respect to this, the children may adopt the parents' eating behaviour and food intake. There is evidence that child and maternal diets are correlated, from early infancy of the child (Lioret et al., 2015).

The lowest Sr/Ca ratio was seen at the weaning period. This finding agrees with the literature, since the ability of gastrointestinal tract to discriminate non-essential Sr is thought to occur with maturity of the system, thus the absorption level of Sr is age-related (Cabrera et al., 1999; Humphrey, 2014).

During late pregnancy, there is a high demand for Zn, and half of the Zn required is transferred to the foetus. Zn from diet and adjustment in Zn homeostasis are two ways to meet high Zn requirement. Low Zn intake from the diet may be encountered by increased Zn absorption in the intestine and or reduce Zn excretion both by the kidneys and in faeces (King, 2000). A previous study in Guatemalan women showed the

increase of Zn absorption was higher in high-phytate than low-phytate consumers from early (8 weeks) to late pregnancy (34 weeks). The absorption level of Zn was calculated based on total Zn concentration of the subject's diet measured using atomic absorption spectrophotometry and the fractional Zn absorption analysed using dual isotope tracer technique as reported by Hambidge et al. (2017). The homeostatic mechanism, however, is no longer effective to compensate very low Zn intake (≤ 6 mg/day) (Donangelo and King, 2012). The present study showed Zn/Ca ratio in HZM was higher than LZM during late pregnancy, where Zn/Ca ratio of HZM was above the average value of Zn/Ca of this study population and vice versa for LZM. Thus, dentine may record and reflect the differences in Zn intake during late pregnancy.

Contrary to this result, the previous study revealed that Zn level was inversely related to Zn dietary intake, because the highest Zn level was observed in prenatal enamel of the children from mothers who consumed low Zn diet during pregnancy (Dolphin and Goodman, 2009). The direction and timing of enamel maturation, which differs to the mineralisation process of dentine (as discussed in section 1.3.2), however, should be considered as a likely reason for different results between these studies. Furthermore, this study did not include individuals who are severely deficient in Zn, as recruited in Dolphin and Goodman (2009) study. Therefore, future work may expand into a larger study to strengthen current results.

At birth until 6 months of age, Zn/Ca ratio of dentine varied in regard to different feeding regimes. The highest Zn/Ca ratio was shown in the BF group at the 0-3-month period compared with other groups. The Zn bioavailability in breastmilk has been identified as higher than formula milk because most of the Zn in breast milk (approximately 85%) binds to whey protein and is thought to be easier to process by the immature gastrointestinal system of infants (Ackland and Michalczyk, 2016). Thus, breast milk is the best source of Zn for infants (Krebs et al., 2014), even though Zn concentration in breast milk is reduced with time (Ackland and Michalczyk, 2016). At the 3-6-month period, the Zn/Ca ratio was also slightly increased compared with 0-3-month period, in the BF group. This is consistent with the previous findings that showed Zn concentration in breast-fed infant serum to be significantly increased at 6 months compared with day 1 and day 28 post birth (Djurović et al., 2017).

One child, who was fed only with formula milk, showed a Zn/Ca ratio in dentine slightly lower than BF group, while Sr/Ca ratio was extremely high compared with the other feeding groups, at 0-3-month period as shown in Figure 5.3B. A previous study revealed Sr contained in formula milk was 4-8 times higher than in breast milk, while the Sr concentration in urine was seven times higher in breast-fed baby compared with formula-fed baby (Widowson et al., 1960). A study in enamel also demonstrated Sr/Ca ratio was increased in formula-fed baby (Humphrey et al., 2007), which parallels the results we observed in this one child. Interestingly, a lower Zn/Ca ratio in dentine of this formula-fed child compared with the breast-fed children was observed. Although Zn concentration of formula milk is about 2-3 times higher than breast milk, as previously mentioned the Zn bioavailability of formula milk is lower than from breast milk due to high levels of casein rather than whey protein (Ackland and Michalczyk, 2016; Rodr´íguez et al., 2000).

Unexpectedly, Zn/Ca ratio in the formula-fed child decreased at 3-6-months old after remaining at a constant level from birth to 3-months old, but Sr/Ca ratio was still above the other feeding groups as seen in Figure 5.3B. The Zn concentration in the human body may be decreased when: the Zn intake is reduced; the Zn absorption is compromised due to high intake of dietary inhibitors and/or gastrointestinal disease; and/or high demands of Zn because of rapid growth of the infant and/or infection (Ackland and Michalczyk, 2016; King, 2000; Krebs et al., 2014). Based on the questionnaire, the FF child was continued to be supplemented with formula milk until 5-years old with no history of severe infection or being hospitalised before 6-month old. A previous study indicated that there is a limitation in individuals who suffer from Zn deficiency to adequately absorb Zn, thus, the efficacy of Zn supplementation will be reduced if a high dose of Zn is progressively administered (Krebs et al., 2014). This finding may explain the reduction of Zn/Ca ratio in the children fed formula after 3 months. Moreover, Zn/Ca ratio of prenatal dentine was lower than postnatal dentine which may indicate a Zn deficiency prior to birth. Thereafter, the Zn/Ca ratio slightly increased at the weaning period when the child was having complementary food alongside formula milk, even though the increase was not as high as BF group.

In mixed-fed infants, the Zn distribution within dentine are interpreted with care since formula milk was given at different times between children. From four mixed-fed children, only two children (Z0961 and Z0581) received formula milk from birth

(including one preterm, low-birth weight baby), while the other two started formula milk at 2- and 5-months of age. A higher Zn/Ca ratio in the two children fed mixed from birth was shown at 0-3-months compared with late pregnancy as described previously in Figure 5.4 B and D, but not in the two children who started formula later (*see appendix C*). Formula milk contains a high concentration of Zn and provides Zn at constant level, which differs to breast milk. Interestingly, the Zn/Ca ratio of MF groups was less than that observed in the BF only group at 0-3-month and 3-6-month periods. It is possible that the combination of formula taken alongside breast milk, could lower the Zn bioavailability of in both, depending on the volume of less bioavailable formula milk consumed (Ackland and Michalczyk, 2016).

A slightly lower Sr/Ca ratio within three months after birth, compared with late pregnancy, was shown in this study. The Sr/Ca ratio in BF and MF groups at 0-3- and 3-6-month periods were markedly lower compared with the FF child. It is consistent that Sr/Ca ratio is expected to be low in breast-fed babies (Humphrey, 2014). In addition to this, Sr/Ca ratio in dentine may relate to the mother's diet, where the Sr level is low in animal based food, vegetables and fruit. In Indonesia, there are cultural recommendations that mothers of new born baby's should consume more green leafy vegetables, such as spinach, papaya leaf, and *katuk* (Saroupus androgynous (L) Merr), to increase the production of breast milk, in rural or urban areas (Muljati and Arnelia, 1995), which may have also contributed to the lower Sr/Ca ratio's measured.

Surprisingly, in the premature baby who was born a low birth weight and mixed-fed from birth, the Zn/Ca ratio increased at 1-month old compared with late pregnancy, but after this period, steeply declined to its lowest level at 4-months old, and then increased at 7-months old when the child started complementary food as presented in Figure 5.4B. This agrees with a previous study that observed Zn deficiency in preterm infants at 3-months of age who had been solely breast fed (Aggett, 2000). However, the differences of Zn concentration in breast milk between the mothers of preterm and term infant are not significant (Fernández-Menéndez et al., 2016). In preterm infants the concentration of Zn binding to hepatic metallothionein is greater than term infants, but the absolute liver size is smaller, thus, they may have a less Zn availability postnatally (Krebs et al., 2014) . Nonetheless, the Zn concentration in serum of preterm infants is greatly decreased within first month after birth, and Zn excretion through urine is extremely high compared with term babies (Terrin et al., 2015).

Excluding the preterm child, only one other child was mixed-fed from birth. Similar to FF children, the Zn/Ca ratio of this MF child did not decline at 30-days after birth, observed in the majority of breast fed children, who showed a steep decline in Zn/Ca ratio (*see appendix C*). Interestingly, when the formula milk was stopped at 4-month old and breast feeding was continued until 24-month old there was a marked decrease in Zn/Ca ratio at 4-month as shown in Figure 5.4B. Although breast milk is robust to meet the Zn requirement in growing infants, other reasons may underlie this. Based on the questionnaire, an iron supplementation was given when the child was 4-month old. The disruption of Zn absorption due to iron supplementation has been studied (King, 2000) and proposed at high concentrations iron can outcompete Zn transport proteins, this may be reflected in dentine as Zn/Ca ratio of the child was decreased immediately after iron supplementation.

Children who prematurely started the complementary food (before 6-month old), either with breast milk (EW+BF) and or formula milk (EW+MF), demonstrated a lower Zn/Ca ratio compared with other groups. In Indonesia, early weaning, by introducing complementary food (solid, semi-solid, or soft food) to the infant before 6-month old is quite common. The main reason for EW is the mothers feel they do not produce enough milk for their baby and/or the milk only is not sufficient to satisfy their baby. A recent study in Nias, an islands in Indonesia, showed the most common foods for young infants were rice porridge, milk porridge, steamed rice, fruit, and manufactured complementary foods (Inayati et al., 2012). Rice porridge, rice, and or soup (water) are also common in diets of older infants (9-11-month old) (Santika et al., 2008). The manufactured complementary foods, which are claimed as fortified foods and provide essential nutrients at adequate level for infant growth, in contrary contain a lower level of Zn and Fe than WHO recommendation (Mauludyani et al., 2014) and were rarely consumed by the infants in this study. A premature introduction of low Zn complementary foods may reduce the frequency of breast feeding (Cohen et al., 1994; Inayati et al., 2012). Zn intake is adequate in breast-fed infants if the volume of milk is adequate (Krebs et al., 2014). As a consequence of reduced milk consumption, the Zn intake will be reduced if the breast milk is complemented or even substituted by complementary foods in young infants (0-6-months old). In sample Z0672, the mother reported that she started solid foods at birth and stopped breast feeding when the child was 3-month old. As seen in Figure 5.4.C, the Zn/Ca ratio remained constant from late

pregnancy until day 60 and then suddenly decreased at day 90, where Zn/Ca ratio of exclusively breast-fed baby, in general, started to increase (see appendix C). This finding may indicate the needs of Zn may not be met by complementary foods alone since less milk will be given and the absorption of Zn derived from solid food may not be as efficient in a very young infant, and breast milk is an adequate source of Zn in exclusively breast-fed infant until 6-month old.

The Sr/Ca ratio level of this group was lower than the FF group, but higher than the BF and MF groups. This result may confirm that the children in both groups have indeed been fed with other foods and not milk only. A previous study suggested the Sr/Ca ratio rose when complementary foods were introduced (Humphrey, 2014).

At weaning, there were differences of Zn/Ca ratio identified between HZI and LZI, but it was not possible for statistical analysis to be carried out, as the sample size of LZI was limited (n = 2). As studied previously, estimating Zn intake at the weaning period is not easy and infants are more vulnerable for Zn deficiency at this stage, especially in cereal-based diet population, as cereal-based based foods have low nutrient density and are a poor source of minerals (Dewey, 2013; Krebs et al., 2014). BF and MF groups, however, demonstrated a slight increase of Zn/Ca ratio at weaning period, not seen in the other groups. This is consistent with recommendation from WHO to continue breast feeding until 1-year of age, alongside complementary foods from 6-months. Furthermore, based on the results of this study, future work may be carried out to obtain more information on Zn/Ca ratio differences between HZI and LZI at weaning period.

5.6 Conclusion

In this study, we present information on Zn and Sr distribution in dentine of deciduous teeth, in relation to dietary Zn intake. Zn is an essential element and has a complex metabolism in human body. Interpretation of the shifts on Zn/Ca ratio within dentine need to consider Zn physiology during pregnancy, lactation, and weaning. Results from this study indicate, for the first time, that dentine may record accurately dietary Zn exposure in early life nutrition and could be a promising tool for monitoring pre-natal and post -natal dietary exposure.

Chapter 6 Expression of human Zn transporters at mRNA level in human dental pulp in response to dietary Zn

6.1 Introduction

Zn is a trace element with essential functions in protein structure, catalytic enzyme, gene regulation and cell signalling (Kambe et al., 2015). Zn homeostasis maintains human body Zn at adequate levels. The total body Zn is, mostly, stored in skeletal muscle and bones, but the stored Zn is not sufficient to replenish Zn levels in Zn deficient condition. Thus, the daily Zn intake from the diet needs to meet the body Zn requirement (Hara et al., 2017).

In intestine, Zn is mainly absorbed and then distributed to body organs and tissues through the circulatory system. The absorption of Zn increases up to 90% in Zn deficiency situation, while Zn secretion increases via the gastrointestinal tract, sloughing mucosal cells, as well as renal Zn excretion in response to excessive Zn intake (Hara et al., 2017; Kambe et al., 2015). As Zn homeostasis plays a crucial role in preventing Zn toxicity, excess Zn is not distributed to sensitive organs, such as the pancreas (Martin et al., 2013). Intracellular Zn homeostasis is regulated by Zn transporter proteins (Hojyo and Fukada, 2016). These proteins are classified into Zn Transporter (ZnT/SLC30) and Zrt-, Irt-related proteins (ZIP/SLC39). ZIP transporters, consists of 14 identified proteins in humans, which play roles in maintaining cytoplasmic Zn at adequate levels by either transporting Zn from extracellular compartment into the cytoplasm across the plasma membrane and or through efflux from organelles into the cytoplasm (Jeong and Eide, 2013). Conversely, ZnTs, which consists of 10 identified proteins, act to reduce cytoplasmic Zn concentrations, by Zn influx from cytoplasm into the organelles and transporting Zn from cytoplasm into the extracellular environment (Huang and Tepaamorndech, 2013). Only a small number of Zn transporters have been experimentally shown to be regulated by dietary Zn intake (Jeong and Eide, 2013).

ZIP1 is responsible for Zn influx from the extracellular space into the cytoplasm and is located in the plasma membrane. *Zip1-KO* mice show abnormalities in embryonal development during pregnancy under Zn deficient condition compared with wild-type mice (Hara et al., 2017). Furthermore, a previous study in mice placenta showed *Zip1* expression at mRNA level decreased in Zn-restricted and Zn-supplemented fed groups

compared with Zn-adequate fed group (Helston et al., 2007). Thus, ZIP1 seems to be responsive to dietary Zn intake. The sensitivity of ZIP1, ZIP2, and ZIP3 to dietary Zn deficiency during pregnancy has been also been demonstrated in previous studies (Jeong and Eide, 2013). The expression of ZIP2 in human monocytic/macrophage THP-1 cells was distinctly increased in Zn-deficient condition (Cousins et al., 2003). *Zip4* is abundantly expressed on the apical membrane of intestinal epithelial cells, in response to Zn-deficient dietary intake (Fukada et al., 2013; Kambe et al., 2015). Furthermore, a previous study in human ileum reported the expression of ZIP4 at mRNA level was not change, while ZIP4 protein decreased and not detected, in Zn-supplemented group compared with placebo (Cragg et al., 2005). In mice, the expression of *Zip4* in enterocyte is rapidly down-regulated after Zn repletion (Cousins et al., 2006). In contrary to ZIP4, the expression of ZIP5 is lower in sufficient Zn intake and associated with less Zn transport from plasma to cell in intestine and kidney (Cousins et al., 2006; Kambe et al., 2015).

The regulation of dietary Zn in ZnT1 and ZnT5 expression has been observed in previous studies (Cousins et al., 2006; Cragg et al., 2005; Fukada et al., 2013; Helston et al., 2007; Kambe et al., 2015). ZnT1 is responsible in transporting Zn from cytoplasm into the extracellular space. The location of ZnT1 is, mainly, on plasma membrane, but it is also observed in cellular vesicle for some cell types. In animal model, dietary Zn supplementation increases the expression of intestinal *ZnT1* at mRNA and protein level (McMahon and Cousins, 1998). In contrast, under low dietary Zn intake, the ZnT1 expression in pancreatic acinar cells decreases and has been associated with pancreatic Zn secretion (Cousins et al., 2006). Consistently, the expression of *ZnT1* along with *ZnT5* at mRNA and protein level, which is localised in villous syncytiotrophoblast of the human placenta and may involve in maternal foetal Zn transfer, decreased significantly in Zn-restricted and Zn-supplemented fed mice (Helston et al., 2007). In human ileum, a reduction in ZnT1 expression at mRNA and protein level after Zn supplementation has been reported, and consistently, ZnT1 mRNA expression decreased in Caco-2 cells treated with higher Zn concentration (200μM) compared with 100uM Zn treatment. The same study demonstrated the reduction of ZnT5 protein, thus, dietary Zn intake may also effect ZnT5 expression (Cragg et al., 2005). ZnT5 is mainly localised in Golgi apparatus and functions in Zn efflux from cytoplasm into organelle cells (Kambe et al., 2015).

Zn is highly deposited in bone, cartilage, and teeth. Zn regulation in bone metabolism through Zn transporter roles has been studied. In humans, lack of ZIP13 expression, associated with Ehlers-Danlos syndrome (EDS), showed delayed growth, short stature, osteogenesis imperfecta, and hypodontia of one or few teeth (Fukada et al., 2013). In animal models, *Zip13-KO* mice, replicating similar conditions to EDS patients, demonstrated abnormal incisor tooth development, less dentine in the root of molar teeth, and decreases in alveolar and mandible bone volume. These clinical signs were associated with disruption of BMP/TGF-β signalling by regulating SMAD nuclear localization. Furthermore, BMP/TGF-β – SMAD complex pathway plays crucial roles in formation and development of cranial neural crest, facial primordia, tooth, alveolar bone, lip and palate (Fukada et al., 2011).

ZIP13, localised in Golgi apparatus and cytoplasmic vesicles, is responsible in Zn influx from organelle cells into cytoplasm and is responsive to dietary Zn intake (Jeong et al., 2012; Kambe et al., 2015). A previous study in rats showed the expression of *Zip13* mRNA in femur, increased significantly after 1-week feeding, using a Zn-deficient diet (Suzuki et al., 2016). Using human cells, ZIP13 has also been reported to be responsive to various extracellular Zn concentrations (Jeong et al., 2012).

In this study, a differential level of Zn incorporation in primary dentine of human deciduous teeth has been observed and is associated with dietary Zn intake level. However, the mechanism of mineral deposition within dentine has not been studied intensively, except for levels of Ca (Linde, 1995). In dentine formation (dentinogenesis), odontoblast cells are responsible for secretion of dentine matrix protein, as well as dentine mineralisation. Thus, the distribution and transport of Ca, as well as other minerals, observed within dentine, may be regulated by these odontoblast cells. Ca is transferred from the vascular circulatory network across the differentiated odontoblast cell layer into the mineralisation front, an interface between non-mineralised pre-dentine and the mineralised dentine, possibly through intracellular and or intercellular route (Linde, 1995).

After the formation of primary dentine is completed, odontoblast cells are localised within dental pulp, a soft connective tissue that acts as a microcirculatory system in tooth, connected to circulatory system of the human body (Nanci, 2013). An excessive dose of Zn administered intravenously in rats, showed a mild inflammatory response

in dental pulp within 6 hours (Scutariu et al., 2016). Thus, Zn may be distributed to dental pulp through the circulatory system. However, the precise mechanism by which Zn is transported from the circulatory system of the pulp to the tooth hard tissue, remains unclear. As Zn transport is not mediated by a passive diffusion mechanism, Zn transporters are essential and may play a role in Zn transport within odontoblast cells during dentine mineralisation. A very recent study using human dental pulp cells (hDPCs), differentiated into the secretory stage, showed the vitality, osteogenic differentiation, and mineralisation capacity of the cells under extracellular Zn exposure, alongside an increased in ZnT1 and ZIP1 expression at mRNA level (An et al., 2017). Therefore, the objective of this study was to elucidate the expression of Zn transporter in human pulp tissue at the mRNA level and relate changes in expression to dietary Zn intake.

6.2 Aims

This chapter aims:

1) To examine mRNA Zn transporter expression in human dental pulp
2) To analyse mRNA Zn transporter expression in vitro, using human dental pulp cell line model, in response to high- and low-extracellular Zn.

6.3 Materials and methods

6.3.1 Expression of Zn transporters at mRNA level in human dental pulp

To identify Zn transporters expressed in human dental pulp, the primers for seven Zn transporters (ZnT type) and housekeeping genes (GAPDH and 18S) were designed as shown in Table 6.1. A conventional end-point PCR technique using BioMix™ Red PCR reagent (Bioline) was performed to check that designed primers were working properly using either human brain RNA (Ambion RNA tissue panel), RNA extracted from Caco-2 (intestinal cell) and SH-SY5Y (neuroblastoma) cells. The efficiency of designed primers were then analysed as described in Chapter 2. All products were sequenced to confirm identity.

Human dental pulp was removed from collected teeth (n = 24) from twenty-one recruited children and prepared for total RNA extraction, then converted to cDNA (see Chapter 2 for specific method). RT-qPCR procedure was carried out to analyse the expression of Zn transporters at mRNA levels in human dental pulp.

Product	Accession number[a]	Primer sequences	Product Size (bp)	Anneal temp. (^{0}C)
ZnT1	NM_021194.2	$_{735}$GCAACTCCAACGGGCTGAAA$_{754}$ $_{883}$ACGCATGTTAAGTTGTCCAGCC$_{862}$	149	60
ZnT2	NM_032513.3	$_{743}$ATCGGCGACTTTATGCAGAGC$_{763}$ $_{848}$TGGAGAAGACGAAGGTGCAGA$_{828}$	106	60
ZnT3	NM_003459.4	$_{655}$ACTGGCATCCTCCTGTACCT$_{674}$ $_{771}$GGCCATTAACAGGTTGGCAC$_{752}$	117	60
ZnT4	NM_013309.4	$_{760}$GACCTAAGCGCCATCATACTC$_{780}$ $_{864}$AGCTGACAAAACCTCTAAGCG$_{844}$	105	60
ZnT5	NM_022902.4	$_{748}$AGCAAAGACAAGGGGAGCTG$_{767}$ $_{895}$GGCAATGGCTGTGTAAAGCA$_{876}$	148	60
ZnT6	NM_001193515.1	$_{276}$CCTGGCTGTATTTGCCTCCA$_{295}$ $_{381}$TCTTCCCGTGTGTATCTCGG$_{362}$	106	60
ZnT7	NM_133496.4	$_{367}$GCTTAGGCTTGATTTCCGACT$_{387}$ $_{504}$CCAGAACTTCCGCTCTAACATAC$_{482}$	138	60
ZnT9	NM_006345.3	$_{1130}$TGGAGTCATGGGATTGCTTCAT$_{1151}$ $_{1230}$CAAGAAGTGTTGCTCCTTCAGA$_{1209}$	101	60
ZnT10	NM_018713.2	$_{836}$TTCGCAAACGTAGCAGGTGA$_{855}$ $_{983}$TGATGACCACAACCACGGAC$_{964}$	148	60
ZIP1	NM_014437.4	$_{698}$TAGTAAGCTGTTTCGCGGGG$_{717}$ $_{812}$AACTGGAGCGTCACGTGCAA$_{793}$	115	60
ZIP2	NM_014579	$_{546}$TTTGGAGTCGCTGGCATTG$_{564}$ $_{671}$TTTGAGGGTGAGGGTAAATGTC$_{650}$	126	60
ZIP3	NM_144564.4	$_{455}$GAGGGAAAAGCTCCAGAAGGT$_{475}$ $_{583}$TTGCGGAAGGTCAGGATCAG$_{564}$	129	60
ZIP4	NM_017767.2	$_{1718}$AGCCCAGAGTTGAGGCTACT$_{1737}$ $_{1867}$TGGCAACTCGTGGCAGAACA$_{1848}$	150	60
ZIP5	NM_173596	$_{209}$GGGGAGAATAGGAGCCAGAA$_{228}$ $_{313}$AAGACGACCCACACACAGAA$_{294}$	105	60
ZIP6	NM_012319	$_{1969}$ACTACCATCATATTCTCCATCATCA$_{1993}$ $_{2100}$CATCACCCATTATCACCATCCA$_{2079}$	132	60
ZIP7	NM_006979	$_{2177}$GTTCTCCAAGGTCCAGTTTCC$_{2197}$ $_{2320}$CCCCAATCACTCCCAAATCAG$_{2300}$	144	60
ZIP8	NM_022154	$_{957}$CTCTCCTCGGATTGATTTTGACT$_{979}$ $_{1086}$AATGCCTCTGGAATAAGTTGGAA$_{1064}$	130	60
ZIP9	NM_018375.4	$_{673}$AGTGGTTGTGGGTGAATAAAGG$_{694}$ $_{792}$GAAATTAACAGCCAAGGGAATGAT$_{769}$	120	60
ZIP10	NM_001127257	$_{2569}$TGGTGATGGTGACAATGAAGAA$_{2590}$ $_{2652}$GCAAATCCAAAGAGCAATCCTAA$_{2630}$	84	60
ZIP11	NM_139177.3	$_{534}$CCATCCGGATAGACAAGAGTGA$_{555}$ $_{641}$GATTCCCTCGAGAAGGCACA$_{622}$	108	60
ZIP13	NM_001128225.2	$_{1254}$ATCGTGGTAATGGTGCTGTTC$_{1274}$ $_{1379}$CCCTCTCTGCCTCTCACATAT$_{1359}$	126	60
ZIP14	NM_015359	$_{2716}$TTGCCGCTTCCATAAATCAAAG$_{2737}$ $_{2842}$AAACCCATCTGTAGCATAATCATTAG$_{2817}$	127	60
GAPDH	NM_001289746.1	$_{185}$TGAAGGTCGGAGTCAACGGATTTG$_{208}$ $_{312}$CATGTAAACCATGTAGTTGAGGTC$_{289}$	128	60
18S	NM_022551.2	$_{340}$GGACCTGGCTGTATTTTCCA$_{321}$ $_{226}$GAGGATGAGGTGGAACGTGT$_{245}$	115	60

Table 6.1 Primer sequences for human ZnTs, ZIPs, GAPDH, and 18S used for RT-qPCR and PCR. End-point PCR was carried out using BioMix™ Red (Bioline), included denaturation step at 94^{0}C and extension at 72˚C, while PCR RT-qPCR was performed using SensiFAST SYBR mix No ROX (Bioline) with initial activation step at 95°C for 2 mins. Cycle parameters (denaturation, annealing extension and number of cycles) for PCR is 60s;60s;60s x 30 cycles and for RT-qPCR is 5s;15s x 40 cycles.
[a] Numbered according to GenBank™

6.3.2 *In vitro study to analyse Zn transporter mRNA expression in human odontoblast-like cell model in response to high- and low-extracellular Zn intake*

HDP-hTERT cell line (Kitagawa et al., 2007), which has odontoblast cells capacity, were used in this study as a model to analyse Zn transporter mRNA expression in odontoblast cells in response to extracellular Zn exposure (*in vitro)*.

HDP-hTERT cells were differentiated using mineral-induced growth medium to obtain the odontoblast-like cells at active-secretory stage. The identification for differentiation process was carried out using Alizarin Red staining and DSPP protein labelling using immunofluorescence technique. The methods for cell differentiation, Alizarin Red staining, and immunofluorescence were described in Chapter 2 (Sections 2.3).

Primers were designed to analyse the expression of Zn transporters (9 ZnTs and 13 ZIPs) (Table 6.1) and validated using the same procedure as explained previously in Chapter 2. The profile of Zn transporters expression was assessed at mRNA level using HDP-hTERT, Caco-2, and SH-SY5Y cells.

The tolerable level of $ZnCl_2$ concentration for HDP-hTERT was assessed using cell viability assays. The odontoblast-like cells were then treated with extracellular $ZnCl_2$ at varying concentrations, based upon the viability assays in order to examine the expression of Zn transporters at mRNA level in response to extracellular Zn exposure. RT-qPCR was then conducted to analyse the mRNA expression level of ZnT1, ZnT5, ZIP1, ZIP13 and ZIP14 relative to GAPDH and 18S (see Chapter 2 for specific methods).

6.4 Results

6.4.1 *Expression of Zn transporters at mRNA level in human dental pulp*

Twenty-one children were recruited, and total RNA was successfully extracted from 5 deciduous teeth originating from 5 children. The RNA extraction for sixteen others were not usable due to less pulp tissue left in the extracted teeth and/or less preserved RNA in pulp tissue after dental extraction procedure. From these five samples, the profile of expression of ZnTs in human dental pulp were assessed as presented in Table 6.2. A differential pattern in ZnT1, ZnT5, and ZnT9 mRNA expression relative to GAPDH was observed in this study as shown in Figure 6.1. These five children, however, were all classified as high-Zn consumers, thus no conclusion could be drawn in relation to diet.

Thus, further attempts were carried out to explore the response of Zn transporter mRNA expression under high or low extracellular Zn exposure using HDP-hTERT cells in vitro.

Zn transporters	Expression in human pulp
ZnT1	✓
ZnT2	X
ZnT3	X
ZnT4	X
ZnT5	✓
ZnT9	✓
ZnT10	X

Table 6.2 Zn transporters expressed in human dental pulp. All primers were assayed for their annealing qualities with RNA from with brain, Caco-2 or SH-SY5Y cells. Only those transporters identified with a ✓, gave a positive result with RNA extracted from all human pulp tissue samples.

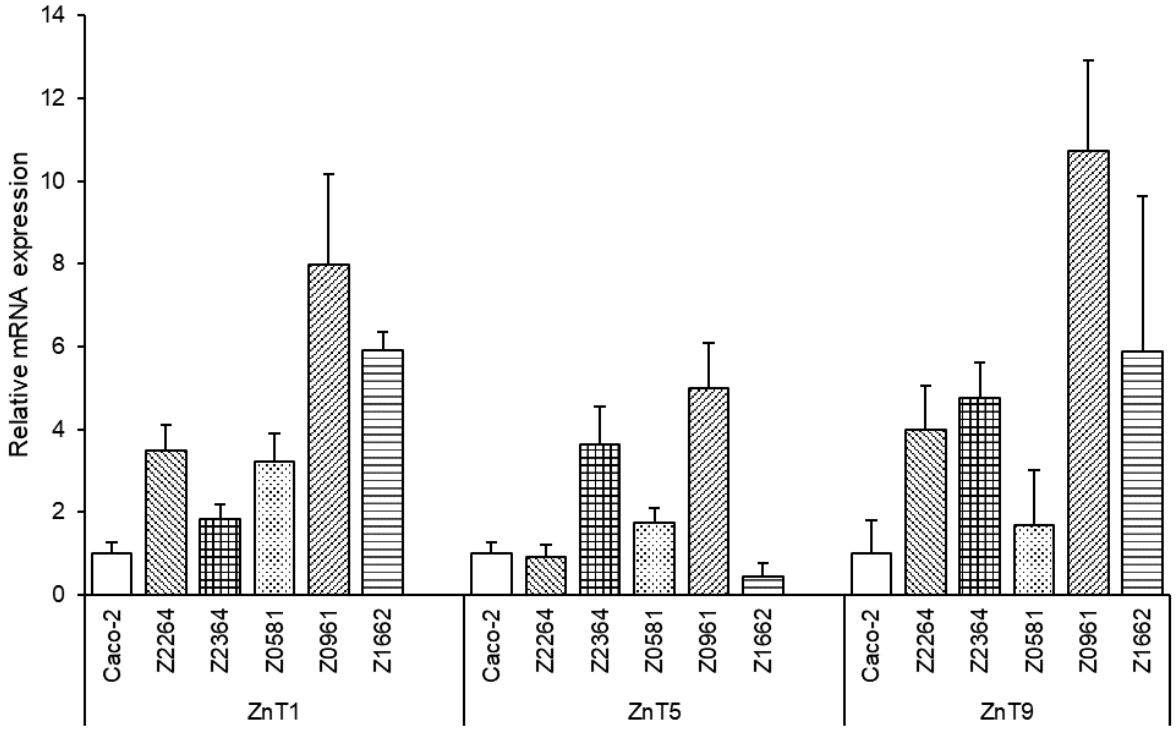

Figure 6.1 Five individual human pulp samples were used and RNA extracted and converted into cDNA. These were used for RT-qPCR using primers and conditions specific for ZnTs and GAPDH as the reference gene (Table 6.1). RT-qPCRs were run in triplicate and mean (±SEM) values relative to GAPDH in the same sample are presented for the ZnTs that were detected in these samples. Relative concentration of target and reference gene were calculated for Ct value using the Pfaffl method (Pfaffl, 2001). Standard curves generated using serial dilution of cDNA from human brain (Ambion RNA tissue panel) and Caco-2 cells were used to ensure the efficiency of primers. To control the quality between plate, Caco-2 cells were used as a calibrator. Standard curves and efficiency for primers are shown in Appendix D.

6.4.2 *Characterization of secretory odontoblast-like cells*

To differentiate HDP-hTERT into odontoblast-like cells at the secretory stage, the cells were grown in mineralisation-induced medium for 3, 7, and 14 days. In all experiments, control, HDP-hTERT were grown in normal growth medium (as described in Chapter 2). The odontoblast cells capacity was determined by assessing the mineralisation activity using Alizarin Red staining in differentiated HDP-hTERT cells after for 14 days. This technique stains mineral nodule formation in differentiated cells, which can be compared with control as shown in Figure 6.2.

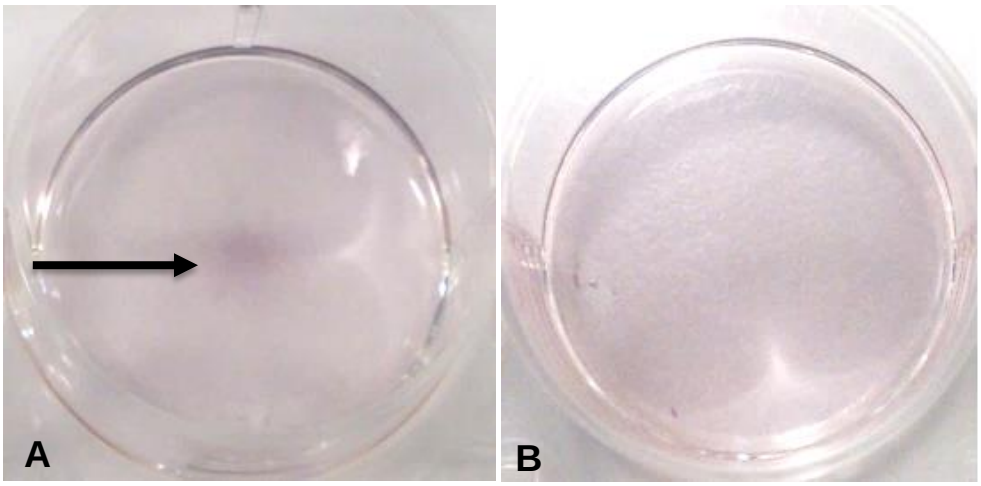

Figure 6.2 Alizarin Red staining in undifferentiated and differentiated HDP-hTERT cells. The cells grown in 24-well plates, seeded at a density of $5x10^4$ cells/well. HDP-hTERT cells were grown with presence of 50 µg/mL ascorbic acid (Sigma Aldrich), 10 mmol/L β-Glycero-phosphate (Cayman), and 0.1 µmol/L dexamethasone (Sigma Aldrich) (A) or growth medium only (B) for 14 days. Cells were washed and treated with Alizarin Red, which stains nodule mineralisation on HDP-hTERT cells, identified by darker staining (arrow).

In parallel an immunofluorescence assay was also carried out using anti-DSPP antibody after the differentiation period (3, 7 and 14 days). DSPP is a specific protein known to be expressed by odontoblast cells during secretory stage. The result showed DSPP protein was expressed after the cells were grown in mineralisation-induced medium after as shorter time period as 3 days compared with control as presented in Figure 6.3.

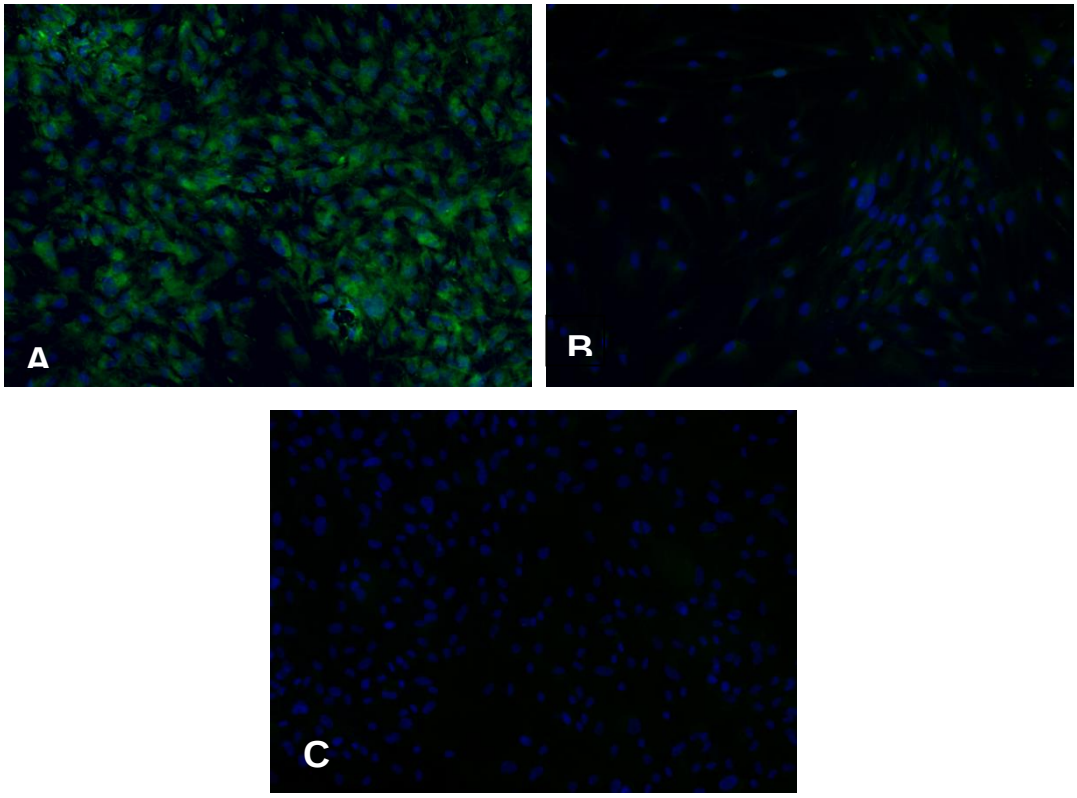

Figure 6.3 Immunofluorescence staining of DSPP in undifferentiated and differentiated HDP-hTERT cells. The cells grown in 24-well plates, seeded at a density of $5x10^4$ cells/well. Grown with presence of 50 µg/mL ascorbic acid (Sigma Aldrich), 10 mmol/L β-Glycero-phosphate (Cayman), and 0.1 µmol/L dexamethasone (Sigma Aldrich) or growth medium for 3 days. Cells were fixed with 100% (V/V) methanol for 5 minutes, washed with PBS, and incubated with DSPP-antibody (Santa Cruz; 1:50 dilution) overnight, and then with anti-IgG-FITC conjugate (Santa Cruz; 1:100 dilution) as secondary antibody for 2 hours. Positive staining for DSPP protein is shown in green. Blue staining represents DAPI nuclear DNA stain.
A) shows differentiated cells treated with DSSP and secondary antibody
B) shows undifferentiated cells treated with DSPP and secondary antibody
C) shows differentiated cells treated with secondary antibody only (control positive)

6.4.3 Viability of HDP-hTERT cells in different concentrations of extracellular Zn exposure

Cells were treated with different concentrations of extracellular Zn (as $ZnCl_2$) for up to 24 hours. Viability was assayed using Alamar Blue at 30-minute, 2, 4 and 24-hour time points. Results are shown in Figure 6.4. The analysis revealed cells were viable up to a maximum concentration of 60µM $ZnCl_2$ at all time points compared with 10µM $ZnCl_2$, take as the physiological extracellular Zn concentration in plasma/serum. Thereafter, for all mRNA expression analysis, differentiated HDP-hTERT cells were exposed to extracellular $ZnCl_2$ at 10 µM (physiological level) and 60µM concentration for either 4 or 24 hours.

6.4.4 Zn transporter mRNA expression in human secretory odontoblast-like cell model in response to extracellular Zn exposure (in vitro)

To analyse whether differentiated HDP-hTERT are a suitable model and express the same Zn transporters as human pulp, these cells were initially screened for expression of the same transporter previous analysed in human pulp and due to sufficient levels of sample, all other known human Zn transporters. Agarose gels showing PCR products are shown in Appendix E and Table 6.3 presents expression in HDP-hTERT cells. Caco-2 cells (intestinal model) and SH-SY5Y cells (neuronal) were used as a positive control to ensure primers and conditions were optimal for successful PCRs. In agreement with human pulp, ZnT1, 5, and 9 were present in the odontoblast-like cells, with ZnT2 and 3 being undetectable. In this cell line model, we were also able to measure expression of ZnT4, differing with our human pulp results.

Zn transporters	HDP-hTERT	Caco-2	SY5Y
GAPDH	✓	✓	✓
18S	✓	✓	✓
ZnT1	✓	✓	✓
ZnT2	X	✓	X
ZnT3	X	✓	✓
ZnT4	✓	✓	✓
ZnT5	✓	✓	✓
ZnT6	✓	✓	✓
ZnT7	✓	✓	✓
ZnT9	✓	✓	✓
ZnT10	X	✓	✓
ZIP1	✓	✓	✓
ZIP2	X	✓	X
ZIP3	✓	✓	✓
ZIP4	✓	✓	✓
ZIP5	X	✓	X
ZIP6	✓	✓	✓
ZIP7	✓	✓	✓
ZIP8	✓	✓	✓
ZIP9	✓	✓	✓
ZIP10	✓	✓	✓
ZIP11	✓	✓	✓
ZIP13	✓	✓	✓
ZIP14	✓	✓	✓

Table 6.3 mRNA Zn transporters, GAPDH, and 18S expressed in HDP-hTERT cells, Caco-2 cells and SH-SY5Y cells (see Appendix E).

ZnT1, ZnT5, ZIP1, ZIP13 and ZIP14 mRNA were analysed using qPCR to measure the expression level in differentiated HDP-hTERT cells in response to extracellular Zn treatment. These Zn transporters were chosen due to their sensitivity to dietary Zn intake and or their expression in mineralised tissue and cells has been observed. No significant changes in expression were seen for all transporters analysed, with the exception of ZnT1 and ZIP13 relative to GAPDH. ZnT1 showed an increase in level of expression after 24-hour treatment with 60µM extracellular $ZnCl_2$ treatment compared with untreated control (Figure 6.6). While, ZIP13 showed a significant increase in mRNA level after 4 hours of treatment at high Zn concentration (10 µM $ZnCl_2$) (Figure 6.6).

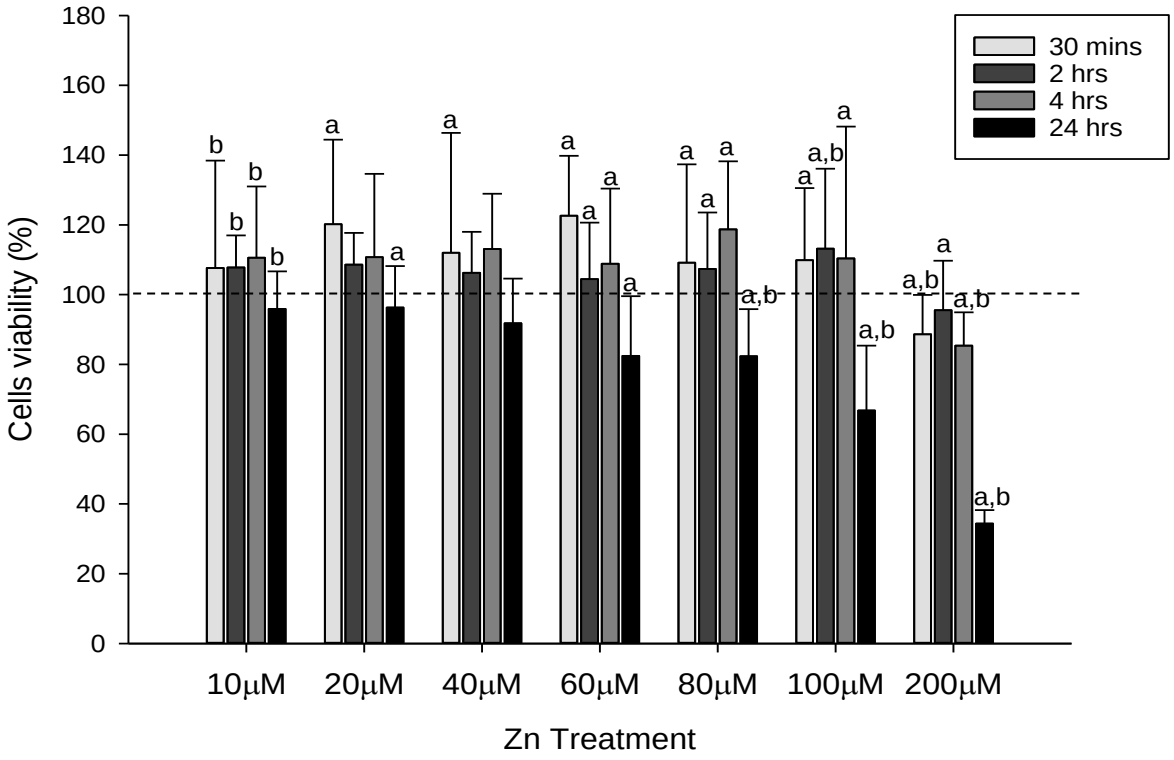

Figure 6.4 Cell viability of HDP-hTERT cells after extracellular $ZnCl_2$ exposure at different concentration and times. Data are expressed as normalised data to cells grown in basal medium as 100% cell viability. Mean values (±SEM) (n=6) are presented for each experiment. The differences between times and concentration were significant analysed using Two-way Repeated Measurement ANOVA (p=0.001). Bonferonni Post-Hoc Test was performed to analyse the differences between times at the same concentration, [a]) $p<0.05$; and the differences between 10 µM $ZnCl_2$ and other concentrations, [b]) $p<0.05$.

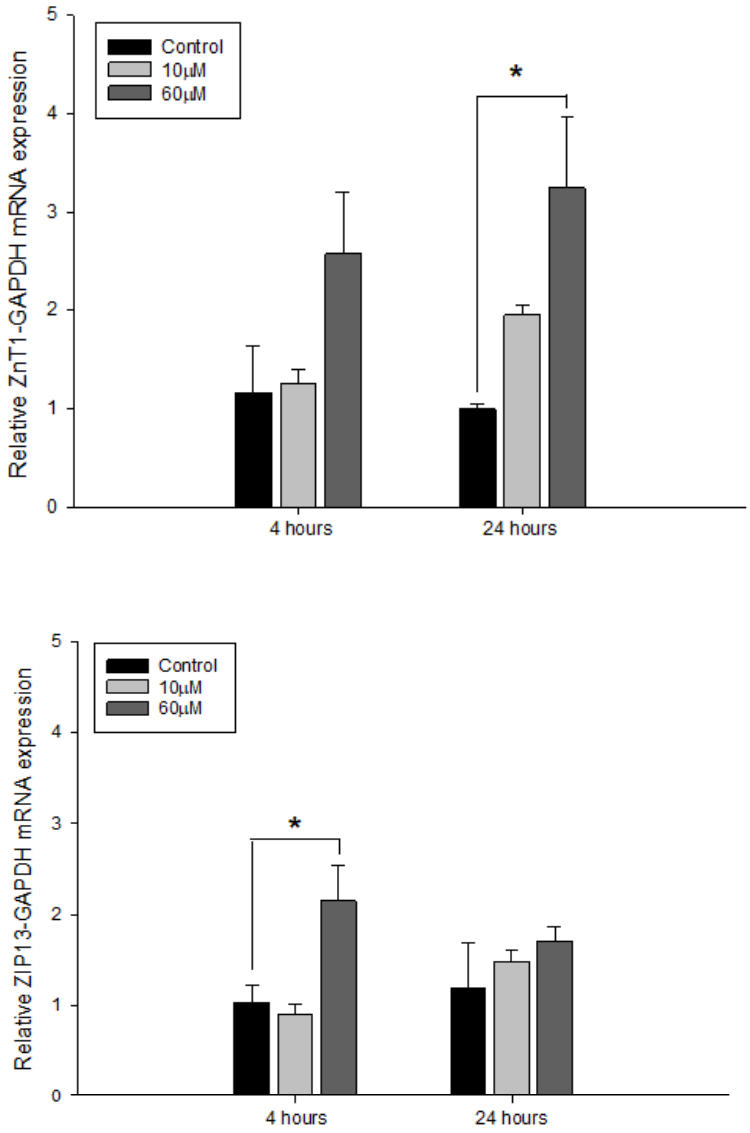

Figure 6.5 Figure 6.5 mRNA expression of ZnT1 and ZIP13 in response to extracellular $ZnCl_2$ treatment. HDP-hTERT cells were grown with presence of 50 µg/mL ascorbic acid (Sigma Aldrich), 10 mmol/L β-Glycero-phosphate (Cayman), and 0.1 µmol/L dexamethasone (Sigma Aldrich) for 7 days. Then, the cells were treated with basal medium (as control), 10 µM and 60 µM $ZnCl_2$. RNA was extracted after 4 and 24 hours, and the expression levels of ZnT1 and ZIP13, relative to the level at the control condition, were measured by RT-qPCR using GAPDH as a reference gene using Pfaffl method. Negative control RT-qPCR reactions were analysed and identical prepared to those yielding the products, but in omission of Moloney murine leukaemia virus reverse transcriptase. Standard curves generated using serial dilution of cDNA from Caco-2 cells were used to ensure the efficiency of primers. To control the quality between plate, Caco-2 cells were used as a calibrator. Standard curves and efficiency for primers are shown in Appendix D. Data is presented in histogram as median (±SEM) from three independent experiments (n=3). The mRNA expression level of ZnT1 and ZIP13 were different significantly, Annova ($p<0.05$). The differences between concentrations and times were analysed using Tukey post Hoc test, *), $p<0.05$.

6.5 Discussion

As this study previously revealed differential levels of Zn incorporation within dentine possibly in response to different levels of dietary Zn intake, determining the mechanism of Zn transport from the vascular circulatory system into dentine is needed. As previously shown in Table 6.2, the ZnT2, ZnT3, and ZnT10 expression at the mRNA level was not observed in human dental pulp. This is consistent with previous studies in that these transporters are not widely distributed in human tissues and or cells (Huang and Tepaamorndech, 2013; Lichten and Cousins, 2009). However, the expression of ZnT1, ZnT5, and ZnT9 at the mRNA level demonstrated in this study may reflect, for the first time, a role for these Zn transporters in dental pulp. It is noted that due to lack of RNA, not all Zn transporters identified in humans, could be analysed in these pulp samples.

A differential expression pattern was observed in ZnT1, ZnT5, and ZnT9 at the mRNA level between the five recruited children, even though these five children were classified into high-Zn consumer group. Taken into consideration that the dietary Zn intake of the recruited children in this study was not controlled, the differential expression of Zn transporters at mRNA level between samples may be expected since Zn homeostasis is tightly controlled to maintain adequate Zn levels in human body (Kambe et al., 2015). Furthermore, the expression of Zn transporters may also be regulated by other stimuli, such as hormones, cytokines, endoplasmic reticulum stress, oxidative stress, and hypoxia in a specific cell and tissue (Hara et al., 2017).

As dental pulp accommodates not only odontoblast cells, but also other cells, such as sub-odontoblastic and stromal fibroblast cells, as well as neuronal, immune and vascular system cells (Goldberg, 2014),using this tissue to measure Zn transporter expression may not be ideal and may as it is not specific for odontoblast cells. Thus, further investigation was carried out using a cell line model. HDP-hTERT cells were chosen due to the odontoblastic capacity that has been demonstrated by Kitagawa, et al (2007).

In the tooth, the odontoblast cells life cycle, known as preodontoblast-secretory-transitional-aged stage, shown different cell morphology, ultrastructure, the marker expression pattern as well as transcriptional control throughout all stages (Couve, 1986; Seino et al., 2017; Simon et al., 2009). In this study, HDP-hTERT cells were

differentiated into the active-secretory odontoblast cells by growing the cells using mineralisation-induced medium supplemented with β-Glycero-phosphate and dexamethasone (Wei et al., 2007). The differentiation step is necessary to ensure the cells are in the secretory stage when primary dentine is actively formed.

In this study, a nodule mineralisation was shown using Alizarin Red S staining in differentiated HDP-hTERT. Thus, the cells are responsive to differentiated medium and show the capacity in mineralisation as reported in previous studies (Kim et al., 2010; Kitagawa et al., 2007). Further confirmation using immunofluorescence technique was also used, DSSP protein was expressed in the cytoplasm when the cells were differentiated (Figure 6.3). This is consistent with previous studies in mice that demonstrated DSPP protein was expressed in the cytoplasm of odontoblast cells. DSPP is a non-collagenous protein as a member of small integrin-binding ligand N-linked glycoprotein (SIBLING) family and a functional marker for secretory activity of odontoblast cells (Quispe-Salcedo et al., 2012). A mutation in human DSPP gene is associated with dentinogenesis imperfecta and dentine dysplasia conditions, which indicated a disruption in dentine mineralisation (Suzuki et al., 2012). Furthermore, DSPP expression is diminished in aged odontoblasts cells where the dentine is not actively formed (Couve et al., 2013). Thus, in this study, HDP-hTERT cells are successfully differentiated into an active-secretory stage.

To assay the viability of HDP-hTERT cells to high levels of extracellular Zn, a viability test was carried out after HDP-hTERT cells were treated with different concentrations of extracellular $ZnCl_2$ at different times to determine the highest tolerable $ZnCl_2$ concentration for HDP-hTERT cells. Results showed that at concentrations higher than 60 µM extracellular Zn, HDP-hTERT cells were less viable. $ZnCl_2$ concentration of 10 µM was applied in the remainder of this study as the physiological level of cellular Zn measured in serum/plasma, although intracellular Zn concentration may range from 10-100 µM and it is not applicable for all cell types (Eide, 2006; Hara et al., 2017). A high level of Zn, at 60 µM $ZnCl_2$ was used in this study, to mimic high concentrations of extracellular Zn exposure, since the differences in viability were not significant up to 60 µM compared with 10 µM $ZnCl_2$ throughout the exposure times. A previous study reported the extracellular Zn exposure, in a range of 10 µM to 80 µM $ZnCl_2$, increased the vitality of human dental pulp cells along the concentration and time increments (1,

3, 5, and 7 days), and may prevent the cells from mitochondrial dysfunction (An et al., 2017).

ZnT1, ZnT5, ZIP1, ZIP13 and ZIP14 mRNA expression were analysed in this study. These Zn transporters were chosen due to their sensitivity to dietary Zn intake and or their expression in mineralised tissue and cells has been observed (An et al., 2017; Cragg et al., 2005; Fukada et al., 2011, 2013; Helston et al., 2007). Furthermore, this study has revealed that ZnT1 and ZnT5 were expressed at mRNA level in human dental pulp from recruited deciduous teeth. The expression of ZnT9 was not analysed, although the expression of ZnT9 was detected earlier in this study. A previous *in silico* study showed ZnT9 has the lowest similarity, based on amino acid protein sequences, with other ZnTs (Seve et al., 2004). As the essential histidine to establish intramembranous Zn-binding site is absence in ZnT9, the function of ZnT9 in Zn transport become questionable, and even, the interaction between ZnT9 with other ZnTs has not been demonstrated yet (Kambe et al., 2015). Thus, in regard to the aims of this study, ZnT9 was not included for analysis.

In response to dietary Zn intake, Zn transporters involved in Zn homeostasis to maintain the adequate Zn levels in human organs, tissues, and cells, since certain organs, such as pancreas, are sensitive to excess Zn. A previous study, using rats as an animal model, showed Zn concentration of plasma, kidney, liver, and femurs was dependently increased in response to dietary Zn levels (24, 1016, 2008, 3000 mg Zn/kg/day) after 10-day exposure, but the mRNA expression of ZnT1 in intestinal epithelium was significantly decreased in higher dietary Zn intake compared with control (24mg Zn/kg/day), which indicates the reduction of ZnT1 roles in transporting Zn from cytoplasm of epithelium cells into the portal vein (Fujimura et al., 2012). In contrast, extracellular Zn exposure (0, 50, 100, and 200 µM $ZnSO_4$) in intestinal epithelial cells-J2 (porcine cell line) increased ZnT1 expression at mRNA levels after 24 hours (Martin et al., 2013).

The up-regulated ZnT1 and ZIP1 mRNA expression observed in differentiated hDPCs after exposure with extracellular $ZnCl_2$ at concentration of 40 µM and 60 µM for 7 days may be evidence of Zn's role in mineralisation of hDPCs (An et al., 2017). Consistent with the previous study, a significant up-regulation of ZnT1 mRNA expression in differentiated HDP-hTERT after 24-hour exposure of extracellular 10 µM and 60 µM

$ZnCl_2$ was demonstrated in this study. Thus, the up-regulated ZnT1 may indicate a role of ZnT1 in differentiated hDPCs during dentine mineralisation, and may related to Zn deposition as ZnT1 responsible in transporting excess Zn into the extracellular space.

Furthermore, regulation of ZnT1 in a secretory pathway involving the tissue non-specific alkaline phosphatase (TNAP) enzyme, has been studied. Using Chicken B lymphocyte-derived DT40 cells, TNAP activity significantly declined when the expression of ZnT1 gene along with metallothionein gene were reduced ($ZnT1^{-/-}MT^{-/-}$ cells) (Fujimoto et al., 2013). A previous study in mice reported TNAP enzyme is essential to reduce pyrophosphate and may affect the odontoblast differentiation, dentine matrix secretion, and mineralisation (Foster et al., 2013). However, when the ALP activity was measured in this study (results not shown), this was not significantly different after extracellular exposure of 10 µM and 60 µM $ZnCl_2$ compared with control. Conversely, however a study in children who suffered from hypophosphatasia condition, a rare inherited disease with reduction activity of TNAP enzyme, reported pyrophosphate, likely, does not affect dentine mineralisation of deciduous teeth (van den Bos et al., 2005). Thus, ZnT1 expressed under the condition of this study may not be related with the secretory pathway of ALP enzyme/activity.

ZIP13 expression at mRNA level elevated significantly under extracellular exposure of 10 µM $ZnCl_2$ compared with control after 4-hour time exposure. The response of ZIP13 due to increase of extracellular Zn intake has also been demonstrated in human kidney cells (293T cells), where the expression of ZIP13 at mRNA level increased after the cells treated with 100µM $ZnSO_4$ compared with untreated control within 6 hours. The authors suggested this may be facilitated by: 1) Zn-sensing molecule expressed in plasma membrane, and or 2) ZIP13 activation by increased intracellular excess of Zn into the Golgi (Bin et al., 2011). Instead of Golgi, the endogenous ZIP13 has also been shown to be localised in intracellular vesicle of human cells and the reduction of ZIP13 expression at mRNA and protein level observed in HeLa cells under the condition of Zn deficiency (Jeong et al., 2012).

6.6 Conclusion

This study showed a differential pattern of Zn transporters expression at mRNA level in both human dental pulp and odontoblast-like cells, which may be extracellular Zn exposure and potentially diet. Using human odontoblast-like cell line as a model, ZnT1

and ZIP13 seem to play major roles in maintaining adequate Zn level in these cells which have secretory odontoblast cells capacity, and may be the transporters involved in Zn incorporation within primary dentine formation.

Chapter 7 Summary and final discussion

The trace element, Zn, is obtained from the diet and has essential functions in human metabolism, which is tightly regulated by Zn homeostasis. Zn deficiency, however, is still prevalence in a worldwide, and shows adverse effects in human growth and development, as the risk of Zn deficiency is higher in pregnant women, lactating mothers, and infants groups. Therefore, it is challenging to detect Zn deficiency in early life due to lack of biomarkers that are suitable for estimating Zn levels in high-risk groups.

Since 1930, trace element deposition in human teeth has been studied and related to environmental exposure, such as pollution, nutrition, and migration, as well as diseases. The tooth formation spans from prenatal until several years after birth, thus, the tooth may act as a bio-archive in documenting human life history (Dean, 2017) . Moreover, the tooth has a special characteristic, known as incremental lines that reflects the gradual process of tooth mineralisation, and is valuable for reconstructing a human life history as age may be assigned using these visible lines.

A previous study has explored the possibility of deciduous tooth enamel recording dietary Zn intake during prenatal and postnatal period, but the results indicate that enamel is not suitable for this purpose (Dolphin and Goodman, 2009), and this may be due to oral environmental effects on Zn levels. Zn distribution in the dentine of human deciduous teeth does not reflect heavy metal exposure from the environment in early life and/, as shown by Pb and or Mn, so other factors may be involved in Zn deposition within human deciduous teeth, which may include dietary exposure (Arora et al., 2017). As the variation of Zn concentration was visible in dentine using the LA-ICP-MS analysis (Shepherd et al., 2012), this study aimed to explore the usefulness of dentine in recording Zn exposure in early life nutrition.

As described in Chapter 3, the shortened FFQ was utilised to estimate the dietary Zn/phytate intake of participants recruited in this study. It was important to develop a FFQ for specific purposes applied in certain populations, and in our knowledge, the information on FFQ to estimate dietary Zn intake in Indonesian population was limited. Taken into consideration, this study was hoping to gain information on Zn intake from three different periods (pregnancy, infancy, and at present), a shortened FFQ (28-food

items) completed with food photos to display portion sizes, has been developed from the full FFQ by focusing on Zn-rich foods, and validated in this study. Using the shortened FFQ developed and validated previously, the results showed rice, milk, plant-based protein, and eggs were major contributors for daily Zn intake in pregnant women, infants, and older children. Red meat is a good source for Zn, but it was less frequently consumed compared with rice and plant-based protein, such as tofu, in all groups of this study, as expected. A previous study reported that rice was the most consumed food in Indonesia (Radix et al., 2012). Typically, women who lived in Jakarta urban areas tend to serve high-carbohydrate, low-protein and -fibre foods (Kolopaking et al., 2011). Consistent with the dietary slogan “4 healthy, 5 perfects”, which refers to staple foods, protein sources, vegetables, and fruits (4 food items) that is completed with milk to be perfect (5 food items), this study showed milk was the major contributor of daily Zn intake and the most frequently consumed food during infancy. The early introduction of solid foods in infants is also reported in this study, and is consistent with the fact that approximately 40 per cent of mothers in Indonesia introduced solid foods to their babies within 1 week after birth (Muslimatun and Wiradnyani, 2016). An interviewed-based shortened FFQ, completed with food photos to display portion sizes, which has been developed and validated in this study, appears sufficient to estimate the relative daily Zn intake of the mother during pregnancy, also the children during infancy and at-present in the Indonesian population.

A method in quantifying Pb levels against the chronological age, as a combination of LA-ICP-MS and dental histological analysis, has been developed (Shepherd et al., 2012). The regulation of Pb deposition within dentine of deciduous teeth, however, is thought to be different to the regulation of Zn deposition. Pb has also shown adverse effects in human health (toxic metal) (Arora et al., 2017). Thus, this study also developed and optimised the method as described by Shepherd et al (2012) for measuring Zn levels within dentine of deciduous teeth as described in Chapter 4. The objective of this stage was to determine the number and location of laser ablation transects within dentine to obtain adequate information on Zn levels. Considering the cusp tips as an obvious landmark in the tooth, the first transect was located closer to the tooth axial plane, and the location of other transect was more cervical compared with the previous transect, but both locations included neonatal line, as it acts as the zero point in age assignment. This study showed a good agreement in Zn levels (Zn/Ca ratio) between two transects from different locations of dentine, but there was a

dramatic increase from the ablation area closest to the dental pulp. This finding has also been reported in previous studies and presumably, due to the development of secondary dentine and or immature dentine, as it allows ions exchange easily and/or more Zn binds in dentine matrix proteins of immature dentine (Kierdorf et al., 2016; Shepherd et al., 2012). However, the secondary dentine and/or immature dentine cannot be distinguished using tooth ground section prepared for this study, thus, a statistical analysis was carried out and suggested the dramatic increase of Zn/Ca ratio was significant in ablation pits is located up to 400 µm from pulp (as pulp is the zero point). Therefore, Zn/Ca ratio obtained from the range of 0-400 µm from pulp was excluded for further analysis in this study as it may present as the product of secondary dentine and does not represent the Zn deposited during the formation of primary dentine in early life. A technique of LA-ICP-MS and dental histology applied in this study seems to be a robust technique to analyse Zn content, both in prenatal and postnatal dentine by considering the structure and formation of dentine.

Furthermore, and consistent with Shepherd et al (2012), the ground section of 100 µm (± 20 µm) was sufficient to analyse the important properties of dentine, such as EDJ, DPC, neonatal line, dentinal tubules, and tertiary dentine, as well as daily von Ebner's lines, Andresen lines, and or other accentuated lines, to carry out dental histological analysis. According to the location of the neonatal line, the laser ablation transect that was located closer to the cuspal axial plane, gave significantly more information (i.e. more laser ablations pits to analyse) on Zn distribution during the prenatal period up to 400 µm from pulp. In comparison with Sr level (Sr/Ca ratio), obtained at the same time, the fluctuation of Zn/Ca ratio was more visible compared with Sr/Ca ratio in this study, as Sr is thought to be a substitute for Ca in crystal apatite of mineralised tissue (Cabrera et al., 1999).

In this study, the average trend of Zn/Ca ratio differs to Sr/Ca ratio in dentine, as the metabolism of Zn and Sr is different in humans. The Zn/Ca ratio in dentine, however, reflects the Zn metabolism in human that has been studied before, in respect to pregnancy, lactation, and infancy periods. The highest absorption of Zn occurs during late pregnancy and most of the Zn is transferred to the foetus (more than 57%) through the placenta and stored in foetal hepatic metallothioneins. The Zn/Ca ratio in dentine demonstrated the highest level in late pregnancy, but it then dropped within three months after birth in breast-fed infants, either exclusively breast-fed or not. The 'drop'

of Zn/Ca ratio did not occur in formula-fed infants at the same time, even though the level of Zn/Ca ratio in formula-fed child did not exceed the level of Zn/Ca ratio of breast-fed baby. The reduction of Zn levels in breast milk with time has been reported previously but formula milk is, of course, supplemented with Zn and reaches values of approximately 2-3 times higher than in breast milk. Surprisingly, the Zn/Ca ratio in dentine of formula-fed infant decreased below the level of breast-fed infant at 3-6-month period. A previous study indicated the ability of Zn-deficient individuals to absorb adequate Zn is limited, thus, the efficacy of Zn supplementation will be reduced if a high dose of Zn is administered progressively (Krebs et al., 2014). This phenomenon of the formula-fed infant, however, was not shown in mixed-fed infant (formula- and breast-milk) and the Zn/Ca ratio level in dentine was even lower in infants who had been introduced to the complementary foods at very early stage (less than 6-month old) at 0-3- and 3-6-month period compared with the other groups. In general, when the infants are fed with the complementary foods, the frequency of milk consumption, either breast milk and or formula milk, is possibly reduced which can affect the Zn intake. Furthermore, the absorption of Zn derived from solid food may not be as efficient in a very young infant.

In respect to high- and low-Zn consumers, the Zn/Ca ratio in dentine was higher in high- rather than low-Zn consumer, but it was the inverse relationship for Sr/Ca ratio in dentine at late pregnancy and weaning period. This finding is reasonable as meat, poultry, vegetables and fruits, which are low in Sr but high in Zn, while cereal, grain, and fish contains higher Sr, otherwise lower Zn (Cabrera et al., 1999; Coelho et al., 2017; Dolphin and Goodman, 2009). Interestingly, the Sr/Ca ratio in formula-fed babies increased dramatically at the 3-6-month period compared with other periods, and the similar finding has been reported in the enamel of the formula-fed baby. Formula milk contains a higher concentration of Sr compared with breast milk (Humphrey et al., 2007) . Moreover, Sr contained in formula milk was 4-8 times higher than in breast milk, while the Sr concentration in urine was seven times higher in breast-fed baby compared with formula-fed baby (Widowson et al., 1960).

Shifts in Zn/Ca and Sr/Ca ratio within dentine observed in this study, for the first time, were interpreted by considering Zn and Sr physiology during pregnancy, lactation, and weaning. Therefore, the technique of LA-ICP-MS and dental histology using dentine developed in this study seems to be a robust technique in analysing trace elements,

especially Zn, deposited within dentine. We understand the participants (n=21) recruited in this study is less than the sample size calculated (as described in Chapter 2, section 2.1) to analyse a significant different in Zn levels between low- and high-Zn consumer (expected total n=34, with 10% dropped out), thus the power of study (80%) was not achieved. This study, however, is valuable as a pilot study to analyse the feasibility of dentine in recording Zn exposure in early life nutrition as well as optimisation and validation of the method (LA-ICP-MS and dental histological analysis) to measure Zn levels within dentine of human deciduous teeth. In regard to the result of this study, sample size of 60 participants may able to identify the differences of Zn/Ca ratio between low- and high-Zn consumer (n=30 for each group) during pregnancy and/or infancy. Thus, a research collaboration with larger scope may be conducted to recruit various subjects, who have a differential level of Zn intake during early life, and or a longitudinal study, either retrospective and prospective, to strengthen the results of this study, that was showing the potency of deciduous teeth in recording Zn exposure during early life, by comparing Zn content of deciduous teeth with other tissues, such as plasma/serum of the mother during pregnancy and lactation, child's umbilical cord, also with the Zn dietary intake obtained using FFQ or diet record.

To elucidate the mechanism of Zn deposition within dentine, the expression of Zn transporters at mRNA level were examined in human dental pulp. As Zn transport is not mediated by a passive diffusion mechanism, Zn transporters are essential and may play a role in Zn transport within odontoblast cells, which are located at the periphery of dental pulp, during dentine mineralisation. A very recent study using hDPCs, differentiated into the secretory stage, showed the vitality, osteogenic differentiation, and mineralisation capacity of the cells under extracellular Zn exposure, alongside an increased in ZnT1 and ZIP1 expression at mRNA level (An et al., 2017). In this study, total RNA was successfully extracted from five deciduous teeth originating from five different children. The expression of ZnT1, ZnT5, and ZnT9 at the mRNA level was demonstrated in this extracted RNA. As the dietary Zn intake of the recruited children in this study was not controlled, the differential expression of Zn transporters at mRNA level between samples is expected since Zn homeostasis is tightly controlled to maintain adequate Zn levels in human body (Kambe et al., 2015) The expression of Zn transporters may also be regulated by other stimuli, such as hormones, cytokines,

endoplasmic reticulum stress, oxidative stress, and hypoxia in a specific cell and tissue (Hara et al., 2017).

As a whole tissue, human dental pulp, accommodates not only odontoblast cells, but also other cells, such as sub-odontoblastic and stromal fibroblast cells, as well as neuronal, immune and vascular system cells (Goldberg, 2014) and therefore it is difficult to assay specific Zn transport attributed to each cell type in the pulp. To model Zn uptake in odontoblasts-like cells specifically an investigation was carried out using hDPCs differentiated into the secretory stage to analyse the expression of Zn transporters at mRNA level in response to extracellular Zn exposure, mimicking dietary Zn intake. ZnT1 up-regulation was observed after 24 hours of extracellular 10 µM and 60 µM $ZnCl_2$ exposure, while the expression of ZIP13 at mRNA level increased after only 4 hours of 60 µM $ZnCl_2$ exposure and it also increased significantly under 10 µM $ZnCl_2$ exposure from 4 hours to 24 hours. In regard to the role of ZnT1, this study indicates an interaction between ZIP13 and ZnT1 in odontoblast-like cells under extracellular Zn exposure. As well as the role of ZnT1 in transporting Zn into extracellular space during dentine mineralisation, it may regulate Zn deposition within dentine. In future work, localisation of Zn transporters in odontoblast cells by immunohistochemistry may provide more information on their function.

In conclusion, the work presented in this study has demonstrated, for the first time, the usefulness of dentine from human deciduous teeth for recording early life exposure of Zn nutrition. As a pilot, this study revealed that the Zn/Ca ratio in dentine of human deciduous teeth may reflect the dietary Zn intake during pregnancy and the infancy period reflecting Zn metabolism in humans. In the future, more studies in this field are expected to strengthen these findings, and it may be useful to determine the reference dietary intake (RDI), especially during pregnancy, lactation, and infancy groups, as they have a higher risk for mild to moderate Zn deficiency for children to develop and reach an optimum growth. Furthermore, this study has made a novel contribution in analysing Zn transporter expression at the mRNA level in human dental pulp and odontoblast-like cells, which may be regulated by dietary Zn intake, and may play a role in Zn deposition within dentine of human deciduous teeth.

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Appendix A Consent form, general questionnaire, long FFQ, shortened FFQ, Zn and Phytate content

CONSENT FORM

I have read the information about the study of “Exploring the Levels and Distribution of Zinc in the Deciduous Tooth as a Tool to Record Early Life Exposure to Zinc Nutrition”, and or the investigator has verbally explained it to me, and I have been given the opportunity to ask questions and discuss with the team of investigator. I understand the aims, benefits, and procedures for this study. Therefore, with mindfulness and without any forces from other parties, I agree for my child to donate his/her extracted deciduous teeth for this study and me to provide information on dietary intake during pregnancy as well as my child’s dietary intake during infancy and at-present.

Mother’s name : Age: (y.o.)

Child’s name : Age: (y.o.)

Address :

Phone number :

Jakarta,

Signature of mother

( )

General Questionnaire

EXPLORING THE LEVELS AND DISTRIBUTION OF ZINC IN THE DECIDUOUS TOOTH AS A TOOL TO RECORD EARLY LIFE EXPOSURE TO ZINC NUTRITION

Personal Information **Code:**

CHILD		**FAMILY**	
Name		Father's Name	
Gender *(tick one of the answer)*	☐ MALE / ☐ FEMALE	Education Level	School/College/University
Date of Birth		Occupation	
Place of Birth		Mother's Name	
Nationality		Education Level	School/College/University
School		Occupation	
Body Weight	_____ kg	Number of Children in family	_____person(s), and the child is number _____
Body height	_____cm		
Childhood Illnesses *(tick one of the answer)*	☐ YES ☐ NO if yes, please to mention it below: _____	Home Address	
Disabilities *(tick one of the answer)*	☐ YES ☐ NO if yes, please to mention it below: _____	Phone / Mobile Phone	
		Annual Household Expense/month *(tick one of the answer)*	☐ < £10,000 ☐ £10,000 - £20,000 ☐ £20,000 - £40,000 ☐ >£50,000

- **PRENATAL AND BIRTH HISTORY -**

1. Mother age during pregnancy	_____y.o.
2. Morning sickness *(tick one of the answer)*	☐ YES ☐ NO
3. Complication during pregnancy (admitted to hospital) *(tick one of the answer)*	☐ YES ☐ NO If YES, details_____
4. Medication/supplement (eg. Folic acid) during pregnancy *(tick one of the answer)*	☐ YES ☐ NO If YES, please mention the type of medication/supplement that was taken: _____ and duration _____week(s)/ month(s)
5. Gestational Age *(tick one of the answer)*	☐ Pre- term, _____ week(s)/ month(s) ☐ At- term, _____ week(s)/ month(s)
6. Labour and Delivery Method *(tick one of the answer)* ☐ Vaginal Birth ☐ Vaginal Birth with intervention (forceps/ventouse) ☐ Caesarian Section	7. Baby weight at birth _____ kg

- POST NATAL HISTORY -

1.	**Feeding Pattern** *(tick one of the answer)*	☐ **Exclusive Breastfeeding,** (please continue to question number 2) ☐ **Mixed Breastfeeding and Formula** (please continue to question number 3) ☐ **Formula** (please continue to question number 4)

2.	**Exclusive Breastfeeding :** From _____week/month to _____week/month

3.	**Mixed Breastfeeding and Formula** **A. Breastfeeding :** From _____week/month to _____week/month **B. Formula :** From _____week/month to _____week/month

4.	**Formula :** From _____week/month to _____week/month

- WEANING HISTORY -

1.	**Age of the baby when you started weaning**	_____month/year
2.	**The first food for your child**	☐ **Homemade baby food,** that was _____ ☐ **Commercially available baby food,** that was _____
3.	**Age of the child when he/she was fully weaned (eating solid food)**	_____month/year

-Vitamin/Supplement for the Child-

Do you routinely give a supplement to your child? *(tick one of the answer)*	☐ YES ☐ NO If YES, please mention the name or content of the supplement : _____
Do you routinely give a vitamin to your child? *(tick one of the answer)*	☐ **YES** *(you can choose more than one option below)* ☐ Multivitamin + Mineral ☐ Certain Vitamin, details :_____ ☐ **NO**

Long -FFQ

HOW OFTEN DOES YOU/YOUR CHILD EAT DURING PREGNANCY / INFANCY / AT-PRESENT: *(cross one of answer)*																
	NEVER	**EVERY MONTH**			**EVERY WEEK**						**EVERY DAY**			**PORTION**			
		1	2	3	1	2	3	4	5	6	1	2	3	A	B	C	D
Rice and cereals																	
Rice																	
Brown rice																	
White bread																	
Wholegrain bread																	
Pasta																	
Breakfast cereal																	
Oatmeal																	
Noodle																	
Red meat																	
Beef																	
Beef sausage/meatballs																	
Cow's offal																	
Veal																	
Veal sausage/meatballs																	
Lamb																	

	NEVER	EVERY MONTH			EVERY WEEK						EVERY DAY			PORTION			
		1	2	3	1	2	3	4	5	6	1	2	3	A	B	C	D
Lamb sausage																	
Lamb's offal																	
White meat																	
Chicken																	
Chicken sausage/nugget																	
Chicken's offal																	
Pork																	
Pork sausage/ham																	
Fish and Seafood																	
Mussels																	
Prawn																	
Crab																	
Squid																	
Fresh-water fish																	
Salt-water fish																	
Eggs														A half egg	1 egg	2 eggs	3 eggs

	NEVER	EVERY MONTH			EVERY WEEK						EVERY DAY			PORTION			
		1	2	3	1	2	3	4	5	6	1	2	3	A	B	C	D
Dairy																	
Yogurt																	
Cheese																	
Milk														<250ml	250	>250ml	
Plant-based protein																	
Tofu																	
Tempeh																	
Kidney beans																	
Mung beans																	
Soy beans																	
Peanuts																	
Cashew																	
Fruits																	
Avocado																	
Apple																	
Guava																	
Orange																	

	NEVER	EVERY MONTH			EVERY WEEK						EVERY DAY			PORTION			
		1	2	3	1	2	3	4	5	6	1	2	3	A	B	C	D
Mango																	
Pineapple																	
Banana																	
Melon																	
Watermelon																	
Papaya																	
Strawberry																	
Grapes																	
Mangosteen																	
Kiwi																	
Apricot																	
Peaches																	
Snake fruit																	
Vegetables																	
Broccoli																	
Spinach																	
Mushroom																	

	NEVER	EVERY MONTH			EVERY WEEK						EVERY DAY			PORTION			
		1	2	3	1	2	3	4	5	6	1	2	3	A	B	C	D
Cabbage																	
Chinese leaf																	
Pak choy																	
Eggplant																	
Pumpkin																	
Bitter gourd																	
Green beans																	
Beansprout																	
Long beans																	
Peas																	
Jackfruit																	
Radish																	
Beetroot																	
Lettuce																	
Watercress																	
Kale																	
Asparagus																	
Zucchini																	

	NEVER	EVERY MONTH			EVERY WEEK						EVERY DAY			PORTION			
		1	2	3	1	2	3	4	5	6	1	2	3	A	B	C	D
Bell pepper																	
Cauliflower																	
Kangkong																	
Cassava leaves																	
Cucumber																	
Potato																	

Shortened -FFQ

HOW OFTEN DOES YOU/YOUR CHILD EAT DURING PREGNANCY / INFANCY / AT-PRESENT: *(cross one of answer)*																
	NEVER	**EVERY MONTH**			**EVERY WEEK**						**EVERY DAY**			**PORTION**			
		1	2	3	1	2	3	4	5	6	1	2	3	A	B	C	D
Protein source																	
Beef /lamb																	
Beef sausage/meatball																	
Chicken																	
Chicken nugget																	
Eggs														A half egg	1 egg	2 eggs	3 eggs
Cow/Chicken's offal																	
Salt-water fish																	
Fresh water fish																	
Seafood																	
Tofu																	
Tempeh																	
Rice and cereals																	
Rice																	
White bread																	
Pasta/noodle																	
Breakfast cereal																	

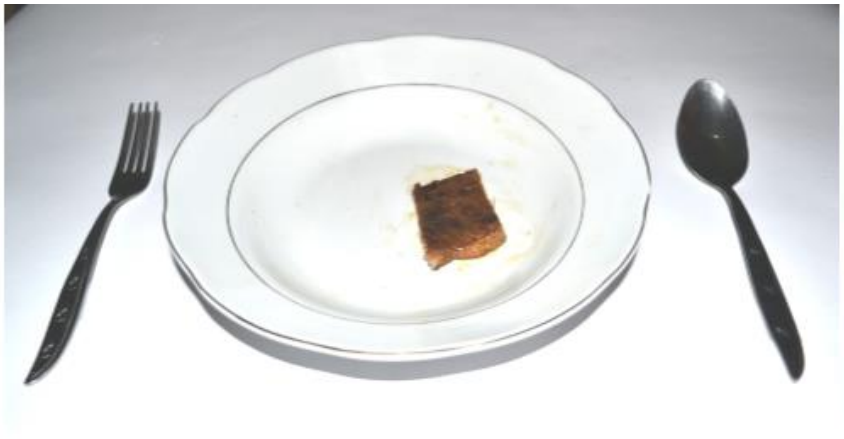
	NEVER	EVERY MONTH			EVERY WEEK						EVERY DAY			PORTION			
		1	2	3	1	2	3	4	5	6	1	2	3	A	B	C	D
Beans and nuts																	
Long beans																	
Mung beans																	
Peanuts																	
Vegetables & fruits																	
Corn																	
Potato																	
Spinach																	
Mushroom																	
Broccoli																	
Other vegetables (carrot, cabbage, etc)																	
Avocado																	
Dairy																	
Milk														<250ml	250	>250ml	
Cheese																	
Yogurt																	

Zn and Phytate content of foods listed in shortened FFQ

		Zn content (mg/100 g)	Phyate (mg/100 g)	Portion 1 (g)	Portion 2 (g)	Portion 3 (g)	Portion 4 (g)
1	Beef/lamb	5.00	0.00	20.00	40.00	80.00	100.00
2	Beef sausage/meatball	2.16	0.00	30.00	45.00	75.00	90.00
3	Chicken	2.00	0.00	30.00	60.00	120.00	150.00
4	Chicken nugget	2.00	0.00	15.00	30.00	60.00	90.00
5	Eggs	0.8/pcs	0.00	0.80	1.60	2.40	3.20
6	Offal	4.4	0.00	40.00	80.00	120.00	160.00
7	Salt-water fish	0.4	0.00	25.00	50.00	100.00	125.00
8	Fresh-water fish	0.8	0.00	25.00	50.00	100.00	125.00
9	Seafood	2.3	0.00	12.00	25.00	37.50	50.00
10	Tofu	2.3	314.00	50.00	100.00	150.00	200.00
11	Tempe	1.25	89.5	10.00	40.00	60.00	80.00
12	Rice	0.35	33.60	50.00	100.00	150.00	200.00
13	White Bread	1.00	0.00	12.50	25.00	50.00	75.00
14	Pasta/noodle	0.43	12.47	30.00	60.00	90.00	130.00
15	Breakfast cereal	2.3	0.00	10.00	20.00	30.00	40.00
16	Long beans	0.67	25.24	25.00	50.00	75.00	100.00
17	Mung beans	0.9	121.00	50.00	75.00	100.00	125.00
18	Peanuts	3.27	2.95	25.00	50.00	100.00	125.00
19	Corn	1.00	152.00	37.5	75.00	112.5	150.00
20	Potato	0.3	60.90	25.00	50.00	75.00	100.00
21	Spinach	0.7	8.91	30.00	60.00	120.00	
22	Mushroom	0.5	0.00	35.00	70.00	105.00	140.00
23	Broccoli	0.6	0.00	40.00	80.00	120.00	160.00
24	Other vegetables (carrot, cabbage, etc)	0.25	0.00	25.00	50.00	100.00	125.00
25	Avocado	0.45	0.00	50.00	75.00	150.00	200.00
26	Milk	1 mg/250ml	0.00	125.00	250.00	>250	
27	Cheese	3.75	0.00	30.00	60.00	90.00	120.00
28	Yogurt		0.00	100ml	125ml	250ml	

Appendix B Food-portion size photographs

1. Red Meat



A

B

C

D

2. Meatballs

A

B

C

D

3. Chicken

A

B

C

D

4. Chicken Nugget

A

B

C

D

5. Salt-water fish

A

B

C

D

6. Fresh water fish

A

B

C

7. Seafood

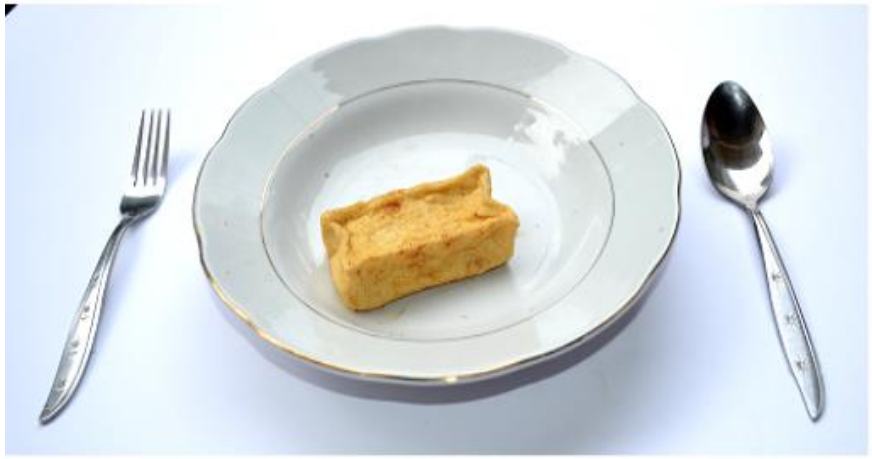
A

B

C

D

8. Tofu



A

B

C

D

9. Tempeh

A

B

C

D

10. Rice

A

B

C

D

11. White bread

A

B

C

D

12. Pasta/noodle

A

B

C

D

13. Breakfast cereal

A

B

C

D

14. Long bean

A

B

C

D

15. Mung beans

A

B

C

D

16. Peanuts

A

B

C

D

17. Corn

A

B

C

D

18. Potato

A

B

C

D

19. Spinach

A

B

C

20. Mushroom

A

B

C

D

21. Broccoli

A

B

C

D

22. Other vegetables

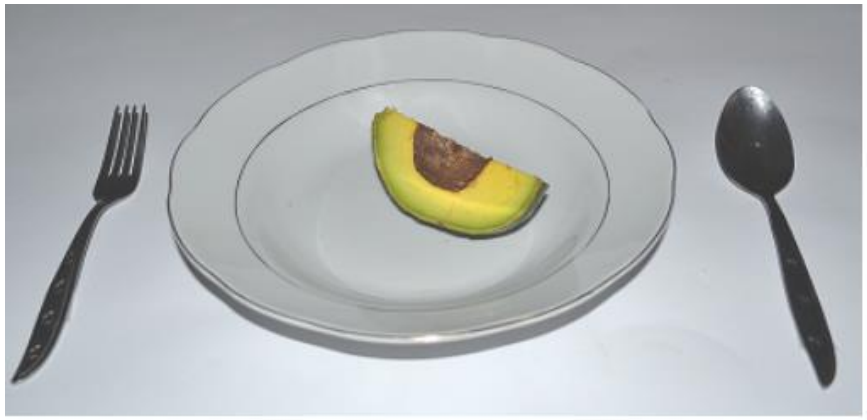
A

B

C

D

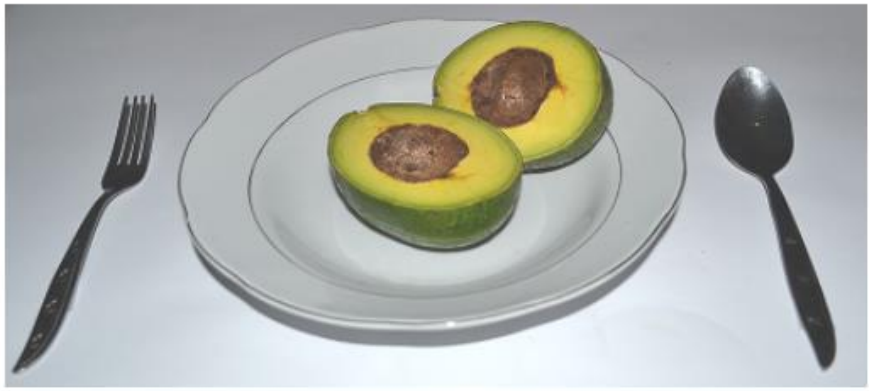
23. Avocado



A

B

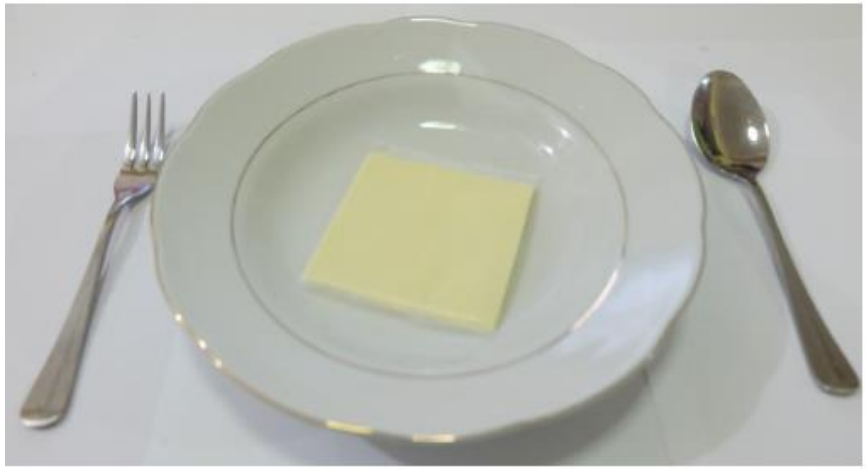
C



D

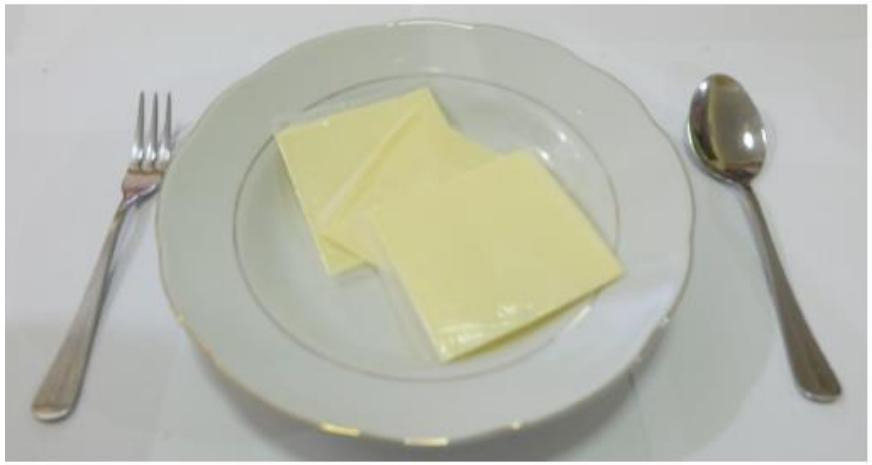
24. Milk (250 ml)

25. Cheese

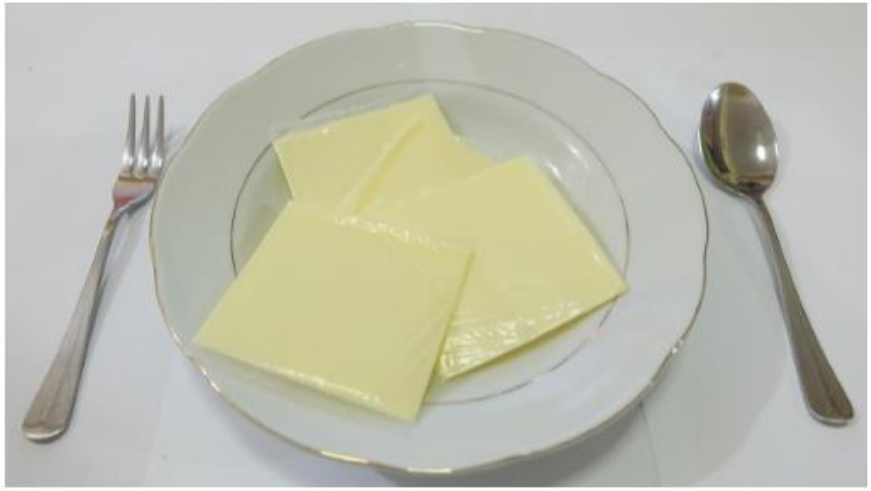


A

B



C



D

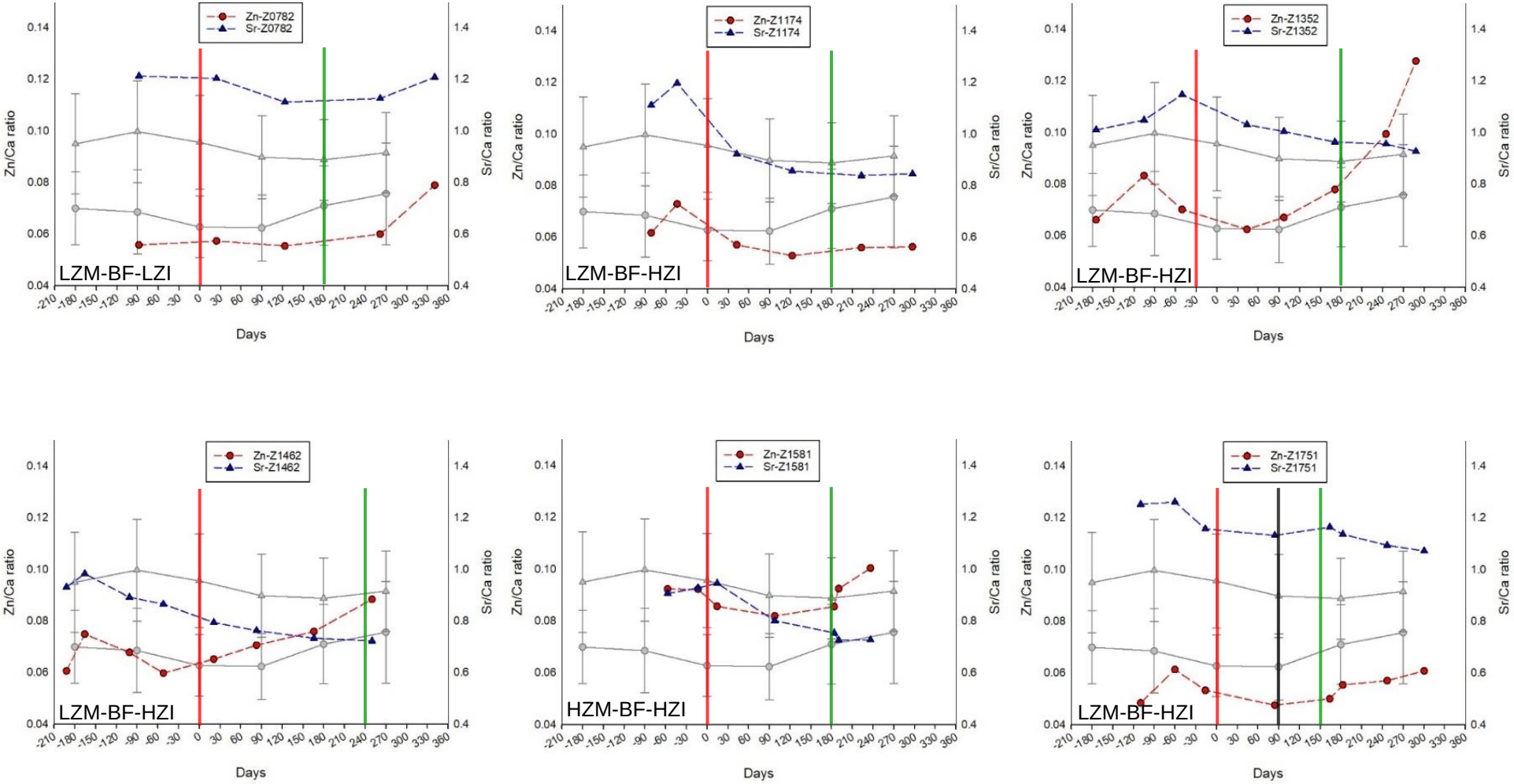
26. Yogurt

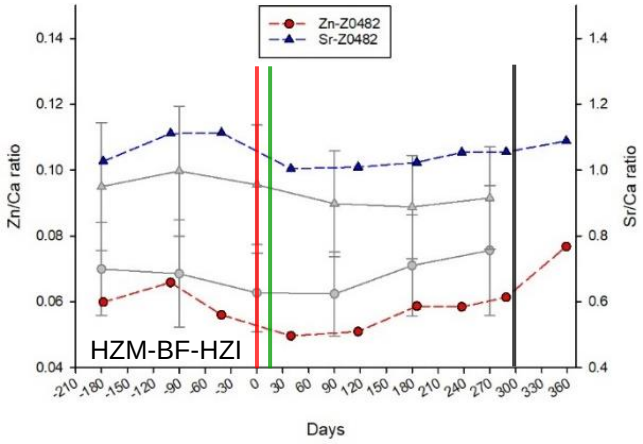
A

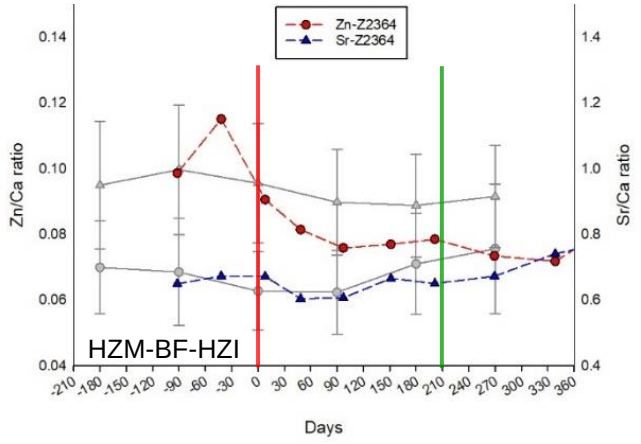
B

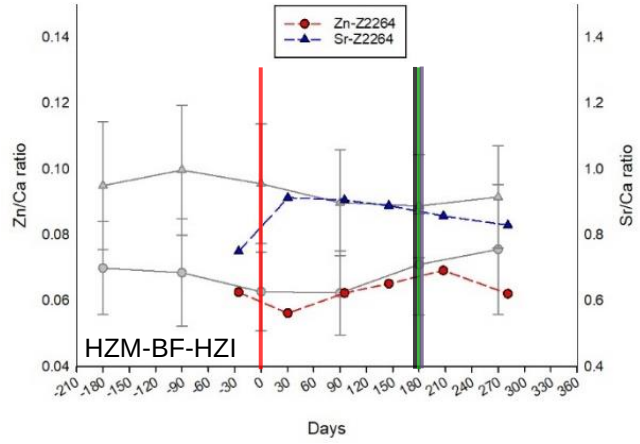
C

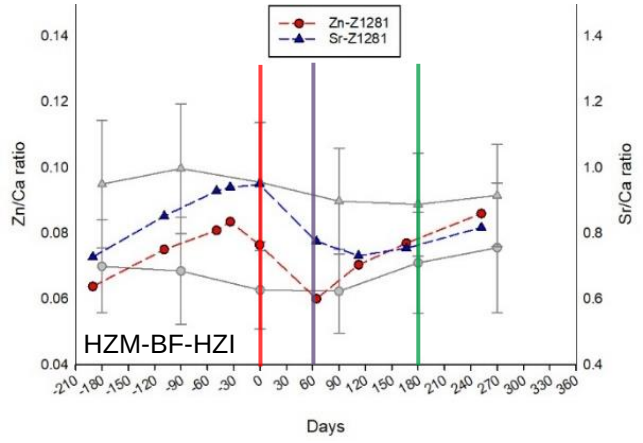
Appendix C Individual trend of Zn/Ca and Sr/Ca ratio

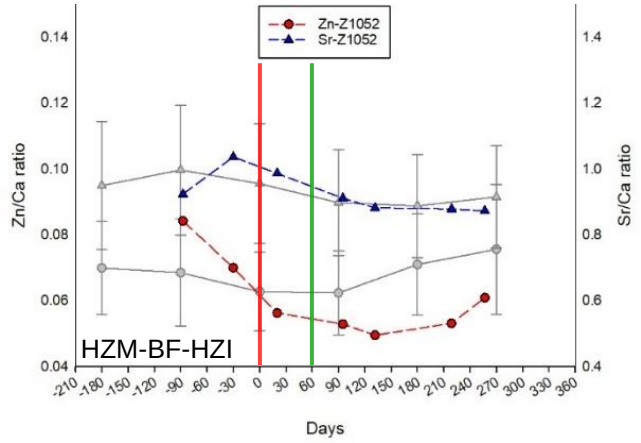


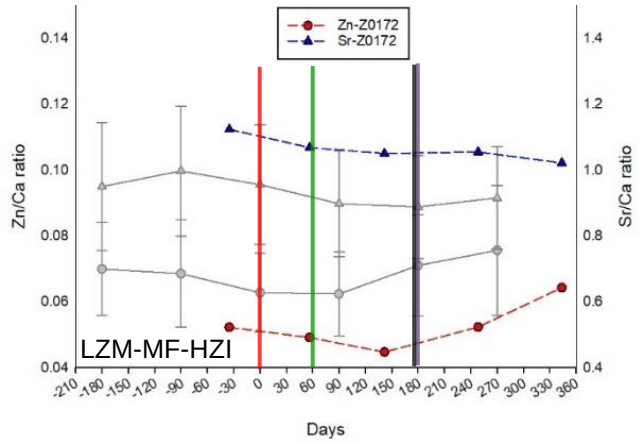

Zn-Z0482
Sr-Z0482
HZM-BF-HZI
Zn/Ca ratio
Sr/Ca ratio
Days

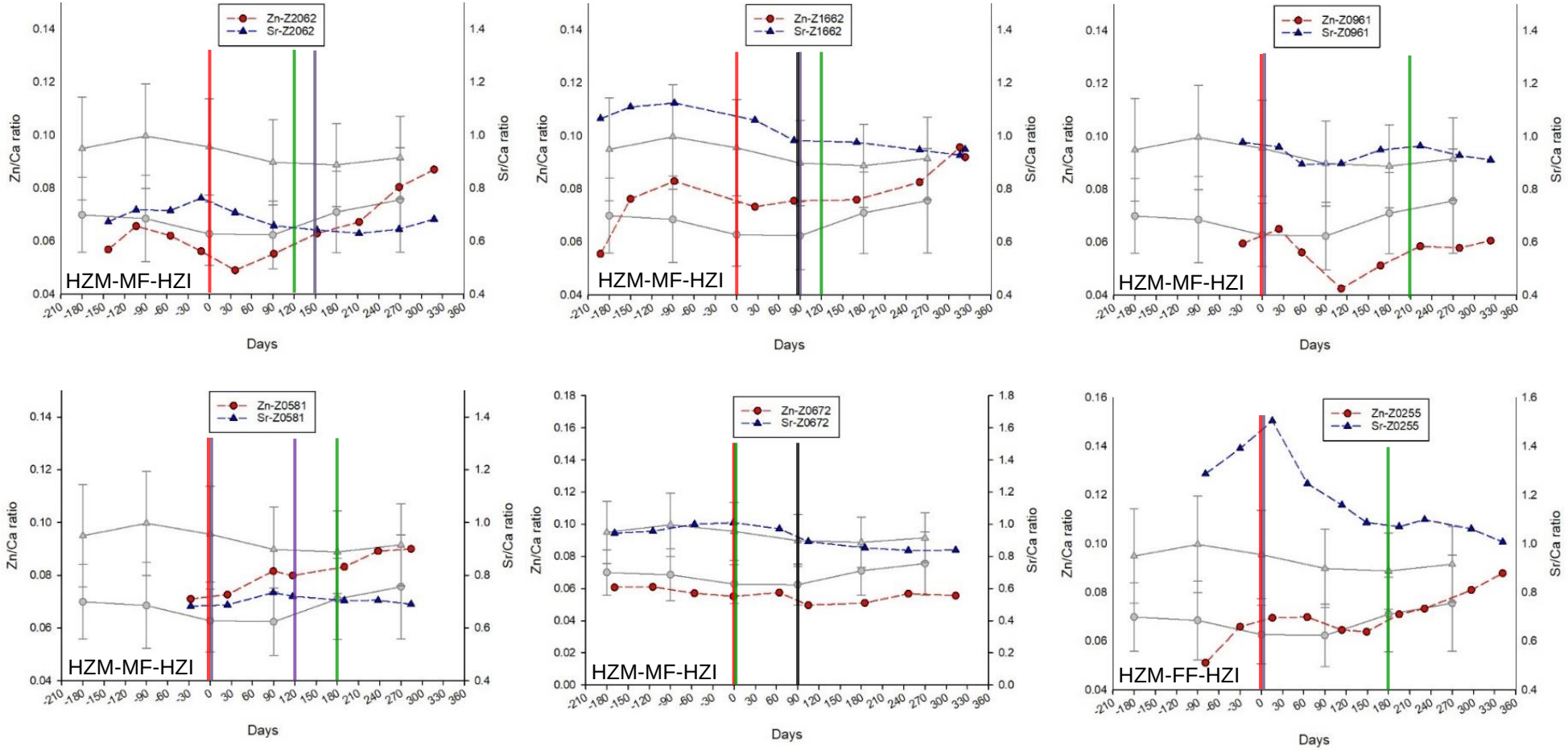

Zn-Z2364
Sr-Z2364
HZM-BF-HZI
Zn/Ca ratio
Sr/Ca ratio
Days


Zn-Z2264
Sr-Z2264
HZM-BF-HZI
Zn/Ca ratio
Sr/Ca ratio
Days


Zn-Z1281
Sr-Z1281
HZM-BF-HZI
Zn/Ca ratio
Sr/Ca ratio
Days


Zn-Z1052
Sr-Z1052
HZM-BF-HZI
Zn/Ca ratio
Sr/Ca ratio
Days


Zn-Z0172
Sr-Z0172
LZM-MF-HZI
Zn/Ca ratio
Sr/Ca ratio
Days



Legends

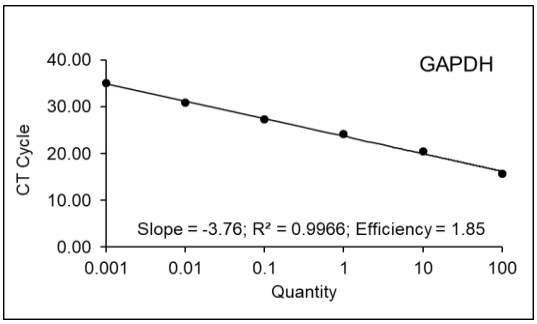
Vertical lines: red = birth as day 0,
brown = the beginning of formula milk,
green = the introduction of solid foods
purple = end of formula milk
black = end of breast feeding

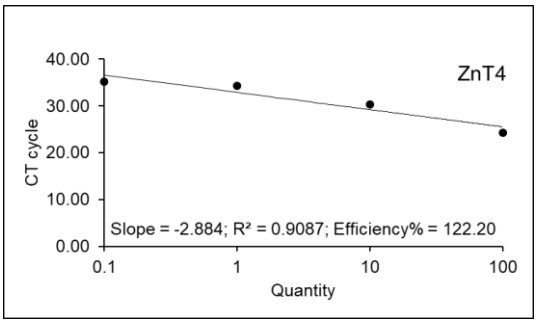
HZM = High-Zn-consumer mother
LZM = Low-Zn-consumer mother
BF = Breast-fed
MF= Mixed-fed
FF = Formula-fed

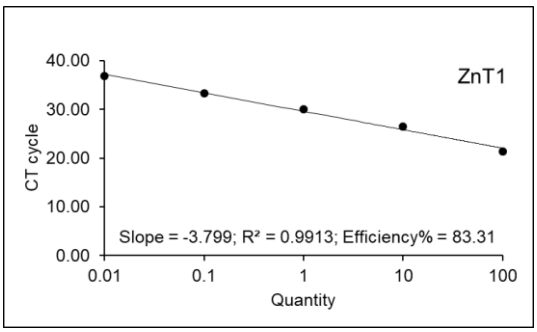
HZI = High-Zn-consumer infant
LZI = Low-Zn-consumer infant

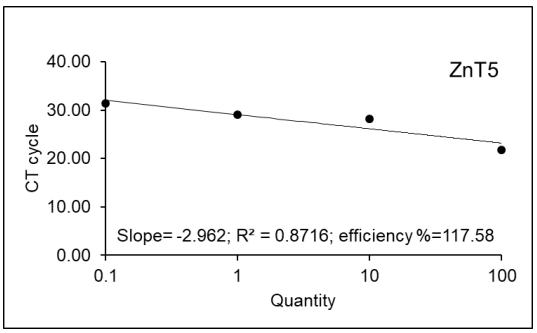
Appendix D Standard curves generated to measure relative levels of ZnTs, ZIPs, GAPDH, and 18S at mRNA level

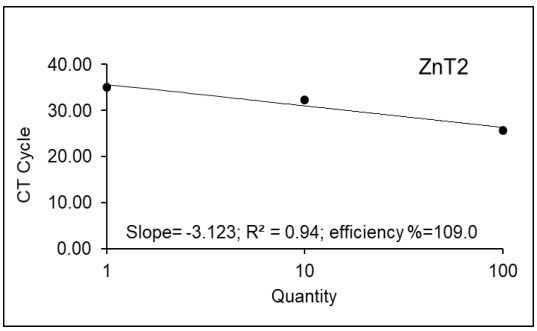
Standard curves generated to measure relative levels of ZnTs, GAPDH, and 18S at mRNA level in human dental pulp tissue using SYBR green fluorescence and the DNA Engine Opticon 2 (MJ Research)

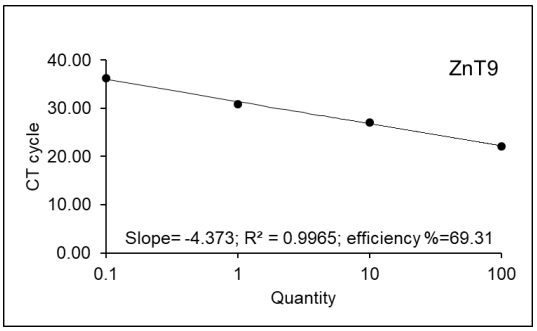


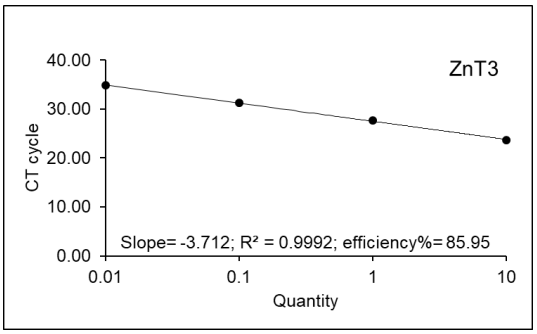


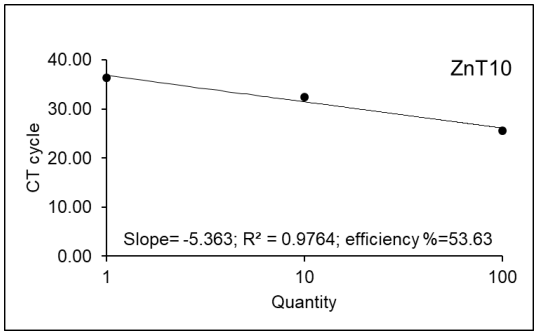




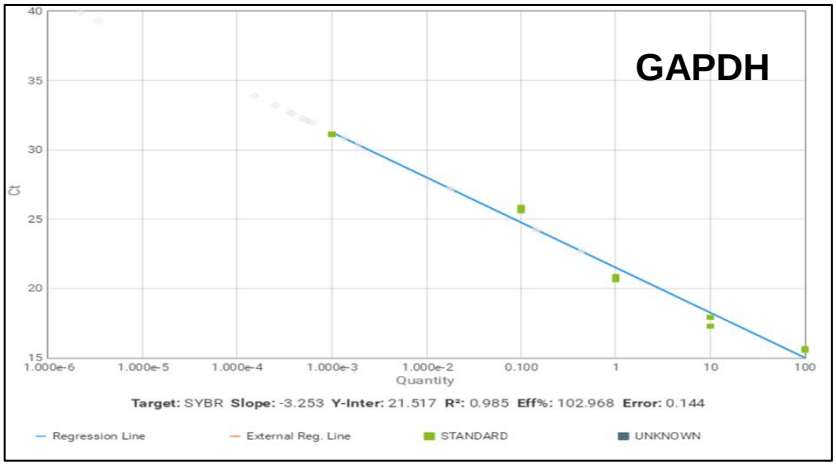


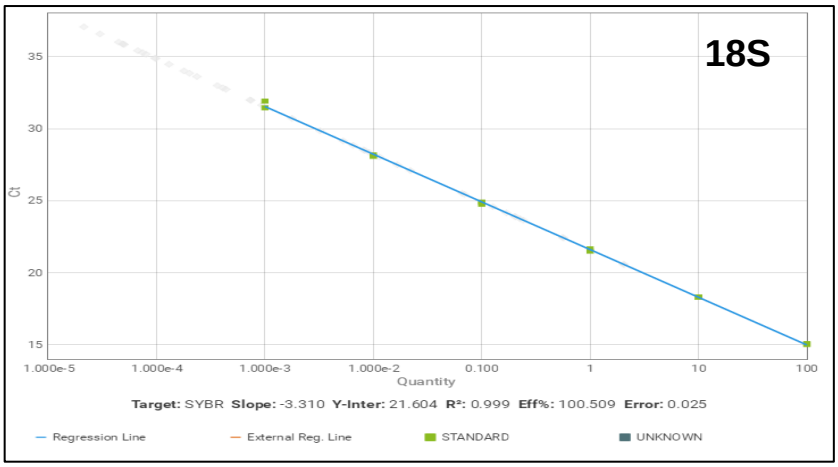


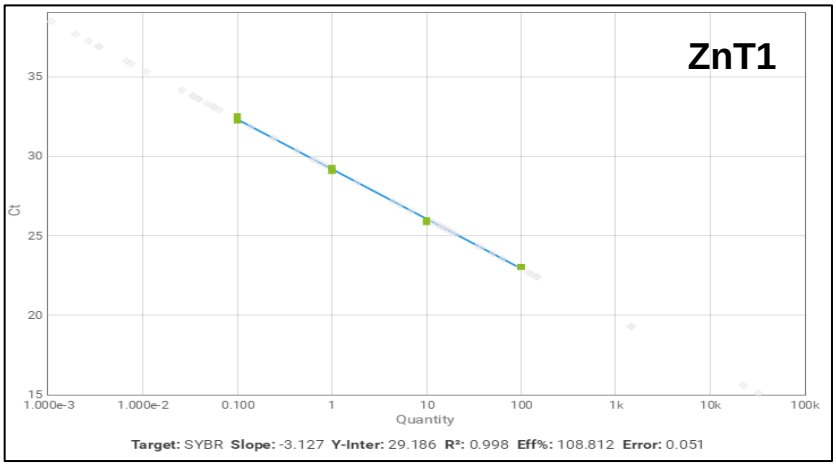


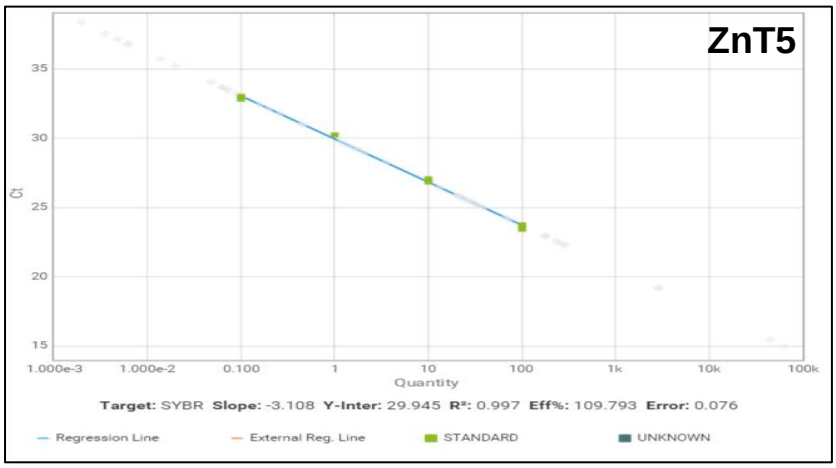


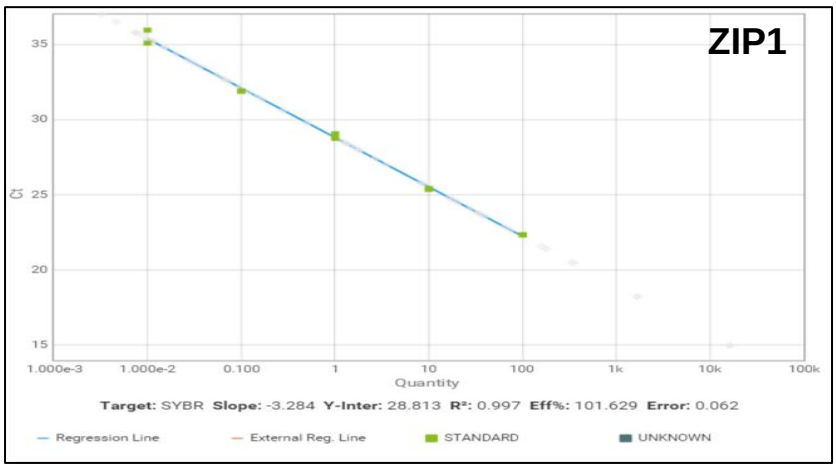
Standard curves generated to measure relative levels of ZnTs and GAPDH and 18S (housekeeping gene) at mRNA level in differentiated HDP-hTERT cells using SYBR green fluorescence and the real-time PCR system QuantStudio 3 (Thermofisher)

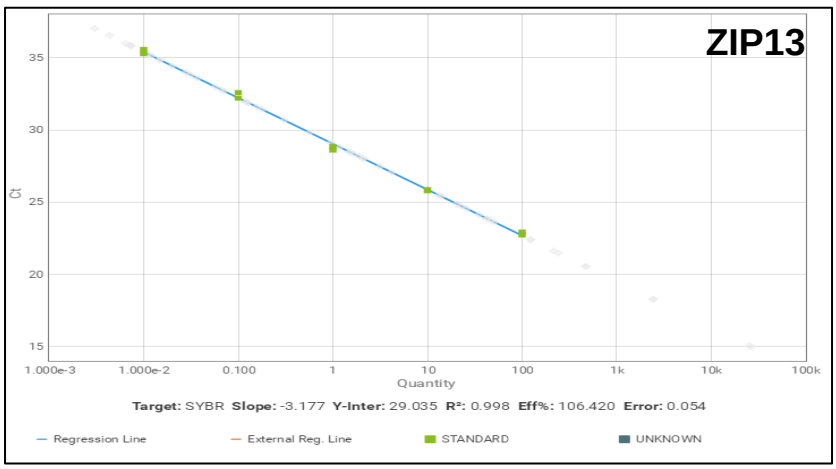


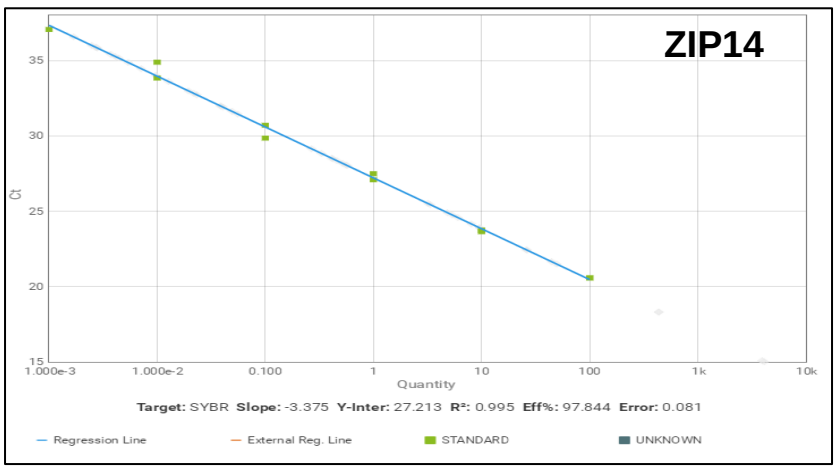




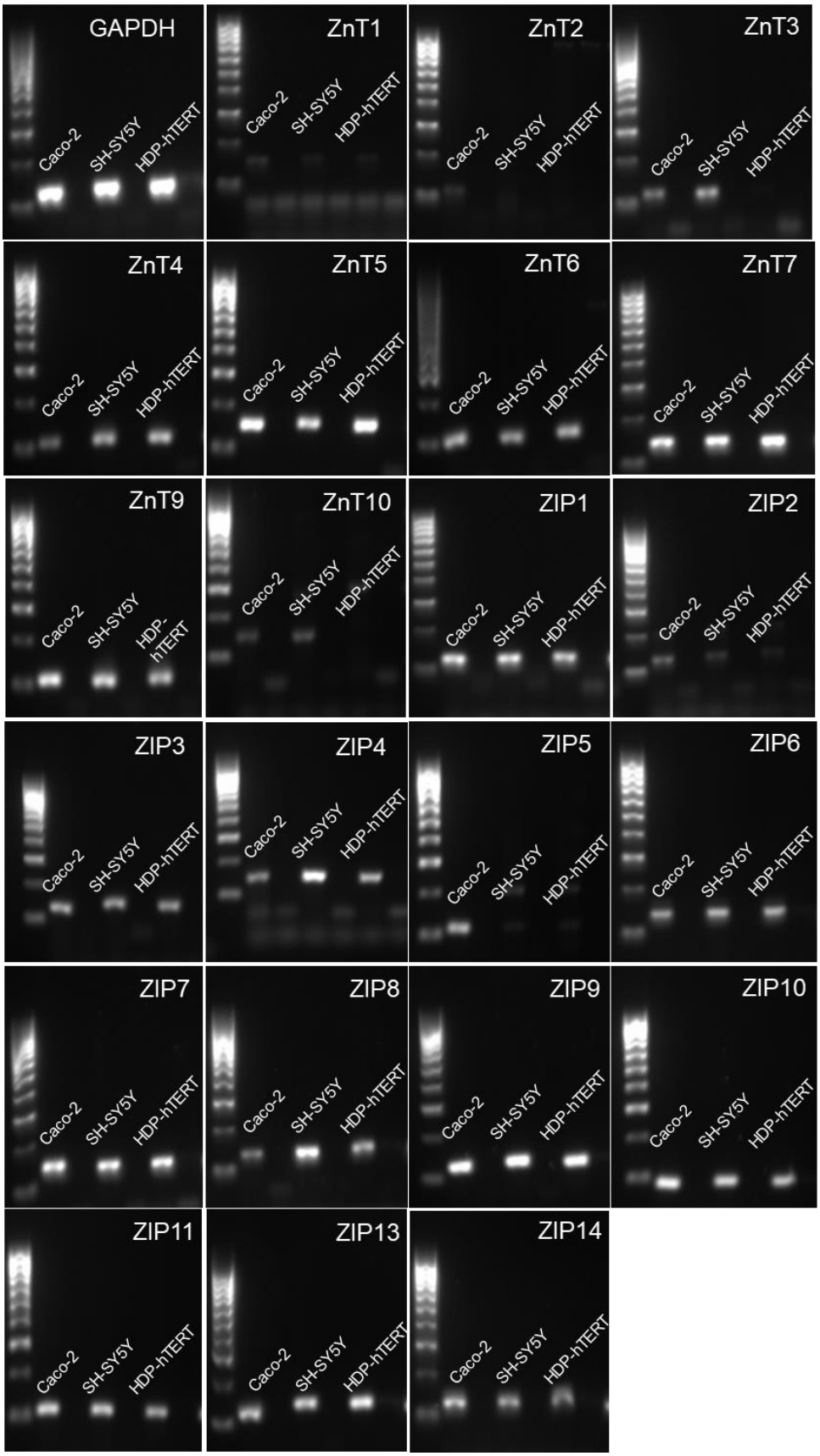

ZnT5
Ct
Quantity
1.000e-3
1.000e-2
0.100
1
10
100
1k
10k
100k
Target: SYBR Slope: -3.108 Y-Inter: 29.945 R²: 0.997 Eff%: 109.793 Error: 0.076
Regression Line
External Reg. Line
STANDARD
UNKNOWN


ZIP1
Ct
Quantity
1.000e-3
1.000e-2
0.100
1
10
100
1k
10k
100k
Target: SYBR Slope: -3.284 Y-Inter: 28.813 R²: 0.997 Eff%: 101.629 Error: 0.062
Regression Line
External Reg. Line
STANDARD
UNKNOWN


ZIP13
Ct
Quantity
1.000e-3
1.000e-2
0.100
1
10
100
1k
10k
100k
Target: SYBR Slope: -3.177 Y-Inter: 29.035 R²: 0.998 Eff%: 106.420 Error: 0.054
Regression Line
External Reg. Line
STANDARD
UNKNOWN


ZIP14
Ct
Quantity
1.000e-3
1.000e-2
0.100
1
10
100
1k
10k
Target: SYBR Slope: -3.375 Y-Inter: 27.213 R²: 0.995 Eff%: 97.844 Error: 0.081
Regression Line
External Reg. Line
STANDARD
UNKNOWN

Appendix E ZnTs and ZIPs expression in Caco-2, SH-SY5Y, and HDP-hTERT cells



Appendix F Element ion intensities (cps)

Sample	Pit	Ca40	Zn66	Mg24	K39	Mn55	Fe56	Cu63	Sr88	Ba137	Pb208
Z0172S	i-1	364849780	19051	2105681	29931	2973	1845	259	410069	2096	1796
Z0172S	i-2	415480454	20405	1969677	37448	3542	1428	372	443825	2886	1586
Z0172S	h-1	428878465	19169	1849097	36983	2682	2374	1107	450379	3238	1800
Z0172S	h-2	396603101	20749	1400404	27018	1813	3623	938	418441	4251	2188
Z0172S	h-3	400299155	25731	1385470	23571	1913	7190	1860	409040	5104	3354
Z0172S	h-4	380252773	29440	1340245	20437	2636	7191	2378	364905	5005	5108
Z0172S	h-5	394489468	35201	1399205	18992	1980	7306	1925	368203	5457	6075
Z0172S	h-6	362780353	62370	1228309	19896	4962	16681	3935	371216	6242	18578
Z0172X	l-1	427294013	26504	2024931	37607	4814	7216	4140	501998	3550	3725
Z0172X	l-2	483382194	26508	2239727	44625	5238	3719	1416	537228	3950	4038
Z0172X	k-1	412208556	23819	1623396	35833	2929	4381	2016	457067	3903	3896
Z0172X	k-2	405608169	24405	1340665	28930	2203	4145	1414	440696	4960	3751
Z0172X	k-3	402806095	30072	1347381	24202	2671	6893	2316	409121	5409	5463
Z0172X	k-4	407665386	33913	1370454	22144	2752	10042	3178	400532	6053	7212
Z0172X	k-5	403683157	39768	1320087	21321	3018	10896	3384	396740	6375	10031
Z0172X	k-6	401030718	84326	1297975	22761	9392	31550	6929	444843	7817	40247
Z0255S	c-1	375265082	18836	2322239	52598	3150	1200	516	482905	2595	1738
Z0255S	c-2	367207635	23417	2430960	57455	4548	785	434	510731	3087	2089
Z0255S	c-3	371425441	24934	2455993	59062	3130	781	561	558783	3752	1867
Z0255S	c-4	371288917	25026	2430605	58584	2414	1313	1182	463006	3312	1650
Z0255S	c-5	361598326	22604	2521524	56996	1295	1193	995	419072	2925	1527
Z0255S	c-6	354501879	21938	2398060	58095	868	1449	914	385538	2462	1665
Z0255S	d-1	357506777	24466	2474203	53243	787	1602	1011	382553	2436	1922
Z0255S	d-2	352813842	24901	2451266	51022	720	1535	1065	387765	2497	2404
Z0255S	d-3	338070528	26216	2493822	46593	517	1554	1366	358679	2321	2486
Z0255S	d-4	348655651	29252	2548921	46345	602	3580	2220	351239	3229	2909
Z0255S	d-5	349970802	32555	2404347	44645	812	2668	2398	382242	2983	3992
Z0255S	d-6	349483831	39890	2277138	41729	1305	6189	4177	450856	3648	4861
Z0255S	e-1	355230248	48482	2202481	39291	1777	9006	6351	479211	4968	6401
Z0255S	e-2	332765214	69413	1725237	33250	2821	19928	10507	500145	7148	13575
Z0255S	e-3	335922251	110890	1614134	35713	3536	25866	13590	567539	10075	41822
Z0255X	h-1	319945822	17511	1376790	44011	1105	562	907	483716	3215	2005
Z0255X	h-2	300850456	18131	1290128	45880	1846	363	484	412916	2900	1304
Z0255X	g-1	322991858	17765	1566367	55530	1716	436	441	401422	2685	1122
Z0255X	g-2	315275865	19254	1737711	52557	535	378	514	365112	2060	1625
Z0255X	g-3	326682175	22944	1895082	51774	385	407	319	369060	1984	2283
Z0255X	g-4	339797987	27485	2245711	52339	351	470	523	387533	3122	2685
Z0255X	g-5	341906882	32981	2219178	48332	632	744	844	460854	2901	3848
Z0255X	g-6	338729826	62517	2162861	39787	976	3680	1643	515849	4079	11347
Z0255X	g-7	342705401	116591	1772903	42716	2310	16706	7149	646345	8202	58337
Z0482S	a4	222126341	13302	2386248	20659	3230	1171	272	228031	704	1948
Z0482S	a5	216188671	14237	2483338	15951	4097	1897	584	240269	949	2141
Z0482S	a6	228812593	12816	2272684	14147	3487	1997	1272	254748	1409	2334
Z0482S	a7	233840184	11605	1924029	16778	2169	1770	1593	234874	1909	2357
Z0482S	a8	242316236	12345	2060792	25479	1971	2390	1774	244563	2024	3777
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Z0482S	b1	244475162	14285	2129081	27887	2504	2435	2173	257648	2358	5991
Z0581S	a2	127047982	9021	577542	21525	3027	825	170	86714	1167	562
Z0581S	a3	126167206	9167	655922	14067	2474	556	97	86727	444	713
Z0581S	a4	115095072	9391	785405	11868	1691	657	126	84453	437	650
Z0581S	a5	110555365	8832	1111122	9614	923	414	111	79639	460	510

Z0581S	a6	117611069	9780	1361670	9977	656	972	95	82818	521	578
Z0581S	a7	112986213	10074	1173937	9552	485	887	220	79631	625	666
Z0581S	a8	110678935	9960	977397	8556	471	703	149	76343	663	557
Z0581S	b1	113371148	11998	569318	9008	435	1164	189	83378	1075	938
Z0581S	b2	108310209	13653	471453	8775	447	1969	245	80707	1493	1247
Z0581S	b3	109523201	16748	433449	10418	657	1530	403	81362	1899	2551
Z0581S	b4	114653892	27557	442920	12862	1830	4795	2579	87467	2244	6642
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Z0581X	a5	122723945	9393	1474569	11336	2154	488	126	80293	407	787
Z0581X	a6	118928175	7480	1520783	10316	461	409	105	72722	387	572
Z0581X	a7	113239385	7318	1547419	8468	315	331	31	74055	441	460
Z0581X	a8	111388840	8118	1224370	6917	266	650	22	75097	568	459
Z0581X	b1	103584390	7952	503191	5872	294	539	39	72964	768	538
Z0581X	b2	103599764	8146	417532	7461	333	940	59	74917	1121	716
Z0581X	b3	113478867	9437	426631	9788	684	1500	363	78403	1699	1112
Z0581X	b4	107716727	9315	388090	11164	1322	1451	707	73126	1852	1396
Z0581X	b5	118785716	20811	430960	16735	4140	10882	5898	84692	2125	4424
Z0672S	a3	202066511	12262	2197834	20823	3742	1299	522	190972	1192	2100
Z0672S	a4	198978775	12131	2366498	20338	4386	1821	596	190456	1344	2830
Z0672S	a5	202504106	11544	2456200	20604	3924	1168	590	202602	1668	3162
Z0672S	a6	197074013	10856	2048781	18767	2199	947	481	199072	1551	3216
Z0672S	a8	187474622	9301	2278763	17757	898	795	424	167332	1648	2927
Z0672S	b1	194005130	9886	2594325	16829	519	1050	562	165599	1309	2975
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Z0672S	b3	190745010	10599	2696064	13732	472	1241	715	160164	1238	3832
Z0672S	b4	190645173	12105	2605990	13407	543	1475	834	171038	1595	4500
Z0672S	b5	186867141	12258	2498016	12515	548	1626	755	180120	1706	5014
Z0672S	b6	184820526	15651	2202974	15707	908	2436	2020	196628	2321	7795
Z0672S	b7	185292558	23758	1835857	12124	1270	4617	3514	211785	3000	20321
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Z0672X	a4	188878567	9414	2042253	18299	1563	1643	354	176451	1124	2708
Z0672X	a5	193782653	9840	2000582	21641	1991	865	390	197849	1864	3027
Z0672X	a6	193172287	9182	2254614	20401	1117	1478	541	174127	1577	2863
Z0672X	a7	191731029	9890	2589845	17443	983	2807	1181	161688	1302	3256
Z0672X	a8	182849343	12641	2453518	14913	791	3641	1886	165302	1624	4328
Z0672X	b1	184934722	13604	2349041	13510	701	4443	1617	183056	2103	4671
Z0672X	b2	181978376	28169	1857177	14709	990	5766	2621	222341	3254	32159
Z0672X	b3	195056381	62629	1459352	16917	1732	7062	5749	306585	5409	67617
Z0782S	a3	203021167	11328	1844741	19883	2197	844	268	246214	712	2237
Z0782S	a4	218457397	12524	1932914	22197	1310	2304	1003	262810	1239	2310
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Z0782S	a6	202542564	12146	1815842	25966	487	2510	343	228037	1644	2382
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Z0782S	a8	200216199	24160	1443484	22544	1036	3645	1718	253528	4279	8662
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Z0782S	b2	216439274	43104	1715731	22775	691	2333	749	314515	4431	49254
Z0782X	a4	217138893	11889	2091946	19278	1771	3964	415	270913	829	2834
Z0782X	a5	216676712	11468	1998063	20252	1310	1519	1041	239897	1086	1914
Z0782X	a6	204603872	11313	2107120	16364	751	3289	1344	203919	1320	1803
Z0782X	a7	212117474	13210	2114557	15299	997	2628	2217	210404	2294	2841
Z0782X	a8	210093023	13892	2142914	14514	748	1553	1451	219015	2864	2993
Z0782X	b1	207828454	17790	2114998	13655	689	2963	1372	246128	3262	4726
Z0782X	b2	220386475	30469	2031140	15294	835	3484	1470	289429	4122	13839
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Z0961S	a4	188316125	11208	1842485	16142	1915	683	152	184283	1278	1532
Z0961S	a5	199815860	12997	1923293	19254	1924	467	192	192012	1897	1448
Z0961S	a6	202092837	11344	2064089	19201	894	416	277	180939	2184	1222
Z0961S	a7	196764175	8349	2240897	14921	328	524	345	176714	2020	957
Z0961S	a8	192445458	9853	2264037	13069	163	487	415	182700	1829	852
Z0961S	b1	191544679	11205	2138266	11171	755	678	531	184709	1933	1008
Z0961S	b2	191076733	11049	2076732	22353	593	565	512	177428	1721	918
Z0961S	b3	189270982	11475	2051918	12689	538	430	392	172601	1551	898
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Z0961S	b5	183489912	17285	1886080	8399	671	716	1390	187158	2406	2336
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Z0961X	a5	89473649	3837	1106703	6892	371	95	12	71041	589	268
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Z2062X	a6	241582781	11133	2902783	16937	1291	549	48	159457	460	1807
Z2062X	a7	248469325	10988	3058703	15793	841	601	61	155096	486	1619

Z2062X	a8	240429865	12319	2959307	14041	806	843	89	151548	608	1826
Z2062X	a9	243800808	14219	2769382	12101	1211	1270	124	162026	974	2114
Z2062X	b1	243275528	12974	2915791	11439	701	959	95	161679	881	2072
Z2062X	b2	255153919	15281	2432684	9833	1027	1274	196	163400	1159	2374
Z2062X	b3	244173799	15445	1874613	7679	1033	1705	234	158961	1459	2228
Z2062X	b4	228289683	16880	1597379	6346	1110	2757	283	150204	1612	2892
Z2062X	b5	252675319	24051	1700745	7018	2691	4067	555	168769	2186	5744
Z2062X	b6	233541606	53544	1631952	7828	5082	9707	1122	170224	2196	37915
Z2264S	a5	176805471	11063	1954788	20522	1187	581	26	132598	1378	1036
Z2264S	a6	188916388	10622	2266221	22079	416	492	31	172567	2141	906
Z2264S	a7	193441219	12057	2448048	20614	198	478	56	175309	2296	865
Z2264S	a8	190688837	12429	2551268	19435	137	506	55	169423	2124	1104
Z2264S	b1	189019790	13082	2697271	18085	439	613	106	162016	2037	1168
Z2264S	b2	191237889	11882	2886858	17589	375	723	151	158658	1875	1292
Z2264S	b3	190411952	12512	2835388	17797	471	1504	159	158524	1977	1593
Z2264S	b4	192847626	14843	2732988	17497	840	18363	547	160509	2147	2644
Z2264S	b5	201795549	15967	2228904	14386	965	3191	495	159589	2235	2605
Z2264S	b6	183812020	16769	1621490	12943	1587	23234	1018	142080	2547	3566
Z2264S	b7	164615279	17071	1275669	15977	3508	33868	3303	127651	2680	6569
Z2264X	a6	172616290	9969	1962472	19294	826	577	49	133539	1338	1172
Z2264X	a7	187862450	8993	2075211	23349	833	501	61	176685	2020	824
Z2264X	a8	192127544	10765	2350282	21806	444	754	76	180159	2210	948
Z2264X	a9	188905455	11550	2495247	19628	408	710	53	165916	2099	1388
Z2264X	b1	192745596	10018	2758098	19600	336	1345	77	158723	1863	945
Z2264X	b2	190000814	10809	2828889	17796	459	755	83	162886	1919	1316
Z2264X	b3	191511784	11482	2793362	22382	558	2135	186	157968	1838	1760
Z2264X	b4	195203397	12278	2692360	16419	612	1526	177	166986	2174	2166
Z2264X	b5	190508477	11888	2022633	13965	1060	2225	428	153961	2134	5102
Z2264X	b6	189976588	12992	1694279	12914	1457	2109	692	153881	2430	3251
Z2264X	b7	181057695	13116	1351092	25050	2947	5669	2325	144066	2804	4240
Z2364S	a3	85987293	8485	711040	8740	1447	230	139	55854	466	436
Z2364S	a4	88735336	10212	978220	9659	2197	254	255	59691	592	584
Z2364S	a5	85397552	7736	1068099	8515	1149	181	111	57367	571	416
Z2364S	a6	81438496	6631	1035342	7745	375	140	139	49212	445	413
Z2364S	a7	83517852	6333	1029114	7638	331	173	220	50702	419	406
Z2364S	a8	84939749	6536	1119440	7700	325	140	288	56526	481	350
Z2364S	b1	82898115	6506	1102178	7061	315	264	501	53909	494	418
Z2364S	b2	81402035	5977	1128684	6819	269	268	542	54729	527	386
Z2364S	b3	83803290	6015	1192772	6771	268	205	275	62071	518	344
Z2364S	b4	74972010	6295	1067784	5828	182	140	152	58754	461	338
Z2364S	b5	76852708	7483	960580	5003	237	365	489	59147	665	574
Z2364S	b6	77730155	11885	794078	4687	290	645	831	54438	844	1443
Z2364S	b7	77431321	28714	657827	4881	645	1056	1089	54199	1359	10983
Z2364S	b8	90588061	37791	718104	7339	1434	20474	6981	62366	1292	16572
Z2364X	a6	65626425	5356	514553	6990	443	196	82	41192	370	407
Z2364X	a7	64964663	5711	480321	7906	731	295	129	43410	424	358
Z2364X	a8	61332944	4471	506636	7538	208	369	148	38033	312	311
Z2364X	b1	60339425	4745	532265	6524	253	375	73	37844	289	279
Z2364X	b2	54369944	4310	522591	5401	178	552	151	35612	314	290
Z2364X	b3	54368903	4497	523605	5280	192	709	251	36300	395	308
Z2364X	b4	50464907	4258	476091	4365	171	310	400	33630	393	286
Z2364X	b5	59776990	4990	539681	4955	209	513	754	42757	532	404
Z2364X	b6	52677997	4819	445242	3898	196	674	856	39818	537	377
Z2364X	b7	58252438	5354	469136	4108	389	490	1222	43579	591	531

Z2364X	b8	57668379	6275	430366	3532	401	724	1539	41165	690	707
Z2364X	b9	60328631	10972	434433	3919	805	4236	4691	40810	778	1586