Zinc Intake, Zinc Status and Expression of Zinc Transporter Genes in Younger and Older Saudi Adults.

Thesis submitted for the degree of Doctor of Philosophy

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Abstract

Background: During ageing, there are changes in many physiological systems including the gastrointestinal tract which may result in decreased absorption of micronutrients such as zinc. Decreased dietary absorption efficiency and/or inadequate zinc intake are contributing factors to the decline in plasma zinc concentration that has been reported in older adults. This decline in plasma zinc concentration may have important implications for health, e.g. through reduced immune function. To date, there is limited information on the relationships between zinc intake and plasma zinc concentration during ageing among adults in Saudi Arabia or on the molecular mechanisms responsible for age-related effects. Thus, the aims of this study were to test the hypothesis that changes in expression of zinc transporters including ZnT1, ZnT5 and ZIP4 are associated with changes in zinc status in young and older Saudi adults.

Methods: Two hundred and two young and older Saudi adults of both sexes were recruited from Jeddah city in the western region of Saudi Arabia. Intakes of zinc and of zinc absorption modifiers in diet were assessed using an FFQ (designed and validated for this purpose) while plasma zinc concentration was determined by ICP-OES. RNA was extracted from white blood cells and qPCR was used to quantitatively measure expression of the ZnT1, ZnT5 and ZIP4 genes at the mRNA level.

Results: Intakes of zinc and of protein were significantly higher in males than in females ($P<0.05$). A substantial proportion of the participants appeared to have inadequate zinc intake with the risk of inadequacy being greatest for young males (15%) and young females (4%). Plasma zinc concentrations in older adults were significantly higher than in young adults ($P<0.05$). Older males had significantly higher ZIP4 expression compared with young adults ($P<0.05$). Although older adults had apparently higher ZnT1 and ZnT5 expression than young adults, these differences were statistically non-significant. Several age and sex-specific correlations between zinc intake, zinc status and expression of zinc transporters were observed. Young and older adults who had apple-shaped fat distribution pattern, had significantly higher expression of ZnT1, ZnT5 and ZIP4 and plasma zinc status compared with participants who had pear-shaped fat distribution pattern ($P<0.05$).

Discussion: Despite the changes in food availability in Saudi Arabia over recent decades, the prevalence of low zinc intake remains substantial. Contrary to reports from elsewhere, the prevalence of low plasma

zinc concentrations was greater in younger than in older Saudi adults. This study also identified relationships between expression of zinc transporters and zinc intake, zinc status and abdominal fatness in Saudi adults.

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Declaration

The work presented in this thesis was carried out by the candidate, with the exception of bio analyzer analysis. RNA samples were sent to the center of excellence in genomic medicine, King Fahad Medical Research Center, King Abdul-Aziz University.

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List of Abbreviation

ALT	Alanine aminotransferase
As	Arsenic
BER	Base Excision Repair
BHK	Baby Hamster Kidney
BMI	Body Mass Index
BMR	Basal metabolic rate
Ca	Calcium
CBC	Complete Blood Count
cDNA	Complementary DNA
Cu	Copper
DIT	Diet-Induced Thermogenesis
DRC	DNA Repair Capacity
EAR	Estimated Average Requirements
EDTA	Ethylene Diamino Tetraacetic Acid
ER	Endoplasmic Reticulum
ER	Estrogen Receptor
FAO	Food and Agriculture Organization
Fe	Ferric
FFQ	Food Frequency Questionnaire
FITC	Fluorescein Iso Thio Cyanate
FR	Food Record
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GI	Gastrointestinal
hsCRP	high sensitive C-Reactive Protein
IgG	Immunoglobulin G
IZiNCG	International Zinc Consultative Group
LRNI	Lower Reference Nutrient Intake
Mg	Magnesium
Mn	Manganese

MRE	Metal Response Element
MT1	Metallothionein 1
MT2a	Metallothionein 2a
mtDNA	Mitochondrial DNA
MUST	Malnutrition Universal Screening Tool
NER	Nucleotide Excision Repair
Ni	Nickel
PA	Physical Activity
PARP-1	Poly (ADP-ribose) Polymerase-1
Pb	Lead
PBS	Phosphate Buffered Saline
PC12	Pheochromocytoma
PCR	Polymerase Chain Reaction
PTP	Permeability Transition Pore
PZR	Phytate-to-zinc molar ratio
ROS	Reactive Oxygen Radicals
SACN	Scientific Advisory Committee on Nutrition
Se	Selenium
SLC30A	Solute-linked Carrier 30
TAE	Tris Acetate EDTA
TEF	Thermic Effect of Food
TM	Transmembrane
UL	Upper Level
UNICEF	United Nations Children's Fund
UNU	United Nations University
WHO	World Health Organization
WHR	Waist-to-Hip ratio
Zip	Zrt- and Irt-like proteins
Zn	Zinc
ZnT	Zinc Transporter
ZTRE	Zinc Transcriptional Regulatory Element

Chapter 1. Introduction and Background

1.1- Definition of Ageing:

"Ageing is a degenerative process that is associated with progressive accumulation of deleterious changes with time, reductions of physiological function and increase in the chance of disease and death" (Lee and Wei, 2012).

Chronological age is the most popular method to define aged or elderly but this approach is contested (World Health Organization, 2001, Balcombe and Sinclair, 2001). The World Health Organization uses 65 years as a numerical cut-off point to refer to older persons (elderly) whereas 80 and over refers to oldest-old. Nevertheless, definitive categorization of older people is difficult because "old" is an individual-, culture-, country- and sex-specific term. For instance, many people in developing countries are functionally "old" in their forties and fifties (World Health Organization, 2001). In Saudi Arabia, 60 years and above refers to older adults as it is considered as retirement age (Almadani, 2005). In 2008, Sanderson and Scherbov proposed an alternative to having a fixed age at which people are categorized as old i.e. to define old age as beginning at some threshold level of remaining life expectancy. Thus, there are at least two ways of defining old age: old-age based on chronological age and one based on prospective age (Sanderson and Scherbov, 2008).

1.2- Demography of Ageing:

Around the world, the number of people aged 60 years and over is on the rise (World Health Organization, 2011). For instance, there were 784 million older people in 2011 which represents 11% of the total population. By 2050, this number is projected to increase by factor of 2.6 to reach around 2 billion (i.e. 22% of the population), with most of the increase in the developing countries. This percentage is expected to reach 30 in 2100 (see Figure 1.1) (World Health Organization, 2011, United Nations, 2011). In Saudi Arabia, the number of older people (>60 years) in 2009 was 907,529 which represents 5.2 % of the Saudi population (Ministry of Economy and Planning, 2009a).

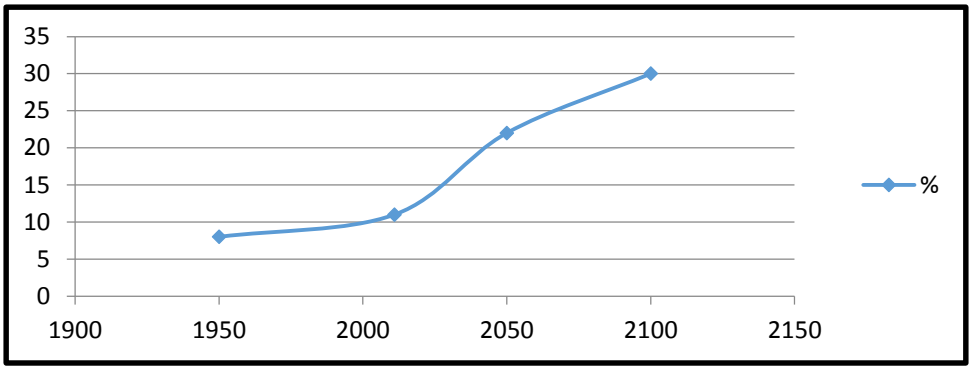


Figure 1.1 Worldwide proportion of people aged 60 and over from 1950 to 2100

Source: United Nations department of economic and social affairs/ population division (2011)

Although there have been notable setbacks in life expectancies in some countries (such as sub-Saharan Africa, the countries of the CEE, Russia and the Baltic States) , the continuously growing proportion of older people is due to increasing longevity (World Health Organization, 2011, Leon, 2011). Table 1.1 shows that life expectancy at birth by years at the world level is on the rise for both sexes (United Nations, 2011). The greatest increases have occurred in east Asia, where life expectancy at birth increased from less than 45 years in 1950 to more than 74 years nowadays (Ministry of Economy and Planning, 2009b, World Health Organization, 2011). Table 1.2 showed the increase in life expectancy in Saudi population over recent years (Ministry of Economy and Planning, 2009b).

Table 1.1 Life expectancy by sex for the world, 2005-2010, 2045-2050 and 2095-2100

Life expectancy at birth (years)					
2005-2010		**2045-2050**		**2095-2100**	
Male	**Female**	**Male**	**Female**	**Male**	**Female**
65.7	70.1	73.2	78	79	83.3

Source: United Nations department of economic and social affairs/ population division (2011)

Table 1.2 Life expectancy by sex for Saudi population, 2006-2009

Life expectancy at birth (years)							
2006		**2007**		**2008**		**2009**	
Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
72.4	74.4	72.5	74.6	72.6	74.8	72.7	74.9

Source: Saudi Ministry of economy and planning/ department of statistics and information (2009)

As can be seen from Tables 1.1 and 1.2, there are sex differences in life expectancy and females have longer life expectancy than males. The reason for this difference is not known but may be because females are less likely to engage in behaviors known to cause chronic health problems and to contribute to premature mortality such as smoking, poor diet and excessive alcohol consumption (McKevith, 2009).

Improvements in life expectancies are part of a major transition in human health around the world. This transition includes a shift in the main causes of death and illness from parasitic and infectious diseases to non-communicable disease and chronic conditions and sustained increases in life expectancy at birth and at older ages (World Health Organization, 2011). Center for disease control and prevention reported ten global health achievements over the first ten years of the 21st century. These improvements include the control of malaria, HIV, Tuberculosis and neglected tropical diseases (Morbidity and Mortality Weekly Report, 2011).

The growing proportion of older people is due not only increasing life expectancy but also to decreasing fertility rate. Globally, the total fertility (average number of children per woman) was 4.45 in 1970-1975. This number declined to 3.04 in 1990-1995 and reached 2.52 in 2005-2010 (United Nations, 2011). Among Saudi women, total fertility rate decreased gradually from 3.6 in 2004 to 3.24 in 2009 and to 2.26 in 2012 (Central Intelligence Agency, 2012, Ministry of Economy and Planning, 2009c).

1.3-The Basic Biology of Ageing:

Ageing is a complex process which involves several mechanisms operating at different levels (von Zglinicki et al., 2001) and is not genetically programmed (McKevith, 2009). In 1956, Harman proposed that reactive oxygen radicals (ROS) cause ageing by damaging membranes, proteins, and DNA. There is a growing body of evidence supporting the idea that the action of ROS is an important theme linking several different kinds of damage (Kirkwood and Mathers, 2009). Cellular DNA is damaged by ROS damage occurring in the nuclear DNA, telomeric DNA and mitochondrial DNA (von Zglinicki et al., 2001, López-Otín et al., 2013).

1.3.1- Nuclear DNA damage and repair:

DNA is subject to damage by exogenous and endogenous damaging agents (Burkle et al., 2002). Oxidative damage is among the most important form of DNA damage as it can occur at any time (Kirkwood and Mathers, 2009). When ROS attacks DNA, a variety of DNA lesions are generated such as oxidized DNA bases, abasic sites and DNA strand breaks, which then lead to genomic instability (Maynard et al., 2009). Repair activities of DNA control the rate of ageing by keeping the DNA damage at low levels (Burkle et al., 2002). In mammals, there are several DNA repair pathways including base excision repair (BER), nucleotide excision repair (NER), double-strand break repair and mismatch repair (Cui et al., 2012). Poly (ADP-ribose) Polymerase-1 (PARP-1) plays a key role in immediate cellular response to free radicals-induced DNA damage by recognizing DNA lesions and flags them for repair (Muiras et al., 1998). In 1992, Grube and Burkle compared the activity of PARP in mammalian species of different maximal life span and found a strong positive relationship between PARP activity and life span (Grube and Burkle, 1992). Similarly, PARP activity was higher in centenarians of a French population than controls aged 20 to 70 years providing further evidence that longevity is correlated with a high poly (ADP-ribosyl)ation capacity (Muiras et al., 1998).

There is considerable inter-individual variation in DNA repair capacity (DRC) even among healthy individuals (Tyson and Mathers, 2007). Tyson et al (2009) found an eleven-fold variation in NER between individuals whilst Caple et al (2010) found a larger variation (41-fold) in BER capacity (Tyson et al., 2009, Caple et al., 2010). This difference is partly due to variations in age as there is sufficient evidence that all DNA repair pathways capacity decreased with increasing age (Xu et al., 2008, Tyson et al., 2009, Gorbunova et al., 2007). DRC can also be influenced by genotype as the majority of genes

encoding proteins important in DNA repair are polymorphic (Mohrenweiser et al., 2002, Tyson and Mathers, 2007). For instance, DRC was modulated by the presence of the A allele of *XPA G23A* (NER gene) polymorphism (Slyskova et al., 2011). Zhu et al (2008) showed that individuals with the variant alleles (AC and CC) of Lys 939 Gln in *XPC*, another NER gene, had significantly higher DNA damage following exposure to benzo[a]pyrene diol epoxide (BPDE) and gamma-radiation. On the other hand, subjects with the variant alleles (CT and TT) of Ala 499 Val had lower DNA damage (Zhu et al., 2008). A significant variation in DNA damage level was also found between 3 genotypes for the Val 16 Ala polymorphism in manganese superoxide dismutase (rs 4880) (Caple et al., 2010).

In addition to age and genotype, diet is also responsible for differences in DRC. Certain dietary factors may enhance DNA repair capacities besides their role in the prevention of oxidative damage. Some examples of these dietary factors are organic products, whole grains, fruits (such as golden kiwifruit) and vegetables (such as broccoli) which are associated with decreased levels of oxidation of DNA bases (purine and pyrimidine) (Riso et al., 2010, Prado et al., 2010, Brevik et al., 2011a). Reduction of DNA single strand breaks (SSB) was more rapid after consumption of mixed carotene capsules while repair activity was enhanced after consumption of cooked carrots (Astley et al., 2004). Similarly, Brevik et al (2011) found a positive effect of consuming anti-oxidant rich plant products on decreasing DNA SSB whereas BER was increased (Brevik et al., 2011b). Thus, dietary factors which limit damage or which enhance repair would be expected to modify the rate of biological aging (Kirkwood and Mathers, 2009). Not only DNA damage but also mutagenesis was reported to increase with age and these contribute to aging by causing cellular dysfunction (Xu et al., 2008).

1.3.2- Telomeric DNA:

Telomeres are repeats of a noncoding sequence of DNA complexed with proteins which protect the end of the chromosomes (Champe et al., 2005). In most human cells, telomeres shorten with each round of DNA replication due to lack of telomerase enzyme. Additionally, oxidative stress accelerates telomere loss and repair of telomeric DNA damage is less effective than elsewhere in the chromosome (von Zglinicki, 2002). Loss of telomeres eventually stops chromosomes replicating and they became senescent. Loss of chromosomal replication limits the capacity for formation of new cells during ageing (Bodnar et al., 1998). Valdes et al (2005) found that telomere length declined constantly with age at a

mean rate of 27 bp/year and was 240 bp shorter in obese than in lean females and smoking accelerates telomere shortening (Valdes et al., 2005). Cawthon et al (2003) suggested that telomere shortening is associated with mortality (Cawthon et al., 2003). In 2008, Starr et al reported that SNPs in *MSRA* and *NDUFA8* genes (oxidative stress-related genes) were associated with variation in telomere length and that nine SNPs in five genes were strongly related to biomarkers of physical ageing (Starr et al., 2008).

Von Zglincki and Martin-Ruiz (2005) proposed that telomere length is a biomarker of ageing and ageing-related morbidity (von Zglinicki and Martin-Ruiz, 2005). However, Mather et al (2011) reexamined this evidence and suggested that this evidence is equivocal. Telomere length does not fully meet the American Federation of Ageing Research criteria that it should reflect life span rather than chronological age and it must monitors a basic process underlying normal ageing at the population level and not the effects of diseases. On the other hand, this biomarker fulfills the criteria that telomere length can be estimated repeatedly without harm and that it can be examined in other mammals (Mather et al., 2011). Recently, Simons (2015) reviewed telomerase knockout and overexpression studies conducted on several model species and find little support that telomeres cause ageing. In addition, the causality hypothesis assumes that there is a critical telomere length at which senescence is induced. This generates the prediction that variance in telomere length decreases with age. In contrast, using meta-analysis of human data, no such decline was found. Inferring the causal involvement of telomeres in ageing from current knowledge is therefore speculative (Simons, 2015).

1.3.3- Mitochondrial DNA:

In 1972, Harman specifically extended the free radical theory by the mitochondrial theory of aging (Harman, 1972). This is based on the concept that mitochondria are the major producers of ROS (as by-product of respiration) in mammalian cells and that mitochondrial DNA (mtDNA) is prone to oxidative damage. mtDNA mutations generated by ROS accumulates in the cell, leading to damaged respiratory chain proteins, thereby generating more ROS, which in turn causes higher mutation rates (Maynard et al., 2009). Several studies showed that mtDNA deletions increased in elderly people compared to young people (Hattori et al., 1991, Yen et al., 1991). In aging human tissue, Nooteboom et al (2010) showed that respiratory chain (RC) deficiency, caused by mtDNA point mutations, attenuates cell proliferation and increase apoptosis in the progeny of RC deficient stem cell, leading to decreased crypt cell population

(Nooteboom et al., 2010). Moreover, high level of mutant DNA in cytochrome C oxidase-deficient fibres were reported in muscle tissues from normal elderly humans (Brierley et al., 1998). Increased 8-oxo-dG (one of the most abundant DNA lesions caused by ROS) in mtDNA is frequently detected in aged tissues (Cui et al., 2012). While mtDNA mutations and deletions increase with age, Welle et al (2003) showed that the amount of mtDNA in muscle of older subjects was 38% lower than in young subjects.

In addition to generating ROS, mitochondria play a critical role in mediating apoptosis (Cui et al., 2012). In many instances, developmental homeostatic and pathological cell death involves a critical step in which mitochondria release proteins that trigger the self-destructive enzymatic cascade that causes apoptosis (Green et al., 2011). Moreover, activation of the permeability transition pore (PTP), which plays a critical role in cell necrosis and apoptosis, is enhanced in spleen, brain and liver of aged mice (Mather and Rottenberg, 2000). Autophagy (a lysosome-dependent cellular degradation process) can specifically target cellular structures such as mitochondria (mitophagy) and, with increasing age, loss of autophagy can lead to accumulation of damaged mitochondria, which promotes cell death and inflammation (Green et al., 2011).

As a result of a wide spectrum of alterations in mitochondria and mtDNA during ageing, Lee and Wei proposed that elevated oxidative stress, subsequent accumulation of mtDNA mutations, altered expression of a few clusters of genes, decreased mitochondrial energy metabolism, mitochondrial dysfunction and apoptosis are important contributors to human ageing (Lee and Wei, 2007, Lee and Wei, 2012b).

1.4- Nutritional and Physical Activity Requirements of Older People:

1.4.1- Energy:

Energy is needed by the body to maintain its fundamental functions (often assessed as Basal Metabolic Rate (BMR)) and for physical activity. Together with the thermic effect of food (TEF), these are the 3 components of energy expenditure (Thompson and Manore, 2009b).

In most people, 60-65% of energy expenditure is used to maintain basic physiologic functions at rest (estimated as BMR) (Thompson and Manore, 2009b). BMR is influenced by body mass, sex (Lazzer et al.), protein turnover (Welle and Nair, 1990), fat free mass, fat mass and age (Johnstone et al., 2005). BMR declines with age, with estimates of 2% and 2.9% decline per decade for normal weight females

and males, respectively (FAO/WHO/UNU, 2004). Aging is associated with changes in body composition, with increases in body fat, while fat free mass decreases, leading to reduced BMR (Guo et al., 1999, McKevith, 2009).

The second component of energy expenditure is the energy cost of physical activity (PA). Typically, this energy represents 15-35% of energy output and it expended on muscular work and movement above basal levels (Thompson and Manore, 2009b). As people age they often become more sedentary and thus energy expenditure is diminished (McKevith, 2009). In addition, the high prevalence of diseases and disabilities affects the daily activities of 39% of persons aged 65-74 years old and 48% of those aged > 75 years (Elia et al., 2000). However, such age-related declines in physical activity are not inevitable. For instance, Evenson et al (2002) observed that, in retirement, American subjects in their study increased their participation in sport and exercise (Evenson et al., 2002). This highlights the fact that PA energy expenditure is the most variable component of total energy expenditure and that it depends on the individual's health, social and cultural features that enhance, or limit, their PA (Elia et al., 2000, FAO/WHO/UNU, 2004).

The thermic effect of food (TEF) (also called diet-induced thermogenesis (DIT)) is the third component of energy expenditure and it represents 5-10% of total energy expenditure. This is energy used to digest, absorb, transport, metabolize and store the nutrients of food (Thompson and Manore, 2009b). The effect of aging on this component of energy expenditure is unclear as results of several studies are inconsistent. For instance, Golay et al (1983) and Thörne and Wahren (1990) found that thermogenesis diminished with age (Golay et al., 1983, Thorne and Wahren, 1990). On the other hand, Poehlman et at (1991) showed that thermic effect of a meal was not related to age (Poehlman et al., 1991). Elia et al (2000) examined the factors which may have been contributed to the different findings about DIT in the elderly. First, the previous studies have been used either protein or carbohydrates meals, or mixed liquid meals and none of which used a mixed meal containing both liquid and solid components. Second, several factors such as body composition, PA level and visceral fat accumulation in women may affect DIT and many studies did not control for these factors (Elia et al., 2000).

Table 1.3 shows the estimated average requirements (EAR) for energy for older people is lower than for younger adults. These values are even lower in cases of immobility or chronic illness (Scientific Advisory Committe on Nutrition, 2011).

Table 1.3 Estimated average requirements for energy (Kcal/day)

Age	Males	Females
19-24	2772	2175
25-34	2749	2175
35-44	2629	2103
45-54	2581	2103
55-64	2581	2079
65-74	2342	1912
75+	2294	1840

Source: (Scientific Advisory Committe on Nutrition, 2011).

1.4.2- Macronutrients:

Dietary recommendations for carbohydrates, dietary fibre and fat for older people are the same for other adults (Department of Health, 1991). However, protein recommendations are slightly changed in older adults compared with young adults. Male requirements are decreased while female requirements increased (see Table 1.4) (Department of Health, 1991). Discussions about the validity of protein requirements based on the FAO/WHO/UNU report are ongoing. Various researchers concluding that these recommendations should be higher because of increased illness, infections, poor wound healing and surgery in this age group (McKevith, 2009).

Table 1.4 Reference nutrient intakes for Protein (g/day)

Age	Males	Females
19-50 **51+**	55.5 53.3	45 46.5

Source: Department of Health (1991).

1.4.3- Micronutrients:

The micronutrients needs of older people are generally similar to those of young adults (see Table 1.5) (Department of Health, 1991, Sentific Advisory Committee on Nutrition, 2015).

Table 1.5 Reference nutrient intake (RNI) for vitamins and minerals for adults over 50 years

Nutrient	RNI	Nutrient	RNI
Calcium (mg/d)	700	**Copper (mg/d)**	1.2
Phosphorus (mg/d)	550	**Iodine (µg/d)**	140
Magnesium (mg/d)	270 female 300 male	**Zinc (mg/d)**	7 female 9.5 male
Sodium (mg/d)	1600	**Iron (mg/d)**	8.7
Potassium (mg/d)	3500	**Vitamin B12 (µg/d)**	1.5
Chloride (mg/d)	2500	**Folate (µg/d)**	200
Selenium (µg/d)	60 female 75 male	**Thiamin (mg/d)**	0.8 female 0.9 male
Riboflavin (mg/d)	1.1 female 1.3 male	**Niacin (mg/d)**	12 female 16 male
Vitamin B6 (mg/d)	1.2 female 1.4 male	**Vitamin D (µg/d)**	10

Source: Department of Health (1991) and Scientific Advisory Committee on Nutrition (2015)

1.4.4- Fluids:

With aging, the water % of total body weight falls. As a result, the body's water reservoir is reduced and the safety margin for staying hydrated become smaller (Brown et al., 2005). Drinking 30 ml/kg of water per day (i.e. 6-8 glasses) is required by adults to prevents dehydration persons, even those who are underweight, should drink at least 1500 ml of fluid per day unless there is renal failure or some other reason to restrict intake (World Health Organization, 2002). Older people should increase their intake if they have fever, diarrhea, living in high environmental temperature or if there is drug- or caffeine-induced fluid losses (McKevith, 2009).

1.4.5- Physical activity:

Regardless of age, sex, stage of life or socioeconomic status, physical activity (PA) has been demonstrated to benefit health (McKevith, 2009). PA recommendations for younger adults are applicable for older adults. In general, adults should achieve at least 30 minutes moderate intensity PA per day on at least 5 days per week. This can be achieved by doing life style activities such as brisk walking or climbing stairs as well as structured exercise or sport. In addition, older people are encouraged to do specific activities that improve and promote coordination, strength and balance (Department of Health, 2004).

The WHO's global strategy on diet, PA and health recommends that individuals should engage in different types and amounts of PA to benefit from different health outcomes. For example, at least half an hour of regular, moderate intensity PA on most days reduces the risk of colon cancer, breast cancer, diabetes and cardiovascular disease (World Health Organization, 2003).

1.5- Dietary Pattern, Nutrient Intake, Nutrient Status and Physical Activity of Saudi Older Adults:

1.5.1- Dietary pattern:

Differences in food patterns have been observed among people of different ages, sexes and cultures. The food patterns of young and older Saudi adults have been investigated by several studies (see Table 1.6). Briefly, the proportion of free-living older adults who consume three meals per day was higher than those of young adults. Sadiq (2005) investigated the number of meals consumed by 50 elderly females in Jeddah city (western province) before and after institutionalization and found that the number who

consumed 3 main meals/day declined significantly after institutionalization (Sadiq, 2005). On the other hand, consumption of snack meals is more common in young than in older adults. Intake of vegetables and fruits (except dates) are less preferred among young than older adults and dates are reported as a favorite fruit for all ages.

Table 1.6 Studies of dietary patterns of young and old Saudi adults

City/ province	Age	Sample size and sex	Observations	Reference
Northern province	60-70 years	404 male	86.2% consume 3 main meals/day	(Alenezy, 2003b)
Riyadh/ central	≥60 years	115 female	57.9% of participants consume 3 main meals/day while only 3% eat snacks more than 3 times/day, 63.2 % drink milk daily, 60% and 44.2 % daily eat vegetables and fruits, respectively.	(Hosa, 2004)
Alqassim / central	All age groups	1804 male 980 female	More than 70% of elderly (≥60) reported preferences for fish, vegetables, fresh fruits and grilled meats while more than 40% of adults (<33) showed the same preferences, more than 40% of all ages groups preferred eating dates	(Midhet et al., 2010)
Abha/ southwestern	17->25	456 male	75% had a snack 1-3 times/day, 42% drinks soft drinks more than 4 times/week	(Al-Gelban, 2008)
Alqassim / central	18-24 years	357 male	31.4% consume 3 meals/day, 11.2% eat daily green, red or yellow vegetables, 4.5% eat daily fruits except dates, 36.1 % consume dates daily	(Al-Rethaiaa et al., 2010)

1.5.2- Nutrient intake:

Studies investigating nutrient intakes in older Saudi adults are rare and have been undertaken mainly in institutionalized rather than among free-living elderly. Average daily energy intake of older Saudi adults are varied according to difference in living situations and sex. For example, mean intake of free-living men (≥50 years) was 1834 kcal (below RNI) (Alissa, 2005), while for men in institutions it was reported to be 2795 kcal (above RDA) (Alnumair, 1998). The latter value seems high and may be affected by measurement errors. However, institutionalized women (55-70 years) had energy intake of 1232 kcal which is below the RDA (Sadiq, 2005a). Figure 1.2 shows the slight differences in the percentage energy in the form of total fat, total protein and carbohydrates between males and females according to different studies results.

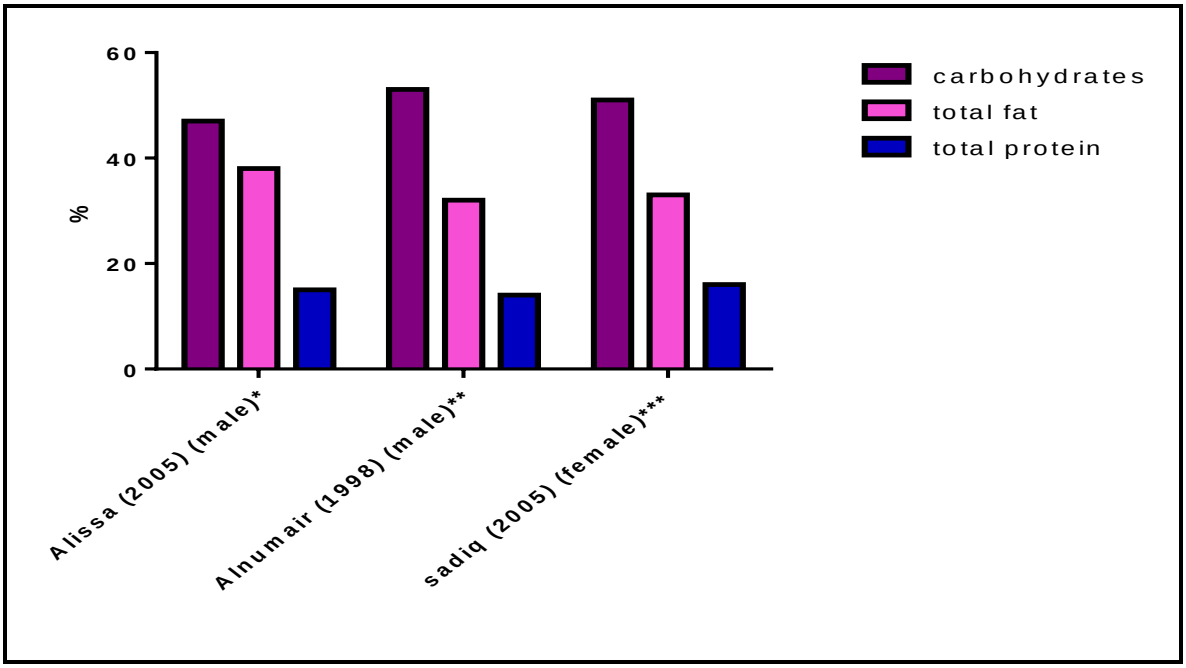


Figure 1.2 Percentages energy from fat, protein and carbohydrates in 3 separate studies of the diets of Saudi elderly. Ages: * (≥ 50 years), ** (50-85 years), *** (55- >75 years)

Mean daily intake of fat, protein and carbohydrates in men were 78 g, 70 g and 213 g respectively (Alissa, 2005). Lower intakes were reported in women, where fat, protein and carbohydrates intake were 47 gm, 47 g and 160 g respectively (Sadiq, 2005). Dietary fibre intakes of male and females living in institutions were below recommendations (RDA) as the intake was 15 g and 4 g, respectively (Sadiq, 2005a,

Alnumair, 1998). Similarly, free-living male showed low fiber intakes (18 g) (Alissa, 2005). Low intakes of a range of micronutrients where highlighted in table 1.7 according to differences in Saudi regions.

Table 1.7 Low intakes of micronutrients by Saudi older adults

City/ province	Age	Sample size and sex	Nutrients with low intake [a] (% of participants with low intake)	Reference
Riyadh/ central	-	- male	Vitamin B12 (34%)* Folate (88%)	(Al-Shitwi, 2006)
Jeddah/ western	55-75	200 female	Niacin Vitamin D** Folic acid Calcium Vitamin C Iron Vitamin A	(Sadiq, 2005a)
Jeddah/ western	≥50	303 male	Selenium (87%)*** Vitamin A (54%) Vitamin E (29%) Vitamin C (32%)	(Alissa, 2005)
Northern province	60-70	404 male	Vitamin A** Vitamin D Vitamin C Vitamin B6	(Al-Gelban, 2008)

a - low has been defined differently. * Nutrients intake below RDI, ** nutrients intake below RDA, ***nutrients intake below EAR

1.5.3- Nutritional status:

In the UK population, it is estimated that one in seven elderly has a medium or high risk of malnutrition when examined using the malnutrition universal screening tool (MUST) (Malnutition Advisory Group, 2003). Morbidity and mortality are predicted by involuntary weight loss. Additionally, excess body weight is associated with increased risks for many age-related diseases (McKevith, 2009). In a number of studies, there were large variations in the proportion of underweight and obese Saudi elderly (Figure 1.3). These studies showed that underweight is more prevalent among institutionalized elderly whereas obesity is high among free-living Saudis.

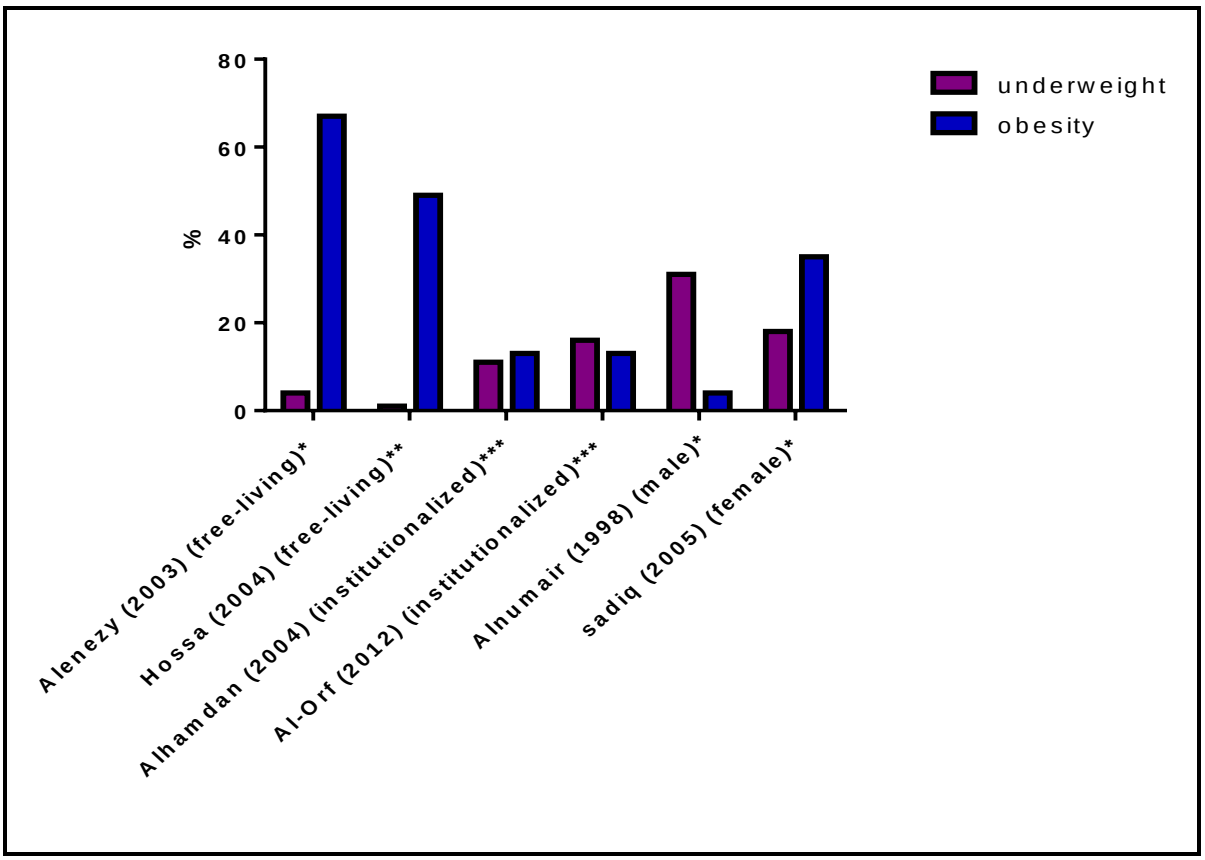


Figure 1.3 Proportions of underweight and obese among free living and institutionalized Saudi elderly. * Criteria of inclusion is not available in the abstract, ** underweight= BMI < 20 kg/m^2, *** underweight= BMI < 18.5 kg/m^2, obese= BMI > 30 kg/m^2

Iron, iodine, zinc, vitamin A and D deficiencies are highly prevalent in the Arab region but the magnitude of the problems differs from country to country (Madani and Kumosani, 2001). In Saudi Arabia, iron and vitamin D are widely studied.

When using WHO criteria for diagnosis of anemia [i.e. Hb < 13g/l in men and Hb < 12g/l in women], the prevalence of iron deficiency anemia in institutionalized men and women in Riyadh was 40% and 32% respectively (Al-Orf, 2012, Alhamdan, 2004). Similarly, the prevalence of anemia among women living in institutions in Jeddah was 32.5% (Sadiq, 2005). In contrast, free-living males and females showed lower prevalence of anemia (12.9%) overall but a higher frequency in females (18%) compared with males (5%) (Alsaeed, 2011).

Vitamin D nutritional status can be determined by plasma 25 hydroxyvitamin D (25OHD) concentration (Ardawi et al., 2011). Decreased vitamin D intake, decreased cutaneous synthesis and other factors contribute to risk of vitamin D deficiency (Holick and Chen, 2008). Although Saudi Arabia is a sunny country, direct exposure to sunlight is restricted for cultural reasons and/or due to excessive heat (Ardawi et al., 2012). Serum 25 OHD concentration decreases significantly with age in both sexes. In men aged (<50 years) mean serum 25 OHD (nmol/l) was 31.25 and declined to 26.84 in older adults (>50 years) (Ardawi et al., 2012). Similarly, mean serum 25 OHD (nmol/l) fell from 42.97 nmol/l to 33.3 nmol/l in pre-menopausal and post-menopausal women, respectively (Ardawi et al., 2011).

1.5.4- Physical activity:

A national epidemiological health survey was carried out in Saudi Arabia between 1995 and 2000 in which 7395 Saudi males and females aged between 30 and 70 years participated. Participants were classified into active and inactive categories based on the intensity, duration and frequency of physical activity. Findings showed that levels of inactivity were high and that significantly more females were inactive (98.1%) than males (93.9%). Inactivity increased with age in males increasing from 89.5% in young men (30-39years) to 97.4% in older men (60-70 years). This highlights the sedentary nature of the Saudi adult population (Al-Nozha et al., 2007).

1.6- Nutritional Role of Zinc:

Zinc is a transition element in the series of the fourth period of the periodic table with the atomic number of 30 and an atomic weight of 65.4, which forms the redox-stable Zn^{2+} cation. In the human body, zinc

is present in all organs and tissues (Gropper et al., 2005) with total body content estimated at 2-3 gm. More than 80% of total body zinc is in muscle, bone, hair and skin. Additionally, the liver, pancreas, kidney and toenails contain high concentrations of zinc (Mahan and Escoot-Stump, 2004, Fairweather-Tait, 1988).

1.6.1- Functions of zinc:

Zinc is an essential cofactor for more than seventy enzymes including DNA and RNA polymerases (Berdanier and Zempleni, 2009) and so it is therefore for nucleic acid synthesis. Equally important, 3-10% of all human proteins bind zinc, including zinc finger proteins of which the majority are transcription factors (Cousins, 1998, Andreini et al., 2006). Recently, it has been discovered that zinc acts as an intracellular signaling molecule, able to communicate between cells, convert extracellular stimuli to intracellular signals and control intracellular events (Fukada et al., 2011). This trace element is also central to other biological functions including inflammation (Prasad, 2009), immunity (Haase and Rink, 2009), bone metabolism (Yamaguchi, 1998), taste perception (Keast, 2003, Wright et al., 1981), spermatogenesis (Yamaguchi et al., 2009), skin health (Rostan et al., 2002) and defense against free-radical attack (Powell, 2000).

1.6.2- Dietary sources of zinc:

Relatively little zinc is stored in the body and, thus, a constant adequate supply of dietary zinc is required. Table 1.8 shows selected zinc-rich foods.

Table 1.8 Zinc content (mg) per common measure of selected foods

Food item and description	Measure	Zinc content (mg/measure)
Oyster (cooked), breaded and fried	3 oz	74.06
Alaska king crab (cooked), moist heat	3 oz	6.48
Baked beans, canned	1 cup	5.79
Duck meat (cooked), roasted	1/2 duck	5.75
Ground beef (cooked)	3 oz	5.31
Turkey meat (cooked), roasted	1 cup	4.34
Sirloin beef steak	3 oz	4.14
Ready- to-eat cereals, Kellogg's all bran	1/2 cup	3.72
Lobster (cooked), moist heat	3 oz	3.44
Ricotta cheese, whole milk	1 cup	2.85
Chickpeas (cooked)	1 cup	2.51
Oats cereal (cooked)	1 cup	2.34
Low-fat plain yogurt	8-oz container	2.02
Mixed nuts with peanuts	1 0z	1.08

Source: USDA National Nutrient Database for Standard Reference, Release 24 (2011)

1.6.3- Zinc in the GI tract:

After consumption of a meal, zinc is liberated from food components as a result of digestive process most likely by proteases and nucleases in the stomach and small intestine. Hydrochloric acid also appears to play a key role in zinc release from the food matrix and/or its absorption (Gropper et al., 2005, Krebs, 2000). Zinc may move across the brush border membrane either as the free ion or as part of a complex. Most evidence supports the latter idea- that free zinc form complexes with ligands such as amino acids, phosphates and other organic acids (Gropper et al., 2005, Krebs, 2000). In addition to dietary zinc (exogenous zinc), endogenous zinc from pancreatic and biliary secretions is released into the gastrointestinal tract (Gropper et al., 2005).

1.7- Zinc Absorption:

Absorption can be considered as the process of influx into the enterocyte, through the apical membrane, and efflux across the basolateral membrane, into the portal circulation (see Figure 1.4) (Krebs, 2000, Wang and Zhou, 2010). Absorption of dietary zinc in humans is in the proximal small bowel, either the distal duodenum or proximal jejunum (Lee et al., 1989, Krebs, 2000). Several studies on rat intestine and human intestinal Caco-2 cell monolayers showed that zinc uptake in the intestine is concentration-dependent and that both carriers mediated (saturable) and non-mediated (non-saturable) pathways are involved (Yasuno et al., 2012, Steel and Cousins, 1985).

Multiple zinc transporters are expressed in the enterocyte including ZnT1, ZnT2, ZnT4, ZnT5, ZnT6, ZnT7, ZIP4 and ZIP5 (see Figure 1.4) (Wang and Zhou, 2010). These mammalian transporters belong to two families – the ZnT and Zip families. The ZnT (solute-linked carrier 30 (*SLC30A*)) proteins decrease intracellular zinc concentration by mediating zinc efflux from cells or influx into intracellular compartments (e.g. endosomes and Golgi apparatus). Conversely, the ZIP (Zrt- and Irt-like proteins (*SLC39A*)) proteins increase cytosolic zinc concentrations (Eide, 2004, Palmiter and Huang, 2004b).

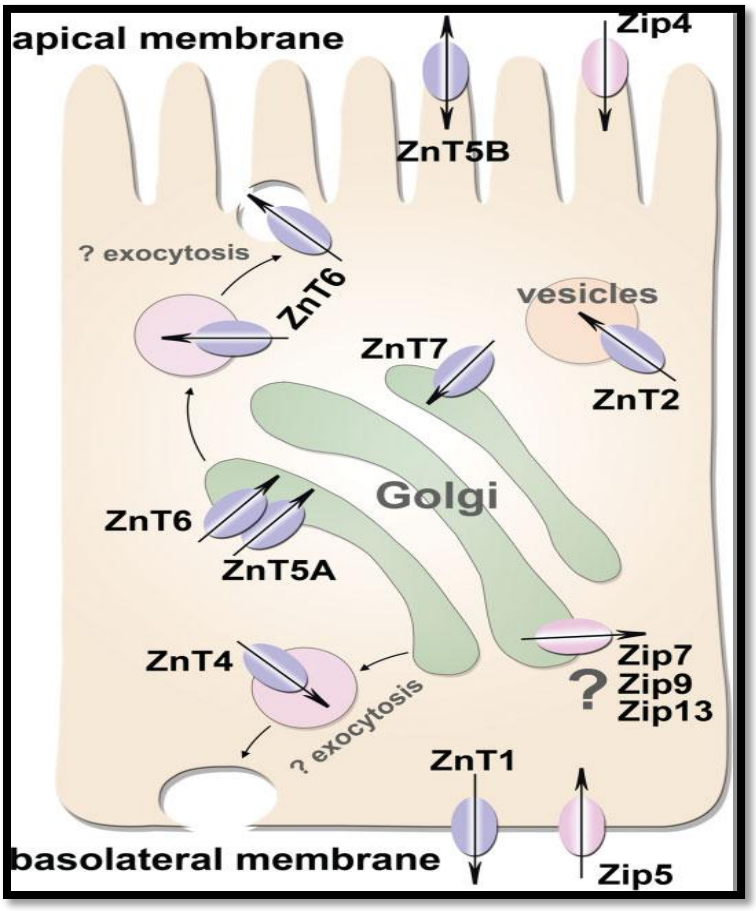
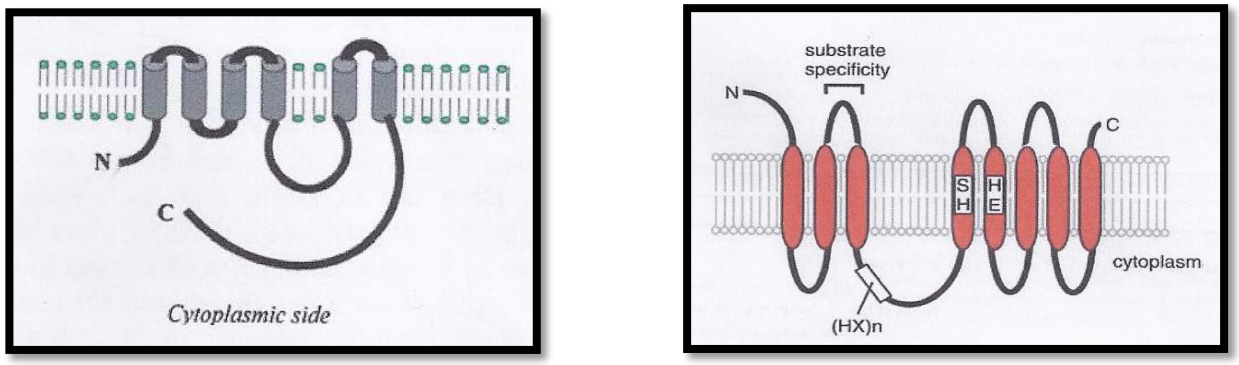


Figure 1.4 Zinc transport pathways in the enterocyte. The question mark indicates a pathway that is not yet well-characterized. Straight arrows indicate the direction of zinc transport. Curved arrows indicate exocytosis. Source: (Wang and Zhou, 2010)

Ten members of the SLC30 family are recognized in mammals. All of them, except ZnT5, are predicted to have six transmembrane (TM) domains with both N- and C- termini on the cytoplasmic side of the membrane, and most have a long C-terminal tail as shown in Figure 1.5A (Palmiter and Huang, 2004b, Lichten and Cousins, 2009). ZnT5 is estimated to have six (in variant A) to twelve (in variant B) additional TM domains and a long N-terminal tail (Cragg et al., 2002). TM domains are connected with small loops with the exception of the loop between domains IV and V which is large (Palmiter and Huang, 2004b, Ford, 2004). This loop was reported to be rich in histidine residues and therefore represents a potential Zn-binding region (Ford, 2004, Paulsen and Saier, 1997).

Fourteen members of the SLC39 family are encoded by the human genome. Most Zip proteins have eight predicted TM domains with extra cytoplasmic N- and C-termini, as shown in Figure 1.5B. Some of the proteins, such as *ZIP4*, have a long N-terminal tail. Loops connecting TM domains are quite short while a longer histidine-rich loop generally occurs between TM domains III and IV (Ford, 2004, Eide, 2004).



(A) **(B)**

Figure 1.5 The Predicted Topology of **(A)** SLC30 Proteins **(B)** SLC39 Proteins. Source: (Palmiter and Huang, 2004b, Eide, 2004).

1.7.1- Zinc transporters involved in zinc uptake into enterocytes:

1.7.1.1- *SLC39A4* (ZIP4):

The human gene *SLC39A4* was identified and characterized by two groups simultaneously (Wang et al., 2002, Kury et al., 2002). This gene is located on chromosome 8 in a region previously identified as being linked to the rare autosomal recessive disorder acrodermatitis enteropathica (AE) (Kury et al., 2002, Wang et al., 2002). Affected individuals suffer from a severe zinc deficiency resulting from impaired absorption of zinc from intestine (Kury et al., 2002). All AE patients studied by (Kury et al., 2002) carry mutations in both alleles of *SLC39A4* which probably affects the function of the protein. Further characterization showed that ZIP4 mRNA was abundantly expressed throughout the small intestine in both humans and mice (Kury et al., 2002, Wang et al., 2002, Dufner-Beattie et al., 2003). Mouse ZIP4 was shown by immunohistochemistry to be localized at the apical membrane of the enterocytes (Dufner-Beattie et al., 2003, Wang et al., 2002) and that the expression of the gene was affected by dietary zinc, exhibiting increased expression under zinc deficiency and rapidly decreased expression with increasing

zinc content in the diet (Dufner-Beattie et al., 2003). All of these findings strongly support a role for ZIP4 in zinc uptake across the apical membrane into the enterocyte (Wang and Zhou, 2010, Lichten and Cousins, 2009).

1.7.1.2- *SLC30A5* (ZnT5 variant B):

SLC30A5 appears to exist as two major splice variants (A and B) (Ford, 2004). The higher-molecular weight transcript encodes ZnT5 variant A (ZnT5A) while the lower molecular weight transcript encodes ZnT5 variant B (ZnT5B), previously named the human zinc transporter like 1 (hZTL1) (Cragg et al., 2002, Ford, 2004). ZnT5B consists of 523 amino acids and has homology with the mouse zinc transporter ZnT1, as shown in Figure 1.6 (Cragg et al., 2002).

In 2002, Cragg et al detected ZnT5 mRNA in a range of mouse tissues (Cragg et al, 2002). At the same time, Kambe et al (2002) reported that ZnT5 Protein was expressed ubiquitously in several human tissues (Kambe et al., 2002). Transfection of a Myc-tagged ZnT5 protein into Caco-2 cells indicated that the protein is expressed on the apical membrane (Cragg et al., 2002). Subsequent confirmation of this location by immunohistochemistry was reported not only in Caco-2 cells but also in human intestine (Cragg et al., 2003, Cragg et al., 2005). In a recent study, localization of ZnT5B to the endoplasmic reticulum (ER) was also reported (Thornton et al., 2011).

Unlike other members of the SLC30 family, ZnT5B is a bidirectional zinc transporter which mediates zinc uptake, as well as zinc efflux. When the protein was expressed in *Xenopus oocytes*, zinc uptake across the plasma membrane increased (Cragg et al., 2002). In addition, the activity of the zinc-responsive metallothionein 2a (MT2a) promoter increased when ZnT5B was co-expressed with an MT2a promoter-reporter construct in Caco-2 cells, indicating increased total intracellular zinc concentration (Valentine et al., 2007). Furthermore, the observed co-localization of ZnT5B with ZIP7 in the ER may indicate a function in uptake of zinc into this compartment for subsequent release by ZIP7, possibly as a component of intracellular zinc signaling (Thornton et al., 2011).

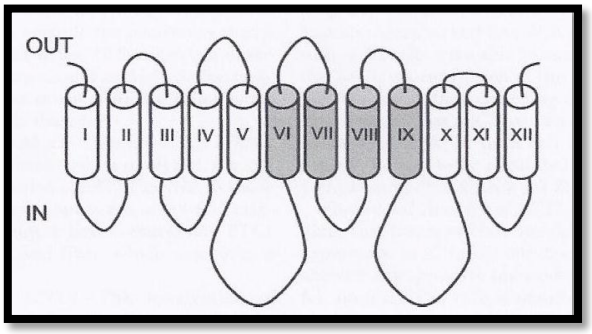


Figure 1.6 The predicted topology of ZnT5B. The shaded area indicates the region of homology with ZnT1. Source: (Cragg et al., 2002)

1.7.2- Zinc transporters involved in intracellular trafficking of zinc within enterocytes:

Mechanisms for intracellular zinc trafficking from the apical cytoplasm to the basolateral cytoplasm of the enterocytes remain largely unknown (Wang and Zhou, 2010). Several intracellular zinc transporters are expressed in enterocytes and their suggested functions in intracellular zinc trafficking is shown in Table 1.9 and Figure 1.4.

In addition to zinc transporters, metallothionein may contribute to zinc transport within the enterocyte. Briefly, this protein binds zinc in two separate clusters with different affinities and has been proposed to play a role in zinc detoxification and as a zinc reserve (Wang and Zhou, 2010, Mahan and Escoot-Stump, 2004).

Table 1.9 Mammalian zinc transporters involved in intracellular trafficking of zinc

Zinc transporter	Sub cellular localization	Function
ZnT2	Vesicles on the apical side of the enterocytes (Liuzzi et al., 2003)	Transport zinc into endosomal/lysosomal-like vesicles and therefore protects cells from zinc toxicity (Palmiter et al., 1996)
ZnT4	Trans-Golgi network, vesicles at the basal cytoplasmic region and endosomes (Murgia et al., 1999, Huang et al., 2002)	When zinc concentration of the medium increased, ZnT4 trans located from Golgi to vesicles (Huang et al., 2002). In addition, a fraction of ZnT4 was detected on the plasma membrane, suggesting with a role in transporting zinc from vesicles to plasma membrane (Henshall et al., 2003). Thus, it is proposed that ZnT4 promotes zinc secretion from Golgi into the blood stream via exocytosis (see figure 4)(Wang and Zhou, 2010)
ZnT5 variant A	Golgi apparatus (Kambe et al., 2002, Thornton et al., 2011, Jackson et al., 2007)	Increases uptake of zinc into Golgi vesicles and thus contribute to the activity of zinc requiring enzymes (Suzuki et al., 2005A, Kambe et al., 2002, Jackson et al., 2007)
ZnT6	Trans Golgi network and apical membrane (Huang et al., 2002, Yu et al., 2007)	• Like ZnT2, ZnT6 was located from Golgi to peripheral vesicles (Huang et al., 2002) indicating that it transports cytoplasmic zinc into Golgi apparatus as well as into vesicular compartments for excretion into the lumen via exocytosis (see figure 4) (Wang and Zhou, 2010, Palmiter and Huang, 2004b) • ZnT6 forms a heteromeric complex with ZnT5A to activate zinc requiring enzymes (Suzuki et al., 2005B)

Zinc transporter	Sub cellular localization	Function
ZnT7	Golgi apparatus (Kirschke and Huang, 2003A)	• Transport zinc from the cytoplasm into the Golgi apparatus (Kirschke and Huang, 2003A, King, 2010) • Like ZnT5 and 6, ZnT7 is required for the activation of zinc requiring enzymes (Suzuki et al., 2005A, Suzuki et al., 2005B)

1.7.3- Zinc transporters involved in releasing zinc to blood circulation:

Zinc must be released from the basolateral membrane of the enterocytes to reach the blood for systemic supply (Wang and Zhou, 2010) and the molecular mediator of this process was suggested to be ZnT1 (Wang and Zhou, 2010). This transporter was the first mammalian zinc transporter to be identified (Palmiter and Findley, 1995). It displays a ubiquitous tissue distribution and is highly expressed in tissues involved in zinc acquisition, recycling or transfer, such as the small intestine (McMahon and Cousins, 1998a, Lichten and Cousins, 2009). Immunocytochemistry with an antibody to a myc epitope on the carboxy-terminus of ZnT1 revealed localization to the plasma membrane (Palmiter and Findley, 1995). McMahon and Cousins (1998) and Yu et al (2007) found that ZnT1 protein was most abundant at the basolateral membrane of enterocytes lining the villi of the rat duodenum and jejunum (Yu et al., 2007, McMahon and Cousins, 1998b). Cragg and Ford (unpublished results) confirmed this localization in the human intestinal Caco-2 cell line (Ford, 2004). ZnT1 location, along with functional evidence for zinc efflux in different cells, such as BHK cells and PC12 cells (Palmiter and Findley, 1995, Kim et al., 2000), support the suggestion that ZnT1 mediates efflux of absorbed dietary zinc from the enterocyte into the blood stream (Ford, 2004, Lichten and Cousins, 2009, King, 2010).

1.8- Factors Influencing Zinc Absorption:

1.8.1- Dietary factors:

The efficiency of zinc absorption in humans responds to changes in dietary zinc intake (Krebs, 2000). Current zinc intake, rather than past zinc intake or status, was inversely related to fractional zinc absorption (Chung et al., 2008). As dietary zinc increases, the total amount of absorbed zinc increases but the percentage of absorption decreases and vice versa (King, 2010). For example, zinc absorption was up regulated to as high as 92% after consuming a low zinc (< 11 mg/d) and low-phytate diet for 4-8 weeks (Hunt et al., 2008).

In addition to zinc intake, the presence of dietary inhibitors and enhancers affects the efficiency of zinc absorption. Phytic acid is the most potent known dietary repressor of zinc absorption as it can bind zinc and form insoluble complexes in the GI tract (Couzy et al., 1998, Kim et al., 2007, Hunt and Beiseigel, 2009). Dietary protein also affects zinc absorption (Krebs, 2000). Generally, higher levels of protein were found to enhance zinc absorption (Sandstrom et al., 1980) but individual protein components can

have different actions. For example casein may inhibit zinc absorption (Lonnerdal, 2000), while amino acids such as methionine and histidine facilitated zinc absorption through a mechanism assumed to be related to zinc binding. Zinc-binding organic acids, such as citrate, had a similar effect (Lonnerdal, 2000).

1.8.2- Age:

Numerous studies have demonstrated that ageing influences intestinal zinc absorption in both humans and rats. Coudray et al (2006 A and B) found that zinc absorption in rat intestine declined significantly with age (Coudray et al., 2006a, Coudray et al., 2006b). Similarly, zinc absorption in human subjects decreased with increasing age (Aamodt and Rumble, 1983). When young men (22-30 years old) and older men (65-74 years old) consumed the same purified formula diet containing 15 mg of zinc per day, mean zinc absorbance was 31% in the young men and fell significantly to 17% in the older men (Turnlund et al., 1986). August et al (1989), reported that zinc absorption from a zinc adequate diet in elderly subjects was 21% compared with 39% in young subjects (August et al., 1989). Couzy et al (1993) reported a trend towards lower zinc absorbance in elderly subjects than in younger adult subjects, but this did not reach statistical significance, which the authors attributed to the small sample size of the study (Couzy et al., 1993a).

1.9- Zinc Transport and Excretion:

Zinc passing from the intestine into the portal blood is transported loosely bound to proteins, predominantly albumin (Mahan and Escoot-Stump, 2004). Up to 60% of zinc is transported by albumin while transferrin, α-macroglobulin and immunoglobulin G (IgG) may transport between 15 and 40% of zinc in the blood (Gropper et al., 2005). The major site of zinc uptake appears to be the liver (Berdanier and Zempleni, 2009).

Zinc is excreted from the human body via three primary routes including the GI tract, kidney and the skin. Most zinc is lost from the body in the feces. Endogenous zinc that is not reabsorbed in the GI tract is also excreted in the feces. In contrast to intestinal zinc losses, renal and dermal losses of zinc, as well as loss in semen and menses, are relatively minor and also more constant (Gropper et al., 2005).

1.10- Zinc Homeostasis:

It has been reported that experimental rats and humans can regulate whole body zinc content, while dietary zinc intakes vary over a 10-fold range (King et al., 2000).

The GI tract is the major site of zinc homeostasis (King et al., 2000) and the mechanisms for zinc homeostasis include adjustments in zinc absorption and endogenous intestinal excretion of zinc (King et al., 2000, Hambidge et al., 2010). With extremely low zinc intakes or with prolonged marginal intakes, changes in urinary zinc excretion, shifts in plasma zinc turnover rates and release of zinc from selected tissues such as bone can occur as secondary homeostatic adjustments (King et al., 2000). Altered regulation of expression of zinc transporters and metallothionein in the intestine is becoming increasingly accepted as critical players in cellular zinc homeostasis in the intestine (Sekler et al., 2007, Fukada et al., 2011). Zinc transporter and metallothionein genes are regulated by increased or reduced zinc availability in both positive and negative manners and some genes show more complex responses to zinc (Jackson et al., 2008).

1.10.1- Regulation of zinc transporters genes:

Several studies report regulatory effects of zinc on ZIP4 expression in the intestine, and details of the underlying regulatory mechanisms are being uncovered. For example, ZIP4 expression in rat intestinal epithelium was up-regulated by excess zinc intake (Fujimura et al., 2012). Zinc supplementation reduced SLC39A4 protein in the ileal mucosa of humans (Cragg et al., 2005), and zinc addition to the medium of cultured cells increased endocytosis of the ZIP4 transporter resulting in reduced cellular zinc uptake (Kim et al., 2004). Similarly, Weaver et al (2007) found that administration of oral zinc caused degradation of ZIP4 in enterocytes while zinc deficiency increased the ZIP4 protein level (Weaver et al., 2007). At the mechanistic level, ubiquitination and degradation of ZIP4 was observed in response to high zinc concentration (Mao et al., 2007) and Kruppel-like factor (KLF4) was found to bind to the mouse ZIP4 promoter under zinc-limiting conditions to increase expression of ZIP4 (Liuzzi et al., 2009).

Unlike ZIP4, ZnT1 responds to zinc in a positive manner in rats and in cell lines but appears to respond differently in humans. McMahon and Cousins (1998) found that dietary zinc supplementation increased ZnT1 mRNA and protein in rat intestine by 50% and 10% respectively (McMahon and Cousins, 1998b). A similar result was reported by Jou et al (2009) (Jou et al., 2009). In cell lines (Mouse Hepa cells and Mouse embryo fibroblasts), treatment with zinc resulted in a rapid (3 hours) and dramatic (12-fold)

increase in ZnT1 mRNA levels. The zinc-responsive transcription factor MTF1 can bind to the ZnT1 promoter and increase gene expression (Langmade et al., 2000). In contrast, ZnT1 mRNA and protein were reduced in ileal mucosa of humans following zinc supplementation, indicating that the response of ZnT1 to dietary zinc supplementation may differ between rats and humans (Cragg et al., 2005).

Regulation of the *SLC30 A5* gene appears to be complex (Jackson et al., 2008). In the human intestinal cell line Caco-2, increasing the zinc concentration of the medium reduced ZnT5 mRNA levels (Cragg et al, 2005) but in the presence of actinomycin D (to prevent transcription) ZnT5 mRNA was increased by zinc, demonstrating stabilization of ZnT5 mRNA (Jackson et al., 2007). Recently, Coneyworth et al (2012) identified a binding site in the *SLC30A5* promoter region which is responsible for zinc-induced transcriptional repression, and which was named the Zinc Transcriptional Regulatory Element (ZTRE) (Coneyworth et al., 2012). Coneyworth et al (2012) also reported that a protein factor whose expression is increased under conditions of high zinc availability binds to the ZTRE and results in abrogated repression of a reporter gene in response to zinc (Coneyworth et al., 2012). This apparently complex mode of regulation may reflect the fact that two opposite pathways -transcriptional repression and increased mRNA stability - operate with increased zinc availability (Jackson et al., 2008).

ZIP5 is another zinc transporter that may be involved in intestinal zinc homeostasis. This transporter was found to be located at the basolateral membrane of enterocytes and was internalized and degraded during zinc deficiency (Dufner-Beattie et al., 2004, Weaver et al., 2007). After zinc-gavage in mice, ZIP5 rapidly resynthesized and targeted to the basolateral membrane (Weaver et al., 2007). Thus, ZIP5 may function in the removal of zinc from the blood stream into the enterocyte for ultimate excretion into intestinal lumen under zinc replete conditions (Dufner-Beattie et al., 2004, Wang and Zhou, 2010).

1.10.2- Regulation of metallothionein gene expression:

Zinc supplementation induces metallothionein (MT) mRNA in both cell lines and humans (Langmade et al., 2000, Cragg et al., 2005). In mice, increased MT1 and 2 gene transcription is mediated through the binding of the transcription factor (MFT1) to the metal response element (MRE) in the promoter region (Heuchel et al., 1994, Coneyworth et al., 2012).

1.11- Zinc Deficiency and Status:

The first cases of dietary zinc deficiency in humans were described in the 1960s in male dwarfs from the Middle East (Sandstead et al., 1967). Clinical features of zinc deficiency included growth retardation, delayed sexual maturation, alopecia, delayed wound healing, impaired appetite and immune deficiencies (Mahan and Escoot-Stump, 2004). The major factor associated with the development of zinc deficiency at the population level is inadequate dietary zinc intake. High physiological requirements for zinc (such as during infancy, adolescence, pregnancy and lactation), decreased dietary zinc absorption efficiency and treatment with certain drugs (e.g. penicillamine, thiazide and glucagon) are additional influencing factors (Gibson et al., 2008).

To assess zinc status and estimate the risk of zinc deficiency at the population level, three types of indicators are recommended by WHO/UNICEF/IAEA/IZiNCG i.e. serum zinc concentration, dietary intake and height-for –age in children aged<5 years (de Benoist et al., 2007). When the prevalence of low serum zinc concentration (less than the age/sex/time of day-specific cutoffs shown in Table 1.10) is more than 20%, interventions to improve zinc status are recommended. Additionally, when the prevalence of inadequate zinc intakes is greater than 25%, the risk of zinc deficiency is considered to be high (de Benoist et al., 2007). Other zinc biomarkers such concentration of zinc in hair, cells, zinc-metalloenzymes, metallothionein (Grider et al., 1990), metallothionein mRNA, ZIP1 transporter levels (Andree et al., 2004) and the size of the exchangeable zinc pool may also be valid, sensitive markers of zinc status (Gibson et al., 2008).

Table 1.10 Suggested lower cutoffs for the assessment of serum zinc concentration in population studies

	Low cutoffs of serum zinc concentration µg/dl (µmol/L)		
	Children aged <10 years	**Females ≥ 10 years**	**Males≥10 years**
Morning fasting	--	70 (10.7)	74 (11.3)
Morning non-fasting	65 (9.9)	66 (10.1)	70 (10.7)
Afternoon	57 (8.7)	59 (9.0)	61 (9.3)
Pregnancy			
1st trimester		56 (8.6)	
2nd trimester		50 (7.6)	

Source: (Hotz et al., 2003b, Gibson et al., 2008)

Table 1.11 provides an overview of zinc status in all age groups in Saudi Arabia. Briefly, low zinc intakes were found in adolescents (Washi and Ageib, 2010) and elderly females (Sadiq, 2005b) but not in males (Alenezy, 2003a). In 1997 in a study of all ages, Kumosani et al found that serum zinc concentration ranged from 0.5 - 13.90 µmol/L, which is lower than the international established standard. More recent studies have shown that low serum zinc concentrations persist in infants and preschool children (Bahijri, 2001, Alshatwi, 2006) but not adults (20-54 years) (Al-Numair, 2006). Pregnant females have also been reported to have lower serum zinc concentrations than non-pregnant women, and that serum zinc concentration decreases as pregnancy progresses (Abdul-Jabbar and Kumosani, 1998). Older adults appeared to have low zinc status compared with young adults. This age-related decrease was also found in other countries (Table1.12).

Table 1.11 Measures relevant to zinc status in different age groups of the Saudi population

Age group	Sample size and sex	Zinc index	Results (mean)	Reference
All age groups	276 males and females	Serum zinc	0-1 year (10.8 µmol/L) 1-3 years (4.6 µmol/L) 3-6 years (4.2 µmol/L) 6-10 years (3.6 µmol/L) 10-14 years (3.5 µmol/L) 14-18 years (9.3 µmol/L) 18-22 years (10.2 µmol/L) 22-50 years (8.6 µmol/L) >50 years (6.1 µmol/L)	(Kumosani et al., 1997b)
Infants and young preschool children	728 Boys And girls	Serum zinc	4-72 months (8.91 µmol/L)	(Bahijri, 2001)
Preschool children	178 boys and girls	Serum zinc Zinc intake	2-6 years (12.1 µmol/L) 2-3 years (5.6 mg/day) 4-6 years (5.8 mg/day)	(Alshatwi, 2006)
Adolescents	239 males and females	Zinc intake	13-18 years (8.68 mg/day)	(Washi and Ageib, 2010)
Pregnant women	123 healthy pregnant and non-pregnant	Serum zinc	Pregnant (3.78 µmol/L) Non-pregnant (8.86 µmol/L)	(Abdul-Jabbar and Kumosani, 1998)

Age group	Sample size and sex	Zinc index	Results (mean)	Reference
Adults	100 females and males	Serum zinc Zinc intake	20-54 years (79.12 µg/dL) 20-54 years (9.40 mg/day)	(Al-Numair, 2006)
Young and older adults	303 males	Zinc intake	<33 years (9.26 mg/day) 34-49 years (8.94 mg/day) ≥50 years (8.75 mg/day)	(Alissa, 2005)
Elderly	404 males	Zinc intake	60-70 (mean zinc intake above RDA (119%))	(Alenezy, 2003a)
Elderly	200 females	Zinc intake	55- >75 years (5.66 mg/day)	(Sadiq, 2005b)

Table 1.12 Age-related decline in serum zinc concentration in different countries

Country	Sample size and sex	Results (mean)	Reference
Tunisia	200 males and females	30-60 years (11.61 µmol/L) >65 years (10.5 µmol/L)	(Sfar et al., 2011)
Thailand	1113 males and females	20-35 years (19.12 µmol/L) 51-75 years (18.05 µmol/L)	(Boonsiri et al., 2006)
Italy	152 males and females	60-90 years (14.51 µmol/L) 91-110 years (11.9 µmol/L)	(Savarino et al., 2001)
Five European countries (Italy, Greece, France, Poland and Germany)	853 males and females	60-69 years (12.99 µmol/L) 70-74 years (12.3 µmol/L) 75-79 years (12.12 µmol/L) 80-84 years (11.91 µmol/L)	(Marcellini et al., 2006)

1.12- Zinc Toxicity:

A tolerable upper level (UL) for zinc intake by adults, including pregnant and lactating women of over 19 years, was set by the US Food and Nutrition Board at 40 mg per day (Institute of medicine, 2001). High intakes (50 to 450 mg) may cause diarrhea, vomiting, headache and other symptoms (Whitney and Rolfes, 2005).

1.13- Project Hypotheses, Aims and Objectives:

My project hypotheses are:

1) Zinc intake and plasma zinc concentrations in Saudi elderly are lower than in young Saudi adults.
2) Ageing changes the expression of zinc transporter genes including *SLC30A1*, *SLC30A5*, *SLC39A4*.
3) Age-related changes in expression of these zinc transporter genes may be associated with changes in zinc intake and plasma zinc concentration in young and older Saudi adults.

My aims are to test these hypotheses by addressing the following objectives:

1) To design and test the relative validity and repeatability of a zinc-specific food frequency questionnaire suitable for use by Saudi adults in order to measure zinc intake.
2) To measure the dietary zinc intake and plasma zinc concentrations of a sample of young and older adults in Jeddah, Saudi Arabia.
3) To examine the effects of ageing on the expression of the zinc transporters *SLC30A1*, *SLC30A5* and *SLC39A4*.
4) To assess whether differences in zinc status between young and older adults are associated with altered expression of the zinc transporter genes.

Chapter 2. Materials and Methods

2.1- Development of the Questionnaire:

The interviewer-administered questionnaire has been designed to collect general demographic information, medical history, smoking habits and socioeconomic status (Appendix A). Questions were derived from previous studies and/or newly developed after consultation with experts in appropriate fields including economics, social sciences, public health and nutrition. In addition, dietary zinc intake was estimated using a specially modified food frequency questionnaire (FFQ). A convenience sample of adults was used to pilot the questionnaire and feedback from this group was used to produce the final version of the questionnaire.

2.1.1- Demographic features:

Demographic features including: sex, age, weight, BMI, waist circumference, hip circumference and waist-to-hip ratio were assessed for participants.

Body weight was assessed by weighing the subject without shoes by using electronic scale (Seca). Height was also measured without shoes using a Seca Portable stadiometer. Measurements of weight and height were made to the nearest 0.1 kg and 0.1 cm, respectively. BMI was calculated by dividing the body weight in kg by the square of the height in m (Kg/m^2). Participants were classified, according to WHO classification, into underweight (BMI= < 18.50), normal (BMI= 18.5-24.99), overweight (BMI= 25-29.99) and obese (BMI $\geq$ 30).

Waist and hips circumferences were measured using a Seca tape for a subgroup of participants only. The maximum circumference over the buttocks was measured as the hip circumference. Waist-to-hip ratio (WHR) was calculated by dividing the waist by the hip circumference. WHR is a simple method to differentiate between fatness in the lower part of the body (hip and buttocks) and fatness in the upper part of the body (waist and abdomen areas). Subjects were indicated as having abdominal fat accumulation if their WHR >0.9 for males and >0.8 for females (Thompson and Manore, 2009a).

2.1.2- Medical history:

Several health problems may affect zinc digestion, absorption, metabolism and excretion. Thus, participants were questioned about health problems including: cancer, high blood pressure, heart disease, liver disease, kidney disease, arthritis, osteoporosis, diabetes and lung disease. Other questions regarding the usage of dietary supplementation, consumption of herbs, dieting were also included.

2.1.3- Smoking habits:

Four categories were used to describe the smoking habits of the participants i.e. smoker, former smoker, passive smoker and non-smoker. Further questions about number of cigarettes and/or happly bubbly (a device used for smoking fermented fruits, glycerin and tobacco in which the smoke passes through water before it is inhaled) usually smoked were also included in the questionnaire.

2.1. 4- Socioeconomic status:

Questions about the education status, occupation and household annual income were included in the questionnaire to describe the socioeconomic status of participants.

2.1.5- Assessment of dietary zinc intake:

A previously-validated semi-quantitative FFQ (Samman et al., 2010) developed in Australia was modified for use in assessing dietary zinc intake by Saudi adults. Modifications were based on a comprehensive list of foods and drinks consumed by 17892 Saudis obtained in a previous survey (Almuhaizie and Albehairy, 2003). This information was supplemented by data from a 24-h recall to collect new and recent data on food and drinks consumption. Saudi mixed dishes e.g. rice with milk and rice with tomato sauce that contain zinc in excess of 0.5 mg /100 g of the edible portion (Musaiger, 2006), according to the food composition tables for Arab gulf countries, were included in the FFQ developed for the present study. Foods with a high zinc content but prohibited or rarely consumed by Saudis e.g. pork, canned fish, raw oysters and quiche were excluded. Phytate-rich foods which inhibit the absorption of zinc and protein-rich foods which enhance absorption were included in the FFQ. Beef, lamb, liver and chicken are examples of high zinc and protein foods in the Saudi diet while chickpeas, broad beans, rice, potato and flafel (beans and vegetables patties) are high in phytic acid. The FFQ collected information on foods eaten alone as well as the same foods when consumed in mixed dishes e.g. white rice alone and rice with milk (saleeq).

Sixty four food items were classified into ten categories: meat (8 items), seafood (4 items), egg (1 item), dairy products (7 items), vegetables (9 items), seeds and nuts (2 items), cereals (19 items), beverages (1 item) and miscellaneous (12 items) (Table 2.1). Relatively simple and unambiguous food items such as egg were placed at the start of the questionnaire since this approach may help study participants to get used to the format of the questionnaire and so decrease reporting errors (Cade et al., 2002). Food items

relatively rich in zinc such as meats and seafood were placed in the FFQ shortly after egg because the accuracy of participants' responses may decline towards the end of the questionnaire due to fatigue or boredom (Cade et al., 2002).

For each food item, a standard serving (medium serve) was expressed in commonly used portions such as grams, cups, table-spoons, slices or pieces (Alissa, 2005, Samman et al., 2010). Participants were asked to recall how often, on average, they had consumed each food over the past year and how their usual serving size differed from that of the standard serve (i.e. small or large). Pictures of food portion sizes were used to aid participant recall (Nelson et al.). The frequency of intake was assessed on an ascending eight-point scale: never, less than once/month, 1-3 times/month, once/week, 2-4 times/week, 5-6 times/week, once/day and twice or more/day. This scale was used because it covers the usual range in frequency of consumption of foods by Saudis; a few foods such as meat or cheese pie are consumed 2-4 times/week whereas others e.g. rice and breads are consumed more than once per day. Finally, participants were asked to record any food items that were usually eaten and which were not included in the list. Between 2 and 6 weeks (average of 4 weeks) after the first administration of the FFQ (FFQ1) all participants completed the FFQ for the second time (FFQ2) to test the repeatability of the FFQ.

Zinc intake from the FFQ was calculated by multiplying the amount of zinc (mg) in a medium serve by serving size factor and frequency factor. Serving size factors were 0.5, 1, 1.5 for small, medium and large serving, respectively. The frequency of intake per day was calculated as follows: 0, 1/60, 2/30, 1/7, 3/7, 5.5/7, 1 and 2 for the eight-point scale respectively. Total daily estimated zinc intake for each participant was calculated by summing the intakes from each food item. Phytic acid and protein intake were calculated in the same way (see appendix B).

An open-ended record of food intake (Food Record; FR) on two sequential week days and one weekend day was used as a reference method for validating the FFQ and were done in the same week after the administration of FFQ1. All participants were given instruction about filling out the FR. In addition, each participant was provided with a booklet of serving size pictures to aid them in recording the quantity of the foods and drinks consumed. Zinc, phytic acid and protein intakes from food records and FFQ were analyzed using the Nutrition Data System for Research software version 2012, developed by the Nutrition Coordinating Center (NCC), University of Minnesota, Minneapolis, MN after entering the analysis of Saudi dishes (Musaiger, 2006).

Table 2.1 Food items (serving size) included in the food frequency questionnaire

Eggs	**Vegetables**	white toast (1 slice; 25 g)
eggs (1 egg)	potato(1 medium; 150 g)	white pasta (1 cup; 100 g)
Meat	broccoli (1 cup; 80 g)	white rice (1 cup; 175 g)
beef (120g)	spinach, silver beet (1/3 cup; 60 g)	rice with milk (saleeq) (1 cup)
lamb (2 chops; 100g)	tabouli (1 cup; 200 g)	rice with tomato sauce (kabsa) (1 cup)
veal (1 chop; 90g)	okra with tomato sauce (1 cup)	rice with lentil (1 cup)
diced meat(kabab or hamburger) (150 g)	vegetables, stuffed (1 piece)	whole meal crisp bread (1; 5 g)
meat shawerma (1 medium sandwich)	chickpeas (1/2 cup; 200 g)	rolled oats (boiled) (1 cup; 230 g)
chicken shawerma (1 medium sandwich)	broad bean (1/2 cup; 100 g)	fruit cake/loaf (1 slice; 75 g)
chicken (1 breast or thigh; 110 g)	**Fruits**	muffin (1; 55 g)
liver (3/4 cup; 150 g)	citrus fruits (1 piece)	biscuits (bran, whole meal) (1; 20 g)
Seafood	**Beverages: Cocoa**	**Miscellaneous**
fish (whole; 1 fillet; 120 g)	drinking chocolate (1tbsp; 9g)	meat pie (sambosak) (1 piece)
oysters (smoked; 1; 5 g)	**Seeds and nuts**	cheese pie (sambosak) (1 piece)
crab/scallops (1/2 cup; 90g)	sesame butter (1 teaspoon)	meat pie (fatayer) (160 g)
lobster/prawn/squid (1/2 cup; 90 g)	nuts (15 g)	cheese pie (fatayer) (160 g)
Dairy	**Cereals**	pizza (150 g)
cheese (1 slice; 20 g)	whole grain corn flakes (1 cup; 30 g)	Beans and vegetables patty (tameya, flafel) (3 pieces)
yogurt (1 cartoon; 200 g)	corn flakes (1 cup; 30 g)	popcorn (1 cup)
cow's milk (1 glass; 200 ml)	brown pita bread (1 slice; 25 g)	chips/ corn chips/ twisties (50 g)
cow's milk (with cereal) (1/2 cup; 125 ml)	white pita bread (1 slice; 25 g)	chocolate (1 bar)
cow's milk (in tea/coffee) (2 tbsp; 40 ml)	brown loaf bread (1 medium size)	custard (1/2 cup)
fermented milk (laban) (1 glass; 200 ml)	white loaf bread (1 medium size)	rice with milk and sugar (mohalabeya) (1/2 cup)
ice cream (2 scoops; 60 g)	brown toast (1 slice; 25 g)	cream caramel (1/2 cup)

2.2 – Clinical Chemistry Tests:

Complete blood count (CBC) and a range of clinical chemistry tests were undertaken to provide an overview of the participant's general health. Numbers of white blood cells, red blood cells and the amount of hemoglobin in fresh blood samples collected in EDTA-tube were measured usinga Beckman coulter instrument. Serum samples, separated from plain tubes, were stored at -80°c for later clinical chemistry measurements including glucose, albumin, creatinine, Alanine aminotransferase (ALT) and C-Reactive protein (CRP) using an Elitech clinical system instrument.

2.3- Analysis of Plasma Zinc and other Minerals:

2.3.1- Reagents and glassware:

Hydrochloric acid was ultra-pure (MERCK). All glassware was decontaminated by soaking it in ultra-pure 20% HCL for 24 hours and rinsed 3-4 times with deionized water.

2.3.2- Preparation of plasm samples and sample diluent:

Three milliliters of blood were collected in trace-element free tubes according to the instruction provided by IZiNCG (IZiNCG, 2007a). Plasma samples were separated and stored at -80ºC for later use. A 1:10 dilution of plasma samples were prepared by adding 0.5 ml of plasma to 4.5 ml of sample diluent. Sample diluent was prepared by adding 8.34 ml of ultra-pure HCl in 2 L volumetric flask and the volume was completed to the mark by adding MilliQ water. Sample diluent was used as blank.

2.3.3- Preparation of standard curve:

A three point standard curve was used for the determination of plasma zinc and other minerals including copper, selenium, magnesium, manganese, iron, calcium, nickel, arsenic and lead. First, a stock solution at a concentration of 50 ppm was prepared by adding 25ml from 100 ppm AccuTrace Reference Standard ICP Quality control standard#1 (Accustandard) to 50 ml volumetric flask and the volume was completed to the mark by milliQ water. Then, another stock solution at a concentration of 10ppm was prepared by adding 10 ml from the first stock to 50ml volumetric flask and the volume was completed to mark by milliQ water. The second solution was used to prepare the three standard solutions. Next, 2.5 ml of glycerol was added to three 25ml volumetric flasks in an attempt to match the matrix of the plasma. From

the 10ppm stock solution, three aliquots of 2.5, 1.25 and 0.625 ml were taken and added to each volumetric flask and the volume were completed by adding sample diluent to prepare the three standard solutions with 1, 0.5 and 0.25 ppm concentrations, respectively (IZiNCG, 2012b).

2.3.4- Analytical method:

Zinc and other minerals were measured in standards, plasma samples and blank (sample diluent) by using inductively coupled plasma- atomic emission spectroscopy. For each mineral, a specific wave length was used as shown in Table 2.2. Concentrations of minerals were calculated automatically by ICP-OES.

Table 2.2 Inductively coupled plasma-atomic emission spectroscopy wavelengths

Mineral	Wavelength
Zinc	213.857
Copper	324.752
Selenium	203.985
Magnesium	279.077
Manganese	259.372
Iron	259.939
Calcium	317.933
Arsenic	188.979
Lead	220.353
Nickel	231.604

2.4- Determination of Plasma Metallothionein 1:

An Enzyme-linked Immunosorbent Assay Kit for metallothionein 1 (MT1) (Uscn Life Science, USA) was used to determine plasma MT1 according to the manufacturer's protocol. Briefly, 100 µl of blank, standards and samples were added to each well in duplicate and incubated for 2 hours at 37°c. Liquid of each well was then removed without washing. One hundred micro-liter of detection reagent A was added and the plate was incubated for 1 h at 37°c. After incubation, the solution was aspirated and the plate washed 3 times by auto washer (PW40, BIORAD) using washing solution. Detection reagent B (100µl) was added and the plate was incubated for 30 min followed by aspiration and washing for 5 times. After that, TMB substrate solution (90 µl) was added and incubated for 20 min. Finally, the enzyme-substrate reaction was terminated by adding 50 µl of stop solution. The yellow color was measured spectrophotometrically at a wavelength of 450nm by microplate reader (PR3100TSC,BIORAD). The concentration of MT1 in the sample was then determined by comparing the average optical density of the samples to the standard curve (Figure 2.1).

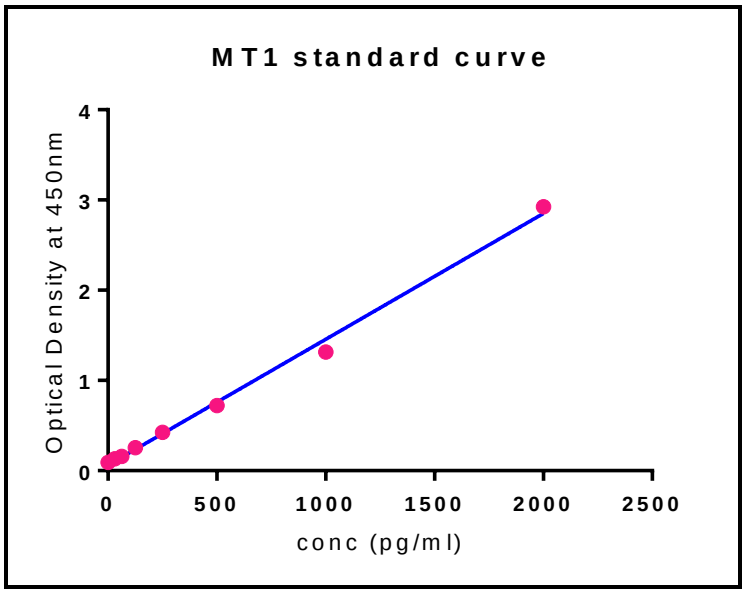


Figure 2.1 Typical standard curve of metallothionein 1 by ELISA kit

2.5- RNA Extraction from Blood:

RNA was extracted from fresh whole blood sample by using QIA amp RNA blood mini kit (Qiagen). The manufacturer's protocol was modified to give better yields of RNA. Three milliliters of fresh blood samples was diluted with an equal volume of normal saline and poured slowly on the wall of a centrifuge tube containing 5 ml of histopaque 1077 (SIGMA, life science). Then, the sample was centrifuged at 700 g for 30 min to separate the buffy coat. The buffy coat was then washed with normal saline and centrifuged at 300 g for 10 min to form the cell pellet. Lysis buffer (600 µl RLT buffer + 6 µl β-mercapto ethanol) was added to the pelleted leukocytes and the sample stored at -80°C for later RNA extraction. Seven hundred microliters of this sample were added to the QIAshredder spin columns and centrifuged for 2 min. at 20000 g. The homogenized lysate was then added to 600 µl of 70% ethanol and transferred to RNease binding spin column. Genomic RNA was bound to the spin column membrane and contaminants were removed by a series of washes using buffer RW1 and RPE. During the purification of RNA, DNA was digested by using RNAase-free DNAase set (Qiagen). Purified RNA was eluted from the spin column in a concentrated form in RNase-free water and stored at -80°c for later use.

2.6- Determination of RNA Concentrations:

Nano drop 2000 spectrophotometer (Thermo Scientific) was used to determine the concentration, absorbance at 260/280 and absorbance at 260/230 of the RNA samples. A 2 to 3 ml sample of whole human blood yields average of 120 ng/µl with an A_{260}/A_{280} ratio of > 1.8 and an A_{260}/A_{230} ratio >0.36 (Lam et al., 2012, Fleige and Pfaffl, 2006).

2.7- Determination of RNA Integrity:

The integrity of the RNA was assessed by using RNA 6000 Nanodrop Bioanalyzer Kit, which resembles RNA agarose gel electrophoresis. The RNA ladder, gel and gel-dye mixture were prepared according to the manufacturer's protocol. Gel-dye mixture, RNA 6000 Nano Marker, RNA ladder and the RNA samples were loaded in a RNA Nano Chip according to the manufacturer's protocol and then inserted into an Agilent 2100 bioanalyzer machine. Good quality, undegraded RNA was indicated by the presence of 2 horizontal bands representing the 28S and 18S ribosomal RNA and RIN values above 8.

2.8- Conversion of RNA to cDNA:

The RNA samples were diluted to approximately 500ng and converted to cDNA by using QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. Briefly, twelve microliter of the RNA samples were added to 2 µl of gDNA wipeout buffer and incubated for 2 min at 42°C. Then, RNA samples were added to tube containing reverse transcription master mix composed of Quantiscript reverse transcriptase, Quantiscript buffer and RT primer mix. For negative samples, RNA was added to reverse transcription master mix containing no transcriptase. Positive and negative samples were then incubated for 15 min at 42°C. Finally, samples were incubated for 3 min at 95°C to inactivate reverse transcriptase. The cDNA produced from the reaction was stored at -20 ºC.

2.9- Quantitative Measurement of Gene Expression:

Reverse transcription RT/PCR is the most sensitive technique to determine the expression of specific transcripts in an mRNA population. QRT/PCR was performed on cDNA samples to test the quality of the cDNA produced by checking the expression of the housekeeping gene (*GAPDH*) and to determine, quantitatively, the expression of specific genes in the study. Each 10µL of cDNA sample was diluted with 10µL of DNase/RNase free water. Two microliter of diluted samples were then loaded in fast optical 96 well reaction plates (0.1ml) in duplicate. Five microliter of Taqman universal master mix (Applied Bio Systems), 0.5 µl of primer of interest (gene assay) and 2.5 µl of DNase/RNase free water were added to each sample. Quantitative RT/PCR was performed using a StepOne plus PCR instrument with hold stage at 50 ºC (for 2 min), initial denaturation stage at 95ºC (for 10 min), denaturation stage at 95ºC (for 15 sec) and annealing stage at 60ºC (for 1 min). The last two stages make one cycle which was repeated for 40 times (40 cycles). Fluorescence was detected for each PCR cycle and the threshold crossing points (CT values) determined. For each sample, expression of targeted genes were normalized with housekeeping gene (*GAPDH*) by subtracting C_T values for *GAPDH* from C_T values of the target gene (ΔC_T). Then, $2^{(\Delta ct)}$ was calculated to determine the relative expression of each targeted gene at the mRNA level.

2.10- Analysis of T cells, B cells, natural killer and monocytes by Flow Cytometery:

Three milliliters of fresh blood sample was used to separate the buffy coat (see section 2.6) which coat was then washed with normal saline and centrifuged at 300 g for 10 min to form the cell pellet. One milliliter of freezing media (50% RPI 1640 + 40% fetal calf serum + 10% DMSO) was added to the

pelleted leukocytes and the sample stored at -80°C for later immunophenotyping of leucocytes by flow cytometery.

At time of analysis, buffy coat samples were thawed at 37°C, washed with 3ml of PBS and centrifuged at 1600 rpm for 10 min at 4°C. The cell pellet was then resuspended in 200µl PBS. From 30 to 40µl of each sample (~ 500,000 cells as counted by VI-CELL cell viability analyzer, Beckman Coulter) was added equally to 3 flow cytometery tubes. The first tube contained 20µl of cocktail monoclonal antibody (CD3/CD16/CD56 antibody, FITC,PE conjugate) (Thermo Scientific). The second tube contained 20µl of another cocktail monoclonal antibody (CD3/CD19/CD45 antibody, FITC,PE, Cy5-PE) (Thermo Scientific). The third tube contained 10µl of each of the following antibodies: CD14 PE, CD15 APC and CD45 FITC (Thermo Scientific). The mixture was incubated in a refrigerator for 30 min. and then washed and centrifuged at 1600 rpm for 7 min. and the pellet was resuspended in 1ml of PBS. Lastly, ten microliter of 7AAD dye (BioLegend) were added to tube 1 and 3 to exclude dead cells from analysis. The samples were analyzed using a Navios Flow Cytometer (Beckman Coulter). The instrument was calibrated before each run by Flowcheck Pro Fluorospheres (Beckman Coulter) to check the laser. Typical dot plot of each tube are shown in Figure 2.2. The percentage of each cell subtype was calculated by using Flowing 2 Software (http://www.flowingsoftware.com/).

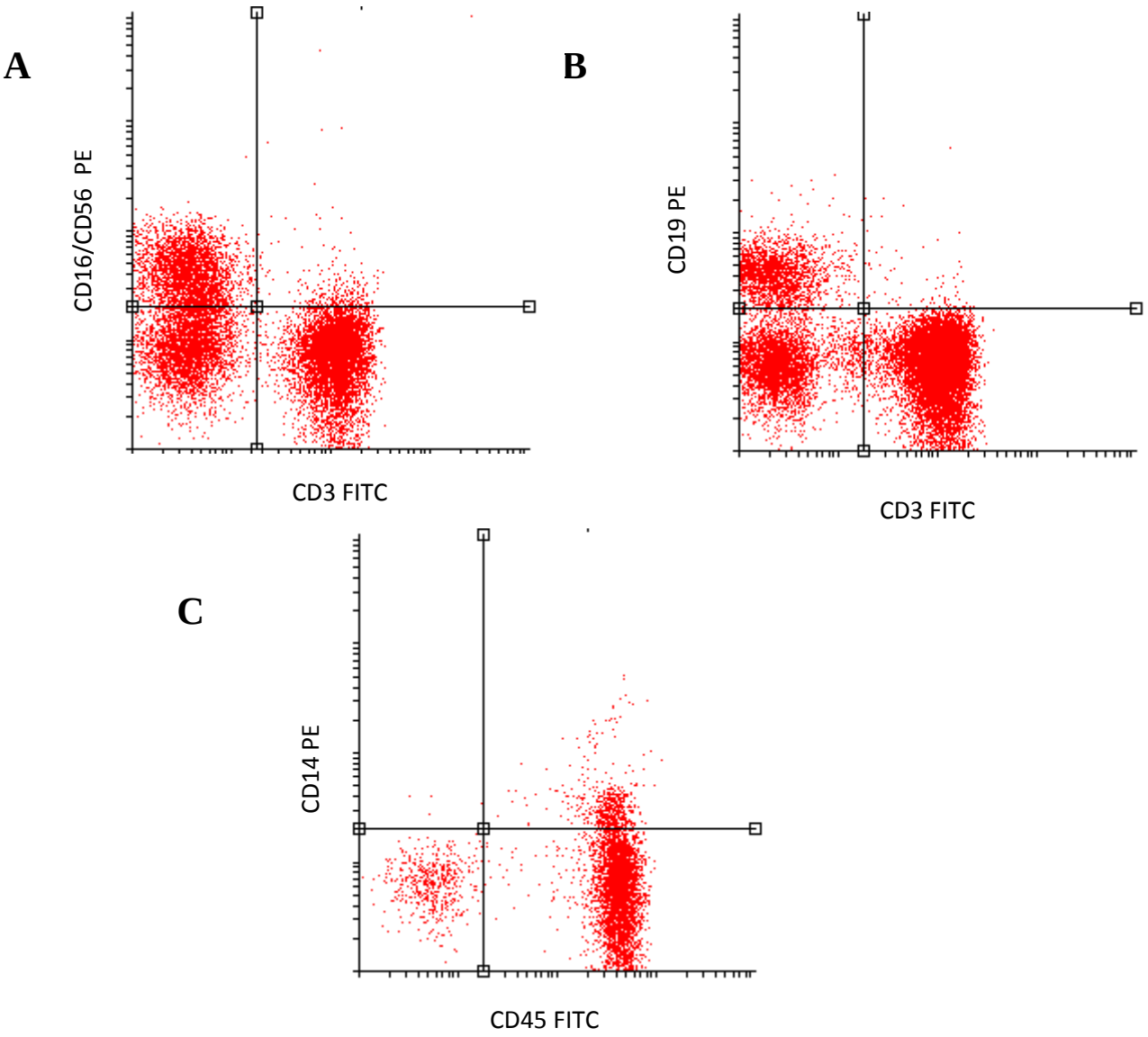


Figure 2.2 Typical dot plot for analysis of (A) CD3+ cells (T cells) and CD56+ cells (natural killer cells (B) CD19+ cells (B cells) and (C) CD14+ cells (monocytes)

Chapter 3. The Relative Validity and Repeatability of a Food Frequency Questionnaire for Estimating Intake of Zinc and its Absorption Modifiers in Young and Older Saudi Adults

3.1- Introduction:

In Saudi Arabia, there have been few studies of zinc intake and zinc status. However, the limited evidence available suggests that older adults have lower serum zinc status than young adults (Kumosani et al., 1997a, Al-Numair, 2006) and the proportion of free-living elderly males with inadequate zinc intake (below the Estimated Average Requirement (EAR)) was higher than among young adults (Alissa, 2005). Additionally, low zinc intakes were found in institutionalized females (Sadiq, 2005a) but not males (Alenezy, 2003b). For free-living older females in Saudi Arabia, there are no data on intakes of zinc or of zinc absorption modifiers. One reason for this evidence gap is the lack of a suitable tool designed specifically for quantifying intakes of zinc and its absorption modifiers by adults of all ages and both sexs in this population group. Most previous studies used either food records (FR) or 24-h recalls (Alenezy, 2003b, Sadiq, 2005a, Al-Numair, 2006); an exception was Alissa (Alissa, 2005). However, these methods requires a high degree of cooperation from participants and analysis of data is labour intensive (Lee and Nieman, 2010). A food frequency questionnaire (FFQ) may provide an adequate assessment of usual intake and has the benefits that the demand on respondents and on researchers is more modest (Lee and Nieman, 2010). Thus, the aim of my study was to design and validate a FFQ suitable for young and older Saudi adults of both sexs.

Objectives:

1) To assess the relative validity and repeatability of a FFQ for estimating dietary intake of zinc and its absorption modifiers in Saudi adults.
2) To use the FFQ to investigate the effect of age and sex on these intakes.

3.2- Materials and Methods:

3.2.1- Subjects:

One hundred male and female participants aged 20 - 30 years (younger adults) and 60 - 70 years (older adults) were recruited from King AbdulAziz University (KAU) students, staff and their families, via personal contact, by email messages and by telephone. This sample size was chosen based on the statistical tests used to assess validity and repeatability. For example, one hundred is desirable for the Bland-Altman test to allow the limits of agreement to be estimated precisely. In addition, one hundred is sufficient for the correlation coefficient tests (Cade et al., 2002). The older age range was chosen from

60 to 70 years because 60 years and above refers to older adults as it is considered as retirement age in Saudi Arabia and 74 is the life expectancy of Saudi adults. Participants were divided equally into four groups according to sex and age (25 persons per group). The study was approved by the Ethical Committee, Faculty of Medicine, King AbdulAziz University and all participants gave written informed consent.

3.2.2- Assessment of dietary intake:

To assess validity, all participants completed the FFQ (FFQ1) and a 3 d food record. After 1 month, the FFQ was administered for a second time (FFQ2) to assess repeatability (see section 2.1.5).

3.2.3- Statistical analysis:

To assess the validity of the FFQ, differences in mean intake of dietary components between the FFQ and FR were examined by using Student's paired t test. Analysis of Variance (ANOVA) was used to test the differences in dietary components intakes between age and sex groups. Correlations between values obtained from the FFQ and the FR were tested using Spearman's correlations because the data were skewed. Bland-Altman analysis was undertaken to investigate the agreement between the two methods for estimating dietary components intakes. Moreover, cross classification was used to evaluate the ability of both methods to classify individuals similarly into equal thirds of the distribution of dietary components intake. The cut-off points were determined separately for the FFQ and the FR.

To test the repeatability of the FFQ, the same statistical tests were performed between FFQ1 and FFQ2 with the exception of the cross classification. Additionally, intra-class correlation coefficients were calculated for each of the dietary components for data from FFQ1 and from FFQ2.

All statistical analyses were performed using IBM SPSS Statistics software version 19 with the exception of Bland-Altman analysis which was performed using MedCalc Statistical software version 12.6.1. Statistical significance was taken as $P< 0.05$.

3.3-Results:

3.3.1- Study participants:

One hundred volunteers participated in this study. All completed two FFQs and a 3-day FR. The majority of the younger participants were healthy while half and one third of the older adults had diabetes and high blood pressure, respectively. The mean body mass index (BMI) was 28.30 kg/m^2. More than 50% of the participants were non-smokers and 40% had a high education level (Table 3.1).

Table 3.1 Characteristics of study participants

	Young Female (n=25)	Young Male (n=25)	Older Female (n=25)	Older Male (n=25)	All Participants (n=100)
Mean age (years)	25.6	22.2	64.9	64.6	
Mean height (m)	1.59	1.71	1.57	1.69	1.64
Mean body mass (Kg)	70.9	82.6	77.3	76.6	76. 9
Mean BMI (Kg/m2)§	27.9	28.0	30.8	26.6	28.3
Health status N (%)					
cancer	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
high blood pressure	0 (0)	0 (0)	9 (36)	8 (32)	17 (17)
heart diseases	0 (0)	0 (0)	4 (16)	3 (12)	7 (7)
liver diseases	0 (0)	0 (0)	0 (0)	1 (4)	1 (1)
kidney diseases	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
osteoporosis	0 (0)	0 (0)	5 (20)	3 (12)	8 (8)
arthritis	0 (0)	0 (0)	7 (28)	2 (8)	9 (9)
diabetes	0 (0)	0 (0)	12 (48)	13 (52)	25 (25)
lung diseases	1 (4)	0 (0)	1 (4)	1 (4)	3 (3)
allergy	2 (8)	0 (0)	4 (16)	0 (0)	6 (6)
gastrointestinal disorders	2 (8)	1 (4)	5 (20)	6 (24)	14 (14)
Cigarettes smoking status N (%)					
non smoker	16 (64)	14 (56)	16 (64)	11 (44)	57 (57)
former smoker	0 (0)	4 (16)	3 (12)	6 (24)	13 (13)
passive smoker	6 (24)	2 (8)	4 (16)	2 (8)	14 (14)
smoker	3 (12)	5 (20)	2 (8)	6 (24)	16 (16)
Shisha/argela smokers $ N (%)	3 (12)	8 (32)	4 (16)	3 (12)	18 (18)
Highest grade in school					
Illiterate (didn't attend school)	0 (0)	0 (0)	1 (4)	0 (0)	1 (1)
primary	0 (0)	0 (0)	6 (24)	1 (4)	7 (7)
intermediate	0 (0)	0 (0)	6 (24)	2 (8)	8 (8)
high school	9 (36)	22 (88)	5 (20)	4 (16)	40 (40)
diploma	0 (0)	0 (0)	1 (4)	3 (12)	4 (4)
college	8 (32)	2 (8)	3 (12)	5 (20)	18 (18)
post graduate	8 (32)	1 (4)	3 (12)	10 (40)	22 (22)
Household annual income N (%)					
less than 50,000 SR	5 (20)	6 (24)	2 (8)	1 (4)	14 (14)
50,000 - < 100,000 SR	0 (0)	2 (8)	5 (20)	4 (16)	11 (11)
100,000 - < 150,000 SR	4 (16)	9 (36)	4 (16)	2 (8)	19 (19)
150,000 - < 200,000 SR	2 (8)	4 (16)	4 (16)	5 (20)	15 (15)
200,000 - < 250,000 SR	3 (12)	2 (8)	4 (16)	1 (4)	10 (10)
more than 250,000 SR	11 (44)	2 (8)	6 (24)	12 (48)	31 (31)

	Young Female (n=25)	Young Male (n=25)	Older Female (n=25)	Older Male (n=25)	All Participants (n=100)
Current Occupation N (%)					
student	9 (36)	23 (92)	0 (0)	0 (0)	32 (32)
unemployed	2 (8)	0 (0)	16 (64)	2 (8)	20 (20)
employed	14 (56)	1 (4)	1 (4)	5 (20)	21 (21)
Self-employed	0 (0)	1 (4)	1 (4)	4 (16)	6 (6)
retired	0 (0)	0 (0)	7 (28)	14 (56)	21 (21)

§BMI, Body Mass Index, $ shisha/argela, consists of tobacco, molasses or honey and dried fruits.

3.3.2- Relative validity:

We assessed the validity of the FFQ by comparing data collected using the first administration of the FFQ (FFQ1) with data from the 3-day FR (raw data for intake of food components are summarized in Appendix C). Table 3.2 shows the mean daily intakes of zinc, phytic acid and protein estimated by the FFQ1 and the 3-day FR. Mean intakes of zinc and protein from FFQ1 were significantly higher than those from the FR ($P< 0.001$ and $P=0.013$, respectively). In contrast, there were no statistically significant differences between estimates obtained by FFQ1 and the FR for the mean intake of phytic acid ($P= 0.792$). Significant correlation coefficients were found between zinc and protein intakes obtained from FFQ1 and the FR (Table 3.3).

Table 3.2 Mean daily intake of zinc, phytic acid and protein assessed by FFQ1, FR and FFQ2 (n=100)

	FFQ1	FR	95% confidence intervals of the difference $		FFQ2	95% confidence intervals of the difference ¥	
Intake(unit/day)	mean	mean	lower	upper	mean	lower	upper
Zinc (mg/day)	10.6	8.2 ***	1.52	3.18	10.0 *	0.08	1.08
Phytic acid (mg/day)	521	511	-61.93	80.94	495	-7.92	60.77
Protein (g/day)	76.4	68.7*	1.66	13.77	72.9*	0.16	6.92

FFQ, Food Frequency Questionnaire, FR, Food Record, $ paired differences between FFQ1 and FR. ¥ paired differences between FFQ1 and FFQ2. Mean values are significantly different from those of FFQ1: * P<0.05, *** p<0.001

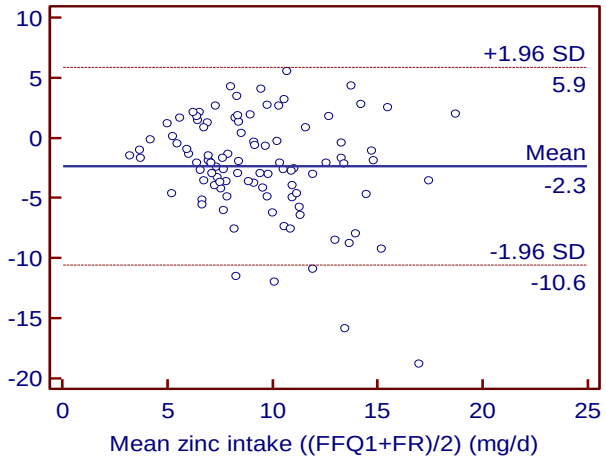
Table 3.3 Spearman correlation coefficients (r) between intakes estimated from FFQ1 and FR (Validity) and between FFQ1 and FFQ2 (Repeatability) (n=100)

Item (unit/day)	r between FFQ1 and FR	r between FFQ1 and FFQ2
zinc (mg/day)	0.410***	0.758***
phytic acid (mg/day)	0.110	0.628***
protein (g/day)	0.429***	0.799***

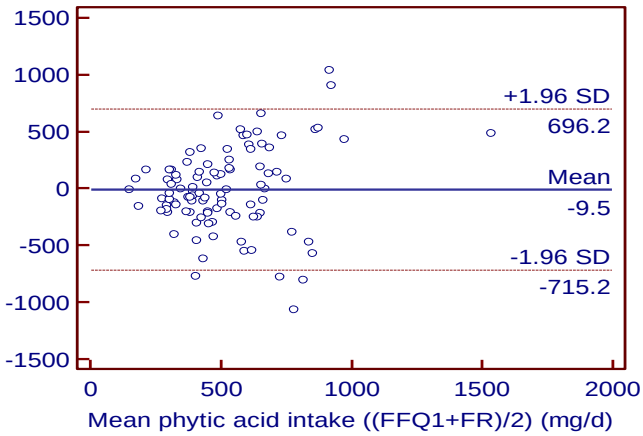
FFQ, Food Frequency Questionnaire, FR, Food Record, r, Spearman correlation coefficient, *** P<0.001

Bland-Altman analysis was performed to reveal if use of FFQ1 compared with the 3 day FR resulted in any bias in measured intake values across the range of values and to obtain values for limits of agreement between the two methods. For this purpose, the differences in the estimates of zinc, protein and phytic acid intake between the two methods (FR-FFQ1) were plotted as a function of the mean zinc, phytic acid and protein intakes estimated by the two methods ((FFQ1+FR)/2). Limits of agreement and mean differences, respectively, were: for zinc -10.6 to +5.9, mean difference -2.3 mg/d (Figure 3.1A); for intake of phytic acid -715 to +696, mean difference -9.5 mg/d (Figure 3.1B); for protein -67.5 to +52.1, mean difference -7.7 g/d (Figure 3.1C). For zinc and phytic acid, the difference in intake estimated by the FFQ1 and the FR increased with increased mean intake (Figure 3.1 A and B, respectively). However, the difference in protein intake as measured by the two different methods was consistent across the full range of intakes (Figure 3.1C). From the Bland-Altman plot (for both zinc and protein) the number of outliers is only 4 or 5 which is not likely to affect the validation of the FFQ (Figure 3.1 A and C).

(A)



(B)



(C)

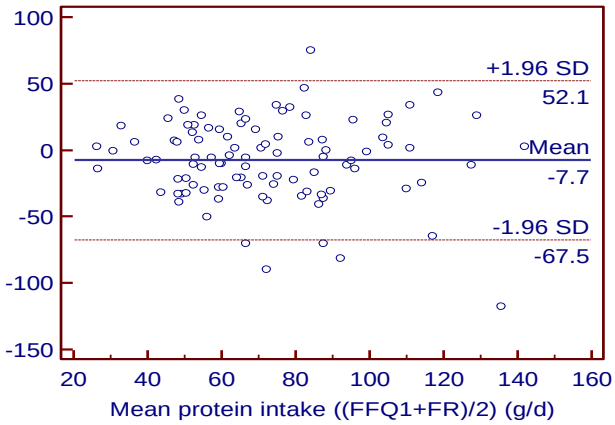


Figure 3.1 Bland-Altman plots showing the relationship between difference in daily intake of (a) zinc, (b) phytic acid, (c) protein estimated by the FFQ1 and 3 days FR, and the corresponding mean daily intake estimated by the two methods. Lines are mean difference and dotted lines are the lower and upper 95% limits of agreements

The extent of agreement between measurement methods in classifying individuals into the same or extreme tertiles of the intake distribution are shown in Table 3.4. The proportion of participants correctly categorized in the same tertile ranged from 55 % (phytic acid) to 62% (zinc). Zinc and protein intake showed the lowest proportion of misclassification (2%), whereas the highest degree of misclassification was observed for phytic acid intake (8%).

Table 3.4 Cross-classification of daily intakes derived from the FFQ1 and the FR (n=100)

Item (unit/day)	Correctly classified	Grossly misclassified
	(same tertile) (%)	**(extreme tertiles)** (%)
zinc (mg/day)	62	2
phytic acid (mg/day)	55	8
protein (g/day)	57	2

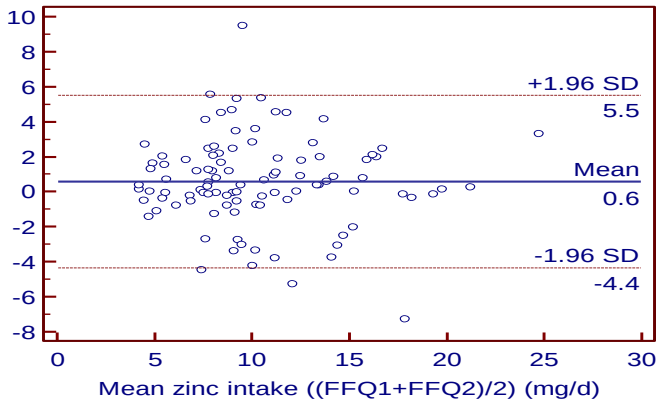
FFQ, Food Frequency Questionnaire, FR, Food Record

3.3.3- Repeatability:

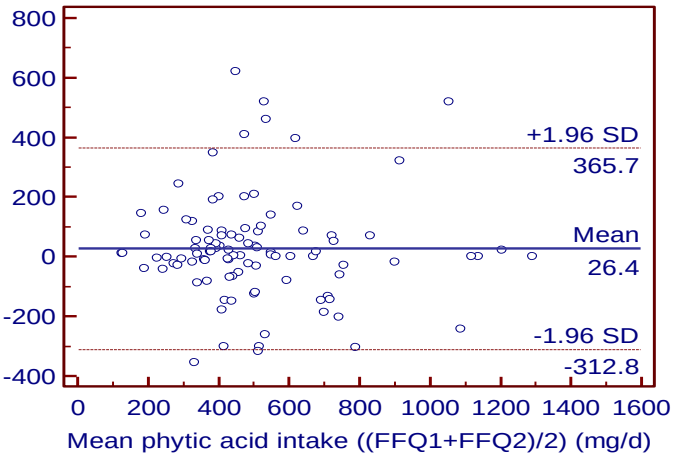
We assessed the repeatability of the FFQ by comparing data collected using FFQ1 and data collected using the second administration of the same FFQ (FFQ2) (Table 3.2) (raw data for intake of food components are summarized in Appendix C). Daily zinc and protein intakes from FFQ2 were slightly but significantly lower than from FFQ1 by ~0.5 mg and ~4 g (P=0.024 and P=0.040, respectively) whereas estimates of phytic acid intake were not significantly different (P= 0.130). Intakes of zinc, phytic acid and protein from FFQ1 and FFQ2 were highly correlated (Table 3.3).

Figure 3.2 presents the limits of agreement and the mean difference in estimated dietary intake obtained from FFQ1 and FFQ2. For all dietary components, the limits of agreement were narrower than those obtained for the comparison between FFQ1 and FR. The mean differences, for zinc and protein, between the two FFQs were smaller and for phytic acid was larger than those obtained between FFQ1 and FR.

(A)



(B)



(C)

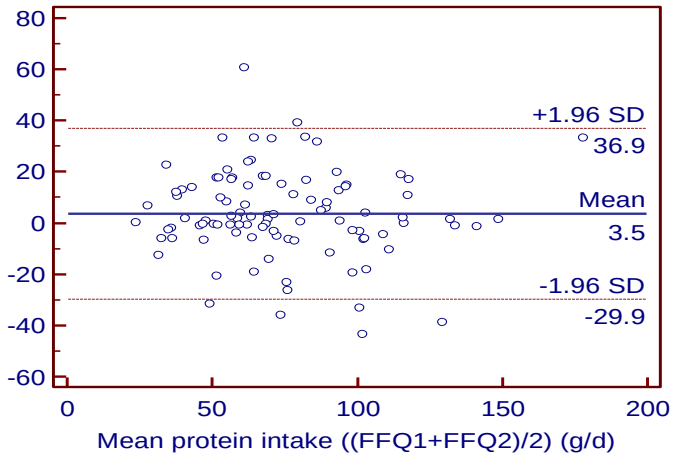


Figure 3.2 Bland-Altman plots showing the relationship between difference in daily intake of (a) zinc, (b) phytic acid, (c) protein estimated by the FFQ1 and FFQ2, and the corresponding mean daily intake

estimated by the two methods. Lines are mean difference and dotted lines are the lower and upper 95% limits of agreements

3.3.4- Effects of age and sex on intakes of zinc and zinc absorption modifiers in Saudi adults

The mean intakes by sex and age groups estimated using each of the two methods are displayed in Table 3.5. Irrespective of whether measured using FFQ1, or the 3-day FR, we observed that young adults consumed more zinc and protein than older adults. There were no detectable effects of age on phytic acid intakes by either dietary assessment method. In contrast, for other dietary components, differences in intakes between groups were statistically significant only when measured by the FR. Intake of phytic acid was 100 mg higher in males than females (P=0.033). Females consumed ~ 2 mg/d less zinc than males (P=0.002). Similarly, female intake of protein was lower, by approximately ~ 22 g/d, than that for males (P < 0.001).

Both FFQs indicated that older adults consumed less zinc and protein than young adults (P=0.002 and P=0.006, respectively) (Table 3.5). Other differences in intakes between groups were statistically significant only when measured by the second FFQ. We observed that males consumed more zinc and protein than females (P=0.021 and P=0.045, respectively).

Table 3.5 Mean daily intake of zinc, phytic acid and protein estimated by FFQ1, FR , FFQ2 by age and sex groups (n=100)

	Male		Female		Pooled SE	Probability of effect		
	Young	**Older**	**Young**	**Older**		**Age**	**Sex**	**Age*Sex**
FFQ1								
zinc (mg/day)	12.9	9.5	10.6	9.3	0.81	***0.005***	0.126	0.157
phytic acid (mg/day)	566	464	567	488	48.54	0.065	0.80	0.813
protein (g/day)	92.6	68.8	75.0	69.1	5.71	***0.011***	0.138	0.12
Food Record								
zinc (mg/day)	10.2	8.2	7.6	6.9	0.63	***0.037***	***0.002***	0.271
phytic acid (mg/day)	579	574	458	436	59.92	0.827	***0.033***	0.891
protein (g/day)	88.4	71.4	59.4	55.5	5.04	***0.041***	***0.00***	0.195
FFQ2								
zinc (mg/day)	12.6	9.3	10.0	8.0	0.82	***0.002***	***0.021***	0.375
phytic acid (mg/day)	555	477	520	428	49.16	0.085	0.395	0.886
protein (g/day)	89.8	67.9	72.6	61.1	5.9	***0.006***	***0.045***	0.376

FFQ, Food Frequency Questionnaire, FR, Food Record

3.4- Discussion:

The 64-item FFQ developed for this study was used successfully to obtain estimates of intakes of zinc, protein and phytic acid by all 100 young and older Saudi adults recruited for this study.

3.4.1- Relative validity:

The FFQ yielded higher estimates of zinc intake than were obtained from the FR. A similar difference in estimates of zinc intake between a 74-item FFQ and a 7-day weighed record was also reported by Samman et al (Samman et al., 2010). Although FFQs can both under- and overestimate nutrient intake, many validation studies have reported that FFQ overestimate nutrient intakes when compared with FR or 24 h recalls (Segovia-Siapco et al., 2007, Sebring et al., 2007, Schaefer et al., 2000). Of course, whether estimates of intake obtained using a FR is reliable cannot be known with certainty but it is evident that our FFQ yielded consistently higher estimates of zinc intake than did the FR (as confirmed by Bland-Altman analysis). Potential reason for this difference is the inclusion of large number of food items focusing on specific dietary components of interest which may lead to overestimates of intakes. Other factors include measurement errors caused by over-reporting of frequency of food consumption and serving sizes (Segovia-Siapco et al., 2007) and the decrease of participants accuracy toward the end of the questionnaire.

The observed correlations coefficients between the FFQ and FR for estimates of zinc, phytic acid and protein intakes compare favorably with those reported in other studies using the same reference method (multiple days of FR) (Barrat et al., 2012, Barrett and Gibson, 2010, Na and Lee, 2012, Lee et al., 2006). The higher correlations between methods reported by Samman et al (Samman et al., 2010) and Heath et al (Heath et al., 2000) may result from the use of a different reference instrument i.e. weighed diet records rather than estimated FR.

In the present study, the limits of agreement between the FFQ and the FR and the mean difference between them were not unreasonable for any dietary components measured. When compared with the results obtained by Samman et al (Samman et al., 2010), the limits of agreement between the two methods in estimating zinc intake were wider and the mean difference was slightly higher. Conversely, our estimate of the limits of agreement for protein intake was narrower than that reported by Pakseresht and Sharma (Pakseresht and Sharma, 2010) and the mean difference was lower. The authors of the latter study chose 24-h recalls as a reference method which shares with FFQs some of the same sources of

potential measurement error including recall bias, conceptualization of portion sizes and distortion of reported diet (Barrat et al., 2012). Bland-Altman plots of phytic acid intake showed that the agreement between the two methods was better for participants with lower phytic acid intake. This indicates that participants with higher intakes may be over-reporting their intake in the FFQ (Pakseresht and Sharma, 2010) or that the FR method does not capture accurately the intakes of high consumers. To our knowledge, this is the first study to use Bland-Altman analysis to evaluate phytic acid intake. The cross-classification analysis revealed that more than 50% of participants were correctly classified into the same third, and less than 10% were misclassified into opposite thirds, of dietary components intake. These results are in line with recommendations (Masson et al., 2003) and consistent with other studies assessing the ability of FFQs and FRs to classify nutrient intakes into tertiles (Lee et al., 2006, Barrett and Gibson, 2010). The favorable tertile classifications that were obtained suggest that our FFQ is suitable for ranking individuals correctly according to their nutrient intake.

3.4.2- Repeatability:

Several studies have reported lower estimates of nutrient intake when a FFQ was used a second time with the same participants (Barrat et al., 2012, Lee et al., 2006). In our study, intake estimates with FFQ2 were consistently lower than with FFQ1 but the differences were small for zinc and for the zinc absorption modifiers investigated (1.0 - 5.5% only) (Table 3). Barrat et al (2012) suggested that such decreases may be due, in part, to a learning effect (i.e. participants responded more accurately in the second FFQ and thus real dietary habits were reflected) (Barrat et al., 2012). Alternatively, the chore of completing the FFQ on a second or subsequent occasion may lead to less careful completion of the questionnaire. Nonetheless, Lee et al (2006) found that correlations between the first FFQ and the best estimate from dietary records (DRs) were slightly lower than those between a third application of the FFQ and the DRs, confirming the possibility of a learning effect (Lee et al., 2006). This trend is confirmed by findings from the present study in which correlations between the second FFQ and the FR were slightly higher than those obtained from the first FFQ (results not shown).

Correlations coefficients, between FFQ1 and 2, observed in the current study were very similar to those reported in a short-term repeatability study by Barrat et al (Barrat et al., 2012) and slightly higher than those reported by Jia et al (Jia et al., 2008), Barrett et al (Barrett and Gibson, 2010), Alissa (Alissa, 2005), Fernandez-Ballart et al (Fernandez-Ballart et al., 2010) and Lee et al (Lee et al., 2006). It should be noted that the time periods between the two FFQs in the latter studies were longer (from 3 months up to 1 year

compared with 1 month in the present study), which increased the likelihood of real changes in dietary habits and thus reduced correlation coefficients (Barrat et al., 2012).

3.4.3- Effects of age and sex on intakes of zinc and zinc absorption modifiers in Saudi adults:

The mean daily zinc intake assessed by the FFQ (10.6 mg) is very similar to the mean values (10.50 mg) obtained by Samman et al (Samman et al., 2010) in Australia and Alissa (Alissa, 2005) (10.23 mg) in Saudi Arabia but substantially lower than that reported by Barrett et al (Barrett and Gibson, 2010) (15.3mg) for Australian participants using the FFQ method. The mean phytic acid intake of Saudi adults (521 mg) was lower than intake of British (917 mg) (Amirabdollahian and Ash, 2010) and New Zealand adults (1498 mg) (Heath et al., 2000) using the 4-7 days weighed intake method and FFQ, respectively. The mean daily intake of protein was 76.4 g which is comparable with the result of another Saudi study (72.53 g) (Alissa, 2005) and lower than those reported in Taiwanese (87 g) (Lee et al., 2006), Spanish (103.1 g) (Fernandez-Ballart et al., 2010) and Australian (105.9 g) (Barrett and Gibson, 2010) studies when using FFQ method.

Several studies found that mean zinc and protein intake were lower in older adults than young adults when estimated by various dietary methods (e.g. FFQ and 24-h recall), in agreement with our findings irrespective of the method of measurement (Alissa, 2005, Adamson et al., 2009). Lower intake of phytic acid, zinc and protein in women than in men, as observed in our study, has been reported previously by Mcdaid et al (McDaid et al., 2007), Amirabdollahian and Ash (Amirabdollahian and Ash, 2010), Coulibaly et al (Coulibaly et al., 2009), and Adamson et al (Adamson et al., 2009).

Chapter 4. Dietary Intakes of Zinc and Its Absorption Modifiers in Young and Older Saudi Adults

4.1- Introduction:

The first cases of dietary zinc deficiency in humans were described in the 1960s in male dwarfs from the Middle East (Gibson, 2005). The major factor associated with the development of zinc deficiency at the population level is inadequate dietary zinc intake. High physiological requirements for zinc (such as during infancy, adolescence, pregnancy and lactation), decreased dietary zinc absorption efficiency and treatment with certain drugs (e.g. penicillamine, thiazide and glucagon) are additional influencing factors (Gibson et al., 2008).

Assessment of dietary intake is one of the three indicators which are recommended by WHO/UNICEF/IAEA/IZiNCG to assess zinc status at the population level. This indicator is important as it provides information on the dietary pattern that may be associated with zinc deficiency or inadequacy and it can aid in identifying populations or subpopulations at elevated risk for inadequate zinc intakes (de Benoist et al., 2007). In addition, dietary assessment may help to identify intervention targets to rectify zinc status. Three stages are involved in assessing the intake of absorbable zinc in the diet. First, calculation of total zinc and phytate intakes. Second, determination of the phytate-to-zinc molar ratio (PZR). Third, selection of the appropriate Estimated Average Requirements (EAR) and their use to determine the prevalence of inadequate zinc intakes. When the prevalence of inadequate zinc intakes is greater than 25%, the risk of zinc deficiency is considered to be high (de Benoist et al., 2007, IZiNCG, 2007b). Detailed information on food contributions to zinc intake and to its absorption modifiers (phytic acid and protein) is of similar importance as these data are useful for the development of food-based dietary guidelines and to make culturally appropriate recommendations to improve dietary quality (Sharma et al., 2013). Another important point to be considered when assessing zinc intake is the effect of non-nutritional factors such as age, sex, BMI, smoking and socioeconomic status. Differences in all of these factors were reported to cause differences in dietary zinc intake or in zinc absorption modifiers in several countries (Alissa, 2005, Adamson et al., 2009, Madej et al., 2013, McDaid et al., 2007, Coulibaly et al., 2009, Kim and Choi, 2013, Alsufiani et al., 2014, Jitnarin et al., 2006, Hur et al., 2011, van Rossum et al., 2000).

Zinc is one of several micronutrient deficiencies which are highly prevalent in the Arab region. Nevertheless, the magnitude of these deficiency problems differs from country to country (Madani and Kumosani, 2001). In Saudi Arabia, there have been few studies of zinc intake and of its absorption

modifiers. Thus, the aim of this study is to assess intake of zinc and its absorption modifiers and the factors affecting them in young and older Saudi adults of both sexes.

Objectives:

1. To quantify the intakes of zinc and of its absorption modifiers in younger and older Saudi adults of both sexes.
2. To estimate the prevalence of inadequate intakes of dietary zinc in different age and sex groups.
3. To assess the impact of phytate intake on the predicted bioavailability of zinc in different age and sex groups by assessing the phytate to zinc molar ratio.
4. To investigate associations between other factors such as BMI, smoking, household annual income and education and intakes of zinc and of its absorption modifiers.
5. To identify food items that most contribute to intakes of zinc and of its absorption modifiers in Saudi adults.

4.2- Materials and Method:

4.2.1-Participants:

Two hundreds and two male and female participants aged 20-30 years (younger adults) and 55-75 years (older adults) were recruited from King AbdulAziz university students, King AbdulAziz university hospital, King Fahad hospital, via advertisements in social media and via personal contact. Due to difficulties in recruiting older adults who were aged from 60 to 70 years, the eligible age range was expanded to be from 55 to 75 years. Exclusion criteria were presence of cancer, liver or kidney diseases, non-Saudis and other age groups. An overview of the recruitment strategy is shown in Figure 4.1. Participants were divided into four groups according to sex and age (53 young males, 51 young females, 49 older males and 49 older females). The study was approved by the Ethical Committee, Faculty of Medicine, King AbdulAziz University and the Health Affairs of Jeddah city and all participants gave written informed consent.

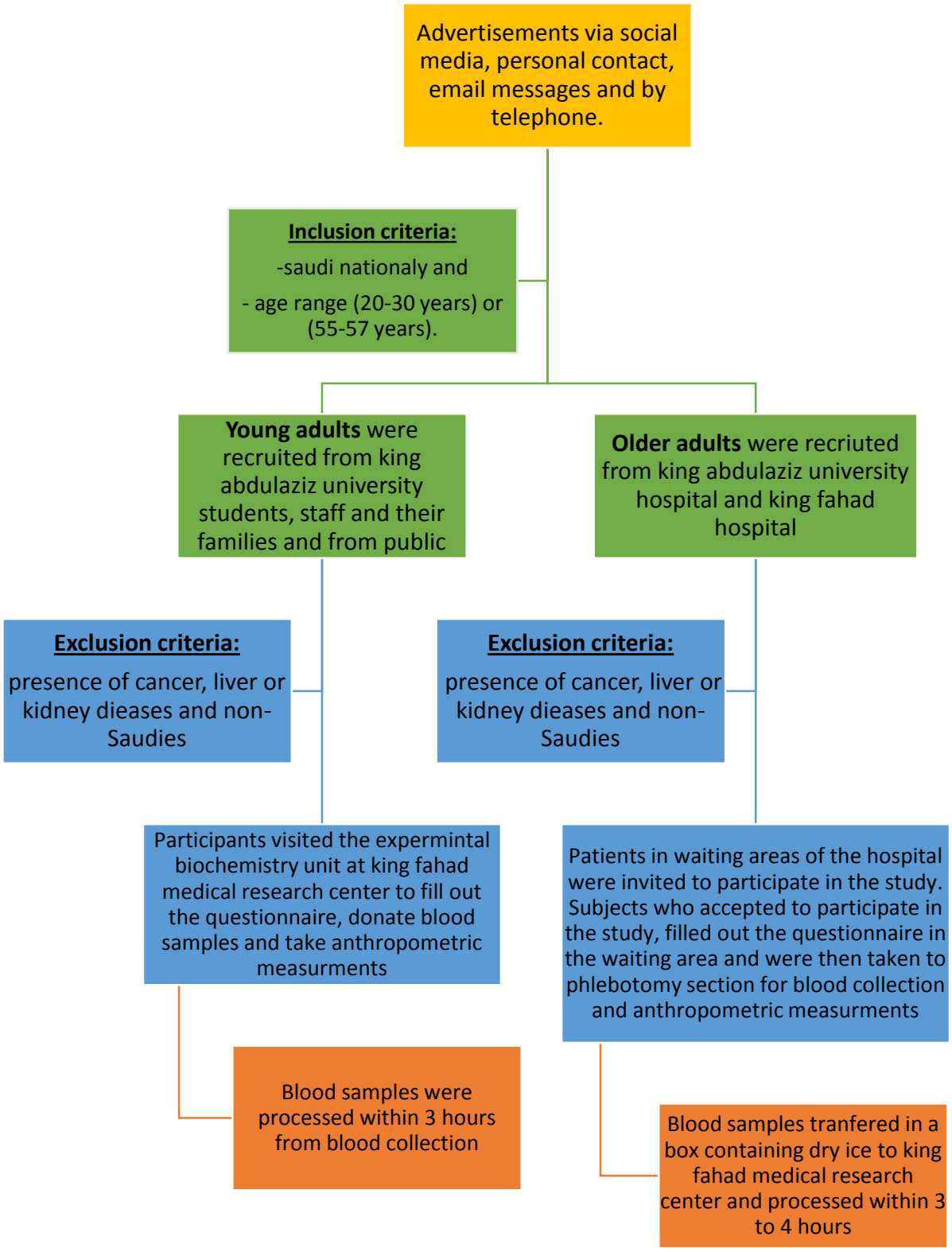


Figure 4.1 Recruitment strategy

4.2.2- Dietary and anthropometric assessment:

All participants completed a questionnaire that has been designed to collect demographic information, medical history, smoking habits and socioeconomic status. In addition, all participants completed a FFQ which had been validated previously to be used in Saudi young and older adults (see Chapter 3). Intake of zinc, phytate and protein were calculated from the FFQ as described previously (see section 2.1.5). Total intakes of zinc and protein were also calculated by summing the intake from diet and from dietary supplements. The UK lower reference nutrient intake (LRNI) cutoff points were used to assess the adequacy of the zinc intakes by young and older adults of both sexes. The prevalence of inadequate intake was determined by calculating the % participants whose zinc intake was below 5.5 mg/d for young and older males or below 4 mg/d for young and older females. The phytate-to-zinc molar ratio (PZR) was calculated according to the following equation:

$$\text{Phytate} - \text{to} - \text{zinc molar ratio} = \frac{\text{Phytate (mg)}/660}{\text{Zinc(mg)}/65.4}$$

PZR in the diet has been used to estimate the absorption of zinc, whereby PZR <5 is associated with relatively high absorption pf zinc, PZR 5-15 with moderate absorption and PZR> 15 with low absorption (Hotz et al., 2003a, IZiNCG, 2007b). The % contributions of each food item to intake of all food components were calculated by dividing the mean intake of each food item by the total intake of the same food component ×100. Body weight, height, waist and hip circumference were measured as described previously in section 2.1.1.

4.2.3- Statistical analysis:

To test the differences in dietary component intake between age and sex groups, two way ANOVA was used. One way ANOVA was used to assess the differences in dietary components between different BMI, smoking, education and household annual income groups. All statistical analyses were performed using the statistical software package IBM SPSS version 19. Statistical significance was taken as $P < 0.05$

4.3- Results:

4.3.1- Study participants:

Two hundreds and two volunteers participated in this study. All completed the questionnaire and donated blood samples. The majority of the younger participants were healthy while half of the older adults had diabetes and high blood pressure. One third of older females had osteoporosis and arthritis (Table 4.1). The overlapping of these diseases was shown in (Figure 4.2). Overall mean body mass index (BMI) was 28 kg/m^2 and older participants had higher BMI compared with younger adults (mean BMI was 24.8, 26, 32 and 29.6 for younger female, younger male, older female and older male adults, respectively). Approximately 60% of the participants were classified as overweight and obese. The prevalence of obesity and overweight was higher among older adults compared with younger adults. Additionally, more than half of the participants had an apple shaped fat-distribution pattern and this body shape was more prevalent in older adults than in young adults. Seventy percent of the participants were non-smokers while 13% were shisha/argela smokers (Table 4.1). Herbal teas were consumed by more than half the sample and the frequency of consumption of the different types of these herbs is summarized in Figure 4.3(A). Thirty four percent of participants consumed dietary supplements including Vitamin D, calcium, multivitamins and minerals (Figure 4.3B). Only 19% of participants were on therapeutic diets currently or during the previous year. The prevalence of these therapeutic diets is summarized in Figure 4.3C.

Table 4.1 Characteristics of study participants

	Young Female (n=50)	Young Male (n=53)	Older Female (n=50)	Older Male (n=49)	All Participants (n=202)
Mean age (years)	26.2	24.1	63.6	61.5	
Mean height (m)	158.2	172.8	156.6	167.6	164.1
Mean body mass (Kg)	62.4	77.8	78.3	83.4	75.3
Mean BMI (Kg/m^2)§	24.8	26.0	32.0	29.6	28.0
BMI Classification N (%)					
Under weight (<18.5)	2 (4)	4 (8)	0 (0)	0(0)	6 (3)
Normal (18.5 -24.99)	27 (55)	22 (41)	4 (9)	13 (28)	66 (34)
Overweight (25-29.99)	11 (22)	17 (32)	16 (36)	20 (44)	64 (33)
Obese (>30)	9 (18)	10 (19)	24 (55)	13 (28)	56 (29)
Mean Waist Circumference (cm)	78.4	91.2	103	106	93.0
Mean Hip Circumference (cm)	102	106	113	111	107
Fat Distribution Pattern N (%)#					
Apple Shape (WHR>0.9 for M and >0.8 for F)	16 (31)	15 (28)	28 (57)	30 (61)	(54)
Pear shape (WHR<0.9 for M and <0.8 for F)	31 (61)	37 (70)	3 (6)	6 (12)	(46)
Health Status N (%)					
cancer	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
high blood pressure	0 (0)	1 (2)	26 (53)	23 (47)	50 (25)
heart diseases	0 (0)	0 (0)	5 (10)	8 (16)	13 (6)
liver diseases	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
kidney diseases	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
osteoporosis	1 (2)	0 (0)	18 (37)	1 (2)	20 (10)
arthritis	1 (2)	0 (0)	18 (37)	6 (12)	25 (12)
diabetes	1 (2)	1 (2)	21 (43)	31(63)	54 (27)
lung diseases	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
allergy	13 (26)	7 (13)	7 (14)	6 (12)	34 (17)
hypothyroidism	2 (4)	0 (0)	8 (16)	1 (2)	11 (5)
Sickle cell anemia	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Blood disorder	0(0)	0 (0)	2 (4)	0 (0)	2 (1)
gastrointestinal disorders	6 (12)	1 (2)	5 (10)	11 (22)	23 (11)
Current intake of Herbs N (%)					
Yes	25 (49)	29 (55)	31 (63)	28 (57)	113 (56)
No	26 (51)	24 (45)	18 (37)	21 (43)	89 (44)

	Young Female (n=50)	Young Male (n=53)	Older Female (n=50)	Older Male (n=49)	All Participants (n=202)
Current intake of Dietary Supplements N (%)					
Yes	11 (22)	10 (19)	34 (69)	13 (27)	68 (34)
No	40 (78)	43 (81)	15 (31)	36 (74)	134 (66)
Dieting currently or during the past year N (%)					
Yes	10 (20)	7 (13)	10 (20)	11 (22)	38 (19)
No	41 (80)	46 (87)	39 (80)	38 (78)	164 (81)
Cigarettes smoking status N (%)					
non smoker	37 (73)	30 (57)	47 (96)	26(53)	140 (69)
former smoker	1 (2)	3 (6)	0 (0)	17 (35)	21 (10)
passive smoker	8 (16)	6 (11)	0 (0)	1 (2)	15 (7)
smoker	5 (10)	14 (26)	2 (4)	5 (10)	26 (13)
Shisha/argela smokers $ N(%)	6 (12)	16 (30)	1 (2)	4 (8)	27 (13)
Highest grade in school					
Illiterate (did not attend school)	0 (0)	0 (0)	20 (41)	4 (8)	24 (12)
primary	1 (2)	0 (0)	13 (27)	9 (18)	23 (11)
intermediate	1 (2)	0 (0)	3 (6)	4 (8)	8 (4)
high school	9 (18)	16 (30)	7 (14)	9 (18)	41 (20)
diploma	3 (6)	21 (40)	0 (0)	7 (14)	31 (15)
college	22 (43)	14 (26)	3 (6)	8 (16)	47 (23)
post graduate	15 (29)	2 (4)	3 (6)	8 (16)	28 (14)
Household annual income N(%)					
less than 50,000 SR	12 (24)	17 (32)	21 (46)	10 (21)	60 (31)
50,000 - < 100,000 SR	11 (22)	15 (28)	7 (15)	14 (29)	47 (24)
100,000 - < 150,000 SR	7 (14)	8 (15)	7 (15)	9 (19)	31 (16)
150,000 - < 200,000 SR	4 (8)	4 (8)	2 (4)	2 (4)	12 (6)
200,000 - < 250,000 SR	6 (12)	3 (6)	5 (11)	6 (13)	20 (10)
more than 250,000 SR	10 (20)	6 (11)	4 (9)	7 (15)	27 (14)
Current Occupation N (%)					
student	20 (39)	24 (45)	0 (0)	0 (0)	44 (22)
unemployed	6 (12)	3 (6)	30 (61)	2 (4)	41 (20)
employed	25 (49)	26 (49)	4 (8)	13 (27)	68 (34)
Self-employed	0 (0)	0 (0)	1 (2)	1 (2)	2 (1)
retired	0 (0)	0 (0)	14 (29)	33(67)	47 (23)

§BMI, Body Mass Index, #WHR, Waist to Hip Ratio, $ shisha/argela, consists of tobacco, molasses or honey and dried fruits

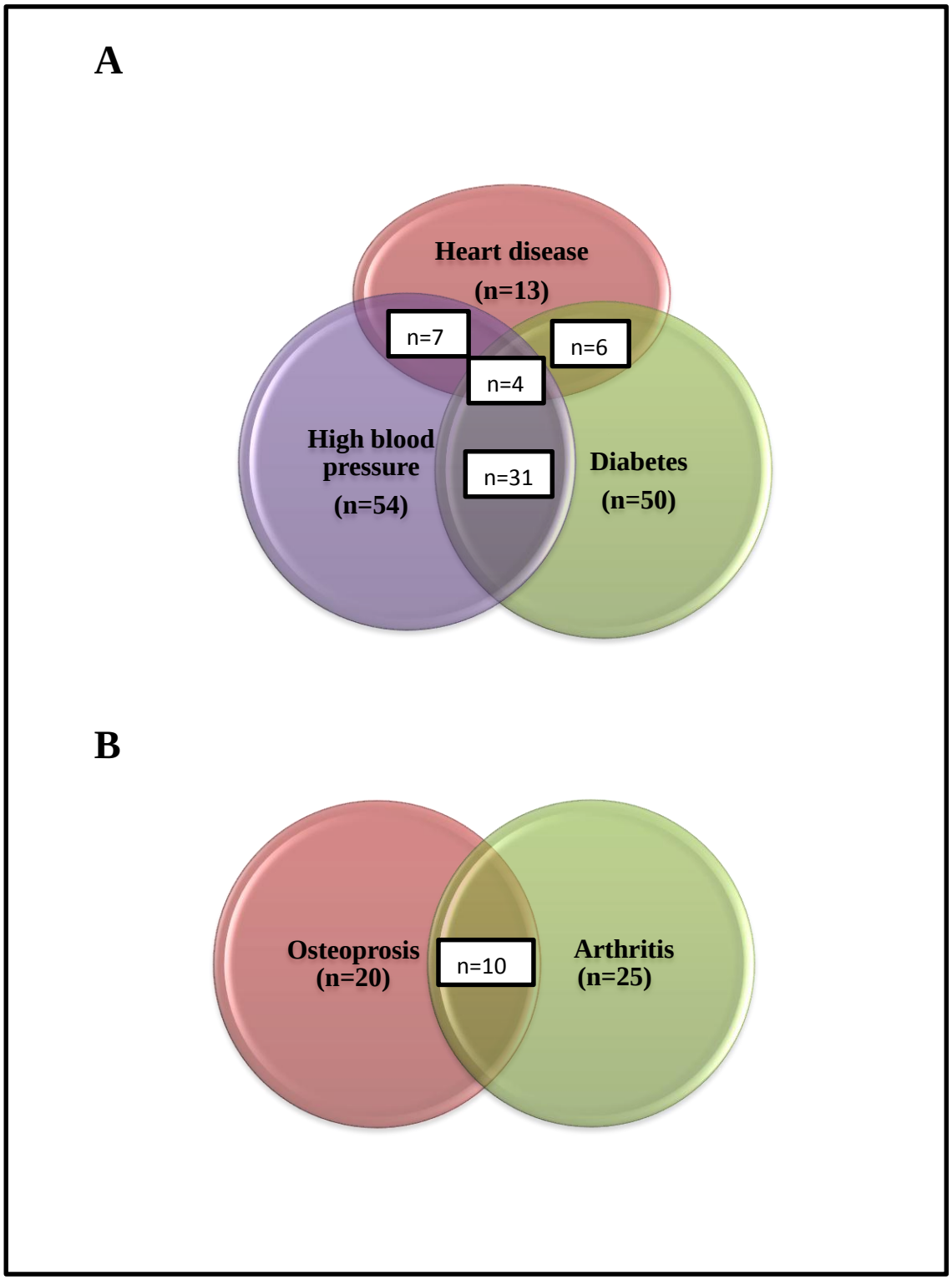


Figure 4.2 Venn diagram showing the overlaps between prevalence of (A) diabetes, high blood pressure and heart disease and (B) osteoporosis and arthritis in older Saudi adults (n=98)

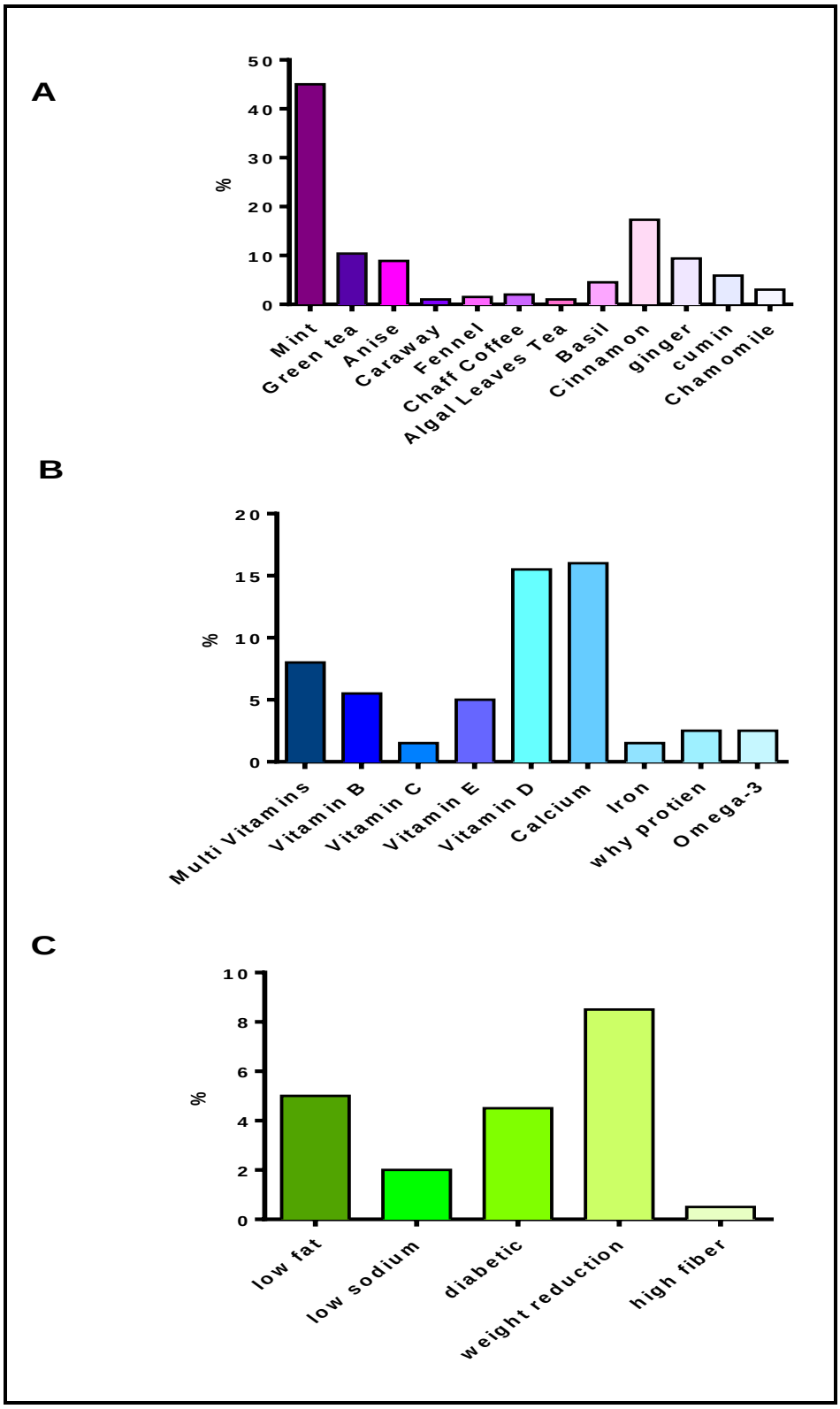


Figure 4.3 Types of (A) Herbal teas (B) Dietary Supplements and (C) therapeutic diets consumed by main study participants (n= 202)

4.3.2-Effects of age and sex on intakes of zinc and of zinc absorption modifiers in Saudi adults:

The mean intakes of zinc and of its absorption modifiers by age and sex groups estimated using the FFQ and from dietary supplements are displayed in Table 4.2 There were no differences in intakes of zinc, phytic acid and protein between young and older adults from diet alone or from diet plus dietary supplements. Intake of zinc from the diet was ~3 mg higher in males than in females (P= 0.002). Similarly, males intake of protein was higher, by approximately ~ 20 g/d, than that of females (P= 0.004). Moreover, total intakes of zinc and protein (from diet and from dietary supplements) were higher in males compared with females (P= 0.01 and P= 0.003, respectively). In contrast, no difference in intake of phytic acid was observed between sex groups. The distribution of zinc intakes estimated using the FFQ are showed in Figure 4.4 (raw data for zinc intake are summarized in Appendix D).

Table 4.2 Mean daily intake of zinc, phytic acid and protein estimated by FFQ and intake from dietary supplements by age and sex groups (n=202)

	All	Male		Female		Pooled SEM	Probability of effect		
		Young	Older	Young	Older		Age	Sex	Age* Sex
FFQ									
zinc (mg/day)	11.1	11.9	13.2	10.1	9.1	0.46	0.901	***0.002***	0.227
phytic acid (mg/day)	508	562	544	507	419	25.2	0.278	0.074	0.465
protein (g/day)	80.3	88.7	91.7	74.0	66.8	3.40	0.756	***0.004***	0.455
FFQ + Dietary Supplements									
zinc (mg/day)	11.8	12.3	13.9	10.5	10.7	0.48	0.360	***0.01***	0.489
protein (g/day)	81.4	92.0	91.7	75.2	66.8	3.44	0.526	***0.003***	0.554

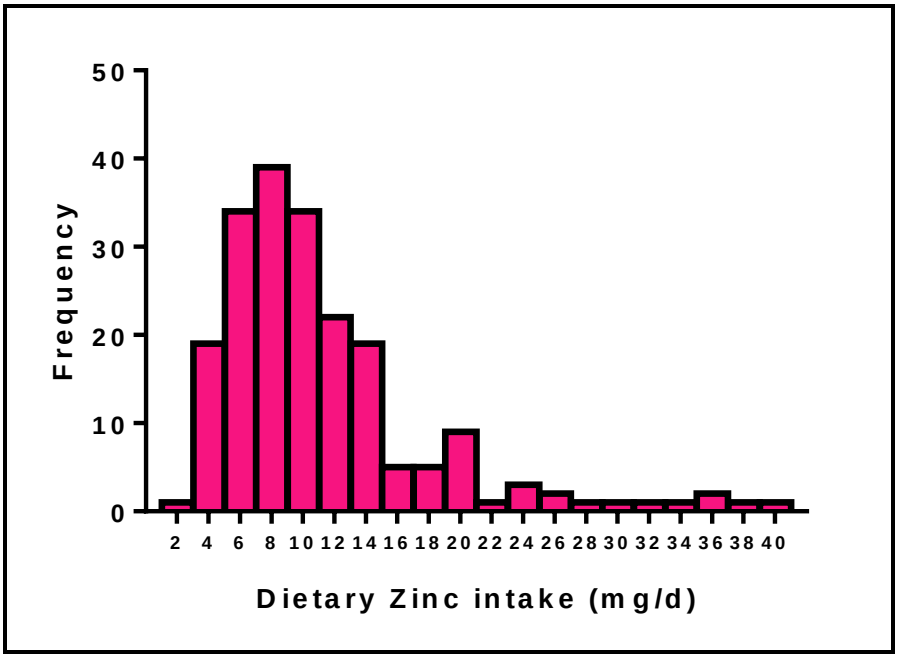


Figure 4.4 The distribution of dietary zinc intake in young and older Saudi adults

4.3.3- Prevalence of inadequate intake of dietary zinc in different age and sex groups:

Figure 4.5 shows the percentages of participants with zinc intake (from diet alone and from diet plus dietary supplements) below the UK LRNI by age and sex. Twelve percent of all participants had inadequate intake of zinc either from diet or from diet and dietary supplements. When zinc estimated from diet only, young males showed the highest percentage of inadequate intake (15%) followed by young females (4%), older males (2%) and older females (2%) (Figure 4.5A). Intake of zinc from dietary supplements did not change the percentages of participants with inadequate intake from any group except young females who showed a small reduction in the prevalence of inadequate zinc intake (Figure 4.5B).

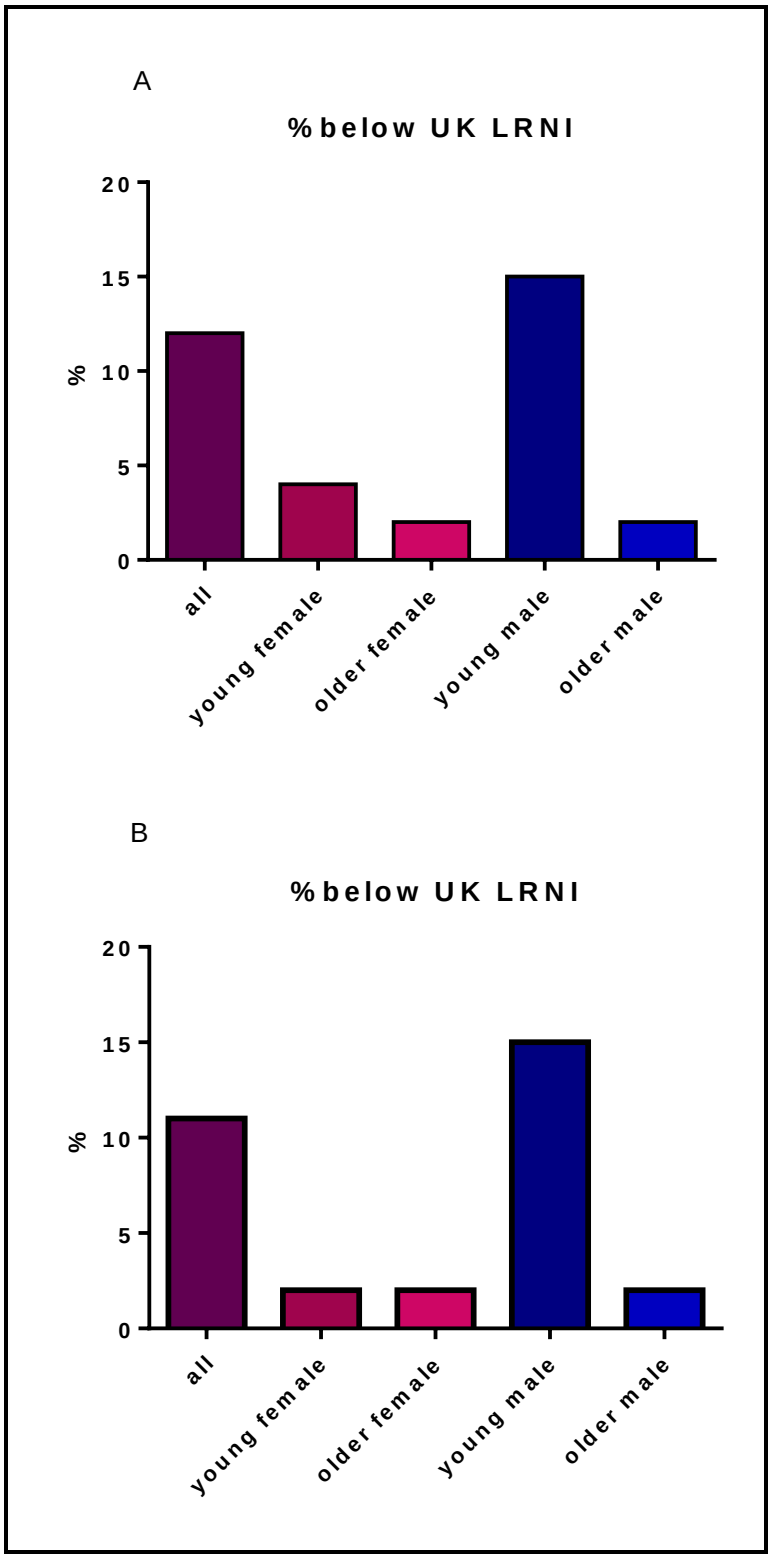


Figure 4.5 Percentage of participants with zinc intakes below the UK Lower Reference Nutrient Intake (LRNI) from (A) diet (B) diet and dietary supplements by age and sex

4.3.4- Effects of phytate intake on the estimated bioavailability of zinc in different age and sex groups:

I examined the potential effect of phytic acid intake on the bioavailability of zinc by calculating the phytate to zinc molar ratio (PZR) in Saudi adults. Table 4.3 presents the mean, median, quartiles and the % of participants with 3 categories of phytate-to-zinc molar ratios. The median molar ratio of phytate-to-zinc for all the participants was 4.40. Older adults had significantly lower phytate-to-zinc molar ratios compared with young adults (P<0.05). No significant differences between men and women were found. More than half the participants were classified as having PZR <5 and none of the participants had PZR > 15.

Table 4.3 Mean, median, quartiles and % of participants with different phytate-to-zinc molar ratios (PZR)

	All	Male		Female		Pooled SEM	Probability of effect		
		Young	Older	Young	Older		Age	Sex	Age* Sex
Mean	4.57	4.84	4.16	4.83	4.45	0.107	***0.014***	0.517	0.480
Median	4.40	4.58	4.07	4.63	4.00				
Quartiles (q25-q27)	(3.41-5.6)	(3.4-5.9)	(3.2-5.2)	(3.6-5.7)	(3-5.5)				
PZR < 5 (%)	62	53	69	65	63				
PZR = 5-15 (%)	38	47	31	35	38				
PZR > 15 (%)	0	0	0	0	0				

PZR, Phytate-to-Zinc Molar Ratio

4.3.5- Associations between BMI, smoking, education and household annual income and intakes of zinc and of zinc absorption modifiers in Saudi adults:

Intakes of zinc, phytic acid and protein by Saudi adults according to BMI category, smoking, education and household annual income are shown in Table 4.4. Intake of all three food components from diet and from the diet plus supplements appeared to be higher in underweight and obese participants but these differences were not statistically significant (P>0.05). Intakes of zinc, phytic acid and protein by smokers were substantially, and significantly, higher than for non-smokers, passive and former smokers. Non-educated (illiterate) and highly educated (post graduate) participants had the lowest intakes of zinc and of its absorption modifiers (non-significant differences) while participants with middle household annual income (150,000 <200,000 SR) had the highest intakes but these differences were not statistically significant (P > 0.05).

Table 4.4 Mean daily intake of zinc, phytic acid and protein by BMI category, smoking, education and household annual income (n=202)

	Dietary zinc (mg/d) Mean ±SD	Total zinc# (mg/d) Mean ±SD	Phytic acid (mg/d) Mean ±SD	Dietary protein (g/d) Mean ±SD	Total protein# (g/d) Mean ±SD
BMI Classification					
Under weight (<18.5)	13.9±9.8	13.9±9.8	609±330	108.7±75.3	108.7±75.3
Normal (18.5 -24.99)	10.7±6.2	11.3±6.3	504±354	76.7±43.9	78.0±44.0
Overweight (25-29.99)	10.4±5.1	11.4±6.1	466±287	77.1±43.8	79.5±46.0
Obese (>30)	12.5±8.5	13.1±8.5	583±444	89±58.4	89.0±58.4
Cigarette smoking status					
non smoker	10.4±5.5	11.2±5.8	470±281	75.0±39.9	76.78±41.2
former smoker	10.8±4.9	11.2±5.5	483±250	79.1±33.0	79.1±33.0
passive smoker	11.3±9.1	12.3±9.0	516±419	82.1±65.3	82.1±65.3
smoker	15.3±10.3*	15.7±10.6*	736±614*	109.4±77.6*	109.44±77.6*
Highest grade in school					
Illiterate (did not attend school)	9.7±3.9	10.9±4.2	408±254	71.6±28.8	71.6±28.8
primary	10.9±7.1	12.0±6.8	499±317	79.5±45.8	79.5±45.8
intermediate	11.0±4.7	13.1±7.8	416±215	78.5±32.1	78.5±32.1
high school	12.2±8.3	12.9±8.4	536±417	88.9±65.8	88.9±65.8
diploma	11.1±6.9	11.3±7.1	561±413	77.7±44.0	77.7±44.0
college	12.0±7.5	12.4±7.7	580±414	88.8±55.9	92.1±56.7
post graduate	9.4±3.5	10.2±4.1	407±167	65.8±24.3	68.7±29.5
Household annual income					
less than 50,000 SR	10.9 ± 6.9	11.7±7.2	526±402	80.9±51.2	80.9±51.2
50,000 - < 100,000 SR	11.8 ± 6.8	12.4±6.8	521±322	83.9±45.0	83.9±45
100,000 - < 150,000 SR	11.0 ± 7.2	11.5±7.0	511±409	81.2±50.3	86.3±51.9
150,000 - < 200,000 SR	13.4±10.8	14.3±12.0	570±517	106.3±94.2	106.3±94.2
200,000 - < 250,000 SR	9.5 ± 4.3	10.3±4.7	448±252	67.8±30.9	68.4±31.5
more than 250,000 SR	11.1 ± 4.7	11.9±4.9	486±264	74.1±29.4	76.4±33.5

#Total zinc and protein were calculated by summing intakes from diet and from dietary supplements, *P<0.05 compared with non-smokers, former and passive smokers

4.3.6- Major food items contributing to intakes of zinc and of its absorption modifiers in Saudi adults:

The percentage contributions by each food group to the daily intakes of zinc, phytate and protein are shown in Figure 4.6 (with details for individual food items summarized in Appendix E). The main source of zinc was the meat group which contributed more than two thirds of the average zinc intake. Within this group, the main contributors were chicken, lamb and diced meat (kebab or hamburger). Cereals (mainly white rice, rice with tomato sauce, brown pitta bread and white pasta) contributed 19% of the mean zinc intake. Dairy products, miscellaneous and vegetables contributed a further 16 %, 13% and 9% to the average zinc intake of Saudi adults, respectively (Figure 4.6A). Meats and cereals were also the top contributors to total daily zinc intake across all participating groups. Dairy products were the third top contributor for older adults while miscellaneous foods were for young adults.

Almost half the intake of phytate (47%) came from the consumption of cereals. White rice, brown pitta and toast breads, whole grain corn flakes and crisp breads, and rice with tomato sauce were the main contributors within this group. A quarter of phytate intake was from vegetables. The top 4 contributors within this group were broad beans, chickpeas, potatoes and tabouleh. Miscellaneous foods (mainly falafel, chocolate, pizza, chips, corn chips and twisties) were the third largest contributor to phytate intake. Seeds and nuts and meats contributed a further 5% and 3% to the total daily intake of phytate, respectively (Figure 4.6B). Cereals were also the first contributor to total daily phytate intake across all participated groups. Vegetables were the second contributor for all groups except for young males for whom miscellaneous foods were the second contributor.

Meats were the top contributors to the average daily intake of protein in Saudi adults. Chicken, lamb and diced meat (kebab or hamburger) were the highest contributors within this group. The second contributor to protein intake was vegetables and most protein came from broad beans, chickpeas, stuffed vegetables and potato. Cereals, dairy products and miscellaneous were the third, fourth and fifth major contributors to daily intake of protein, respectively (Figure 4.6C). Meats and cereals were the top contributors to total protein intake across all participating groups. Dairy products were the third top contributor for older adults while miscellaneous foods were for young adults.

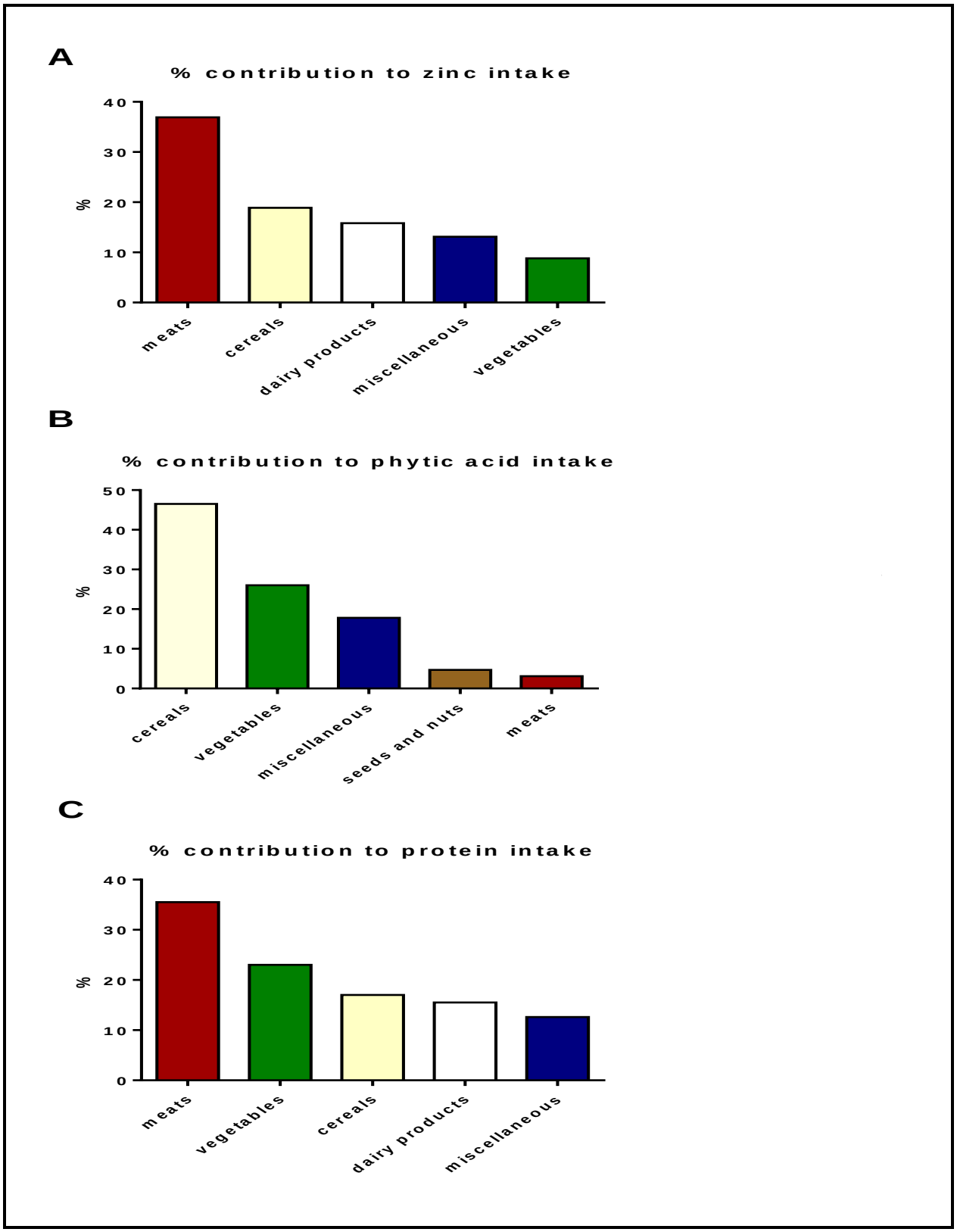


Figure 4.6 Percent food group contribution to intake of (A) Zinc (B) Phytic acid and (C) Protein in Saudi diet

4.4- Discussion:

4.4.1- Effects of age and sex on intakes of zinc and zinc absorption modifiers in Saudi adults:

The mean daily zinc intake assessed by the FFQ (11.1 mg) is similar to the mean values (10.50 mg) reported by Samman et al (Samman et al., 2010) in Australia and by Alissa (Alissa, 2005) (10.23 mg) and Alsufiani et al (10.6 mg) (Alsufiani et al., 2015) (see Chapter 3) in Saudi Arabia but substantially lower than that reported by Barrett et al (Barrett and Gibson, 2010) (15.3mg) for Australian participants by using FFQ method. The mean phytic acid intake of Saudi adults (508 mg) was similar to results from a previous Saudi study by Alsufiani et al (Alsufiani et al., 2015) (521 mg) (see Chapter 3) and much lower than intakes of British (917 mg) (Amirabdollahian and Ash, 2010) and New Zealand adults (1498 mg) (Heath et al., 2000) estimated using 4-7 days weighed intake method and FFQ, respectively. The mean daily intake of protein was 80.3 g which is comparable with the result of other Saudi studies (72.53 g) (Alissa, 2005) and (76.4 g) (Alsufiani et al., 2014) and lower than those reported in Taiwanese (87 g) (Lee et al., 2006), Spanish (103.1 g) (Fernandez-Ballart et al., 2010) and Australian (105.9 g) (Barrett and Gibson, 2010) studies when using either FFQ or 24h recall methods.

In contrast with the findings from the present study, several studies have observed that mean zinc and protein intakes were lower in older adults than in young adults when estimated by FFQ, (Alissa, 2005, Adamson et al., 2009). This may due to the higher BMI of older males in the present study which was associated with higher zinc and protein intakes than for the older males in my previous study (see Chapter 3). Higher intakes of zinc and protein in men than in women observed in the present study confirms the results that I reported in Chapter 3 and those of Madej et al (Madej et al., 2013), Mcdaid et al (McDaid et al., 2007), Coulibaly et al (Coulibaly et al., 2009) and Kim and Choi (Kim and Choi, 2013).

4.4.2- Prevalence of inadequate intake of dietary zinc in different age and sex groups:

Dietary zinc deficiency in humans was first reported in male dwarfs from the Middle East in the 1960s (Sandstead et al., 1967). An estimated 17% of central Asia, North Africa and Middle East's population is at risk of inadequate zinc intake (Wessells and Brown, 2012). In the Arab region, low zinc intake still persists in different age groups (Al-Daghri et al., 2013, al-Shawi, 1992, Naem et al., 2014, Baroudi et al., 2010). In the present study, the prevalence of zinc intakes below the UK LRNI was calculated by using 4 and 5.5 mg zinc/d as cut-off points for females and males, respectively. The LRNI is estimated to be adequate for only 2.5% of the population. For all participants in the current study, the percent of participants whose zinc intake (estimated by FFQ) below the UK LRNI was 12 %. The risk appeared to be greatest for young males (15%) followed by young females (4%) (Figure 4.5 A). Even when zinc from supplements was included, 15 % of respondents in the young males group had total zinc intakes below the UK LRNI (Figure 4.5 B).

4.4.3- Effects of phytate intake on the predicted bioavailability of zinc in different age and sex groups:

The median daily phytate-to-zinc molar ratio (PZR) in Saudi adults (4.40) observed in the present study is lower than those reported for Chinese (11.1) (Ma et al., 2007), South Korean (15.9) (Kwun and Kwon, 2000), Iranian (10-13) (Abbaspour et al., 2013), young Indian women (26) (Herbst et al., 2014), British young adults (9.66) and British older adults (8.70) (Amirabdollahian and Ash, 2010). The higher PZR may be due to higher phytic acid intake in those populations compared with Saudi adults. Amirabdollahian and Ash (2010) found that young women (19-24 years) had a significantly lower PZR than older women in other age groups (25-34 years, 35-49 years and 50-64 years). Amirabdollahian and Ash (2010) also reported that youngest age group had significantly lower phytate intake than the older age groups (Amirabdollahian and Ash, 2010). In contrast, our results showed that older adults had lower PZR compared with young adults and this may due to the lower phytic acid intake of older adults in comparison with young adults. In the present study, more than half the participants had PZR< 5 which indicates the potential for relatively high absorption of zinc and the remaining participants had potentially moderate absorption of zinc (PZR 5 - 15) (Hotz et al., 2003a).

4.4.4- Associations between BMI, smoking, education and household annual income and intakes of zinc and of zinc absorption modifiers in Saudi adults:

Although the differences in food components intake between different BMI categories were non-significant, under- weight and obese participants appeared to have higher intakes of zinc, protein and phytate (Table 4.4). This may due to higher food intake of obese individuals than who is normal weight or over weight. In contrast, it is possible that underweight individuals were over-reporting their intakes. These potential explanations were supported by plasma zinc status measurements reported in Chapter 5 (see section 5.3.5). A similar difference in total protein intake was also reported by Shay et al (2012) who found higher intakes of protein across higher BMI categories in middle-aged US men (Shay et al., 2012).

Dietary differences in intake between smokers and non-smokers were previously reviewed by Dallongeville et al (1998). Their results showed that, in general, smokers have less healthy patterns of nutrient intake and with higher intakes of energy, total fat, cholesterol, saturated fats and alcohol while intakes of protein and carbohydrates did not differ between smokers and nonsmokers (Dallongeville et al., 1998). In present study, smokers had higher intakes of zinc and its absorption modifiers when compared with other smoking groups. These results are consistent with Jitnarin et al (2006) who reported that adult smokers living in the central region of Thailand had higher intakes of total protein and zinc in comparison with non-smokers (Jitnarin et al., 2006). In another study with Italian women, the authors found that dietary pattern of current smokers were characterized by higher intakes of macro and micronutrients from animal sources (D'Avanzo et al., 1997). These differences could be due to the smoking-related changes in taste which lead smokers to prefer certain foods. Moreover, nicotine intake from smoking may increase metabolic rate and lead to greater energy intakes (Dallongeville et al., 1998, Jitnarin et al., 2006).

Socioeconomic status, including education and income, is associated with differences in food and nutrients intakes in several countries (Hur et al., 2011, van Rossum et al., 2000, D'Avanzo et al., 1997). In this study, non-educated (illiterate) and highly educated (post graduate) participants tended to have the lowest intake of zinc and its absorption modifiers while participants with middle household annual income (150,000 <200,000 SR) had the highest intake, these differences were not statistically significant ($P > 0.05$). These results are consistent with another Saudi study conducted by Al-Numair (2006) on adults living in Riyadh city who found no significant associations between zinc intake and

socioeconomic status of the participants (Al-Numair, 2006). D'Avanzo et al (1997) also reported no meaningful differences in protein intake due to variation in education level (D'Avanzo et al., 1997). On the other hand, less educated Dutch elderly showed lower protein intake as proportion of energy (van Rossum et al., 2000) lower-income Korean men and women had lower intakes of protein compared to high income participants (Hur et al., 2011).

4.4.5- Food items which contributed most to intake of zinc and its absorption modifiers in Saudi adults:

Meats were the main sources of zinc for the Saudi adults and the majority came from lamb, chicken and diced meats. Zinc intake from these foods is high as they contain a high amount of zinc (e.g. 100 g of lamb contains 5.8 mg zinc) and they are frequently consumed in the Saudi diet. The second largest source of zinc was from cereals mainly white rice, rice with tomato sauce, brown pitta bread and white pasta. The zinc contents of these food items are not particularly high, but their frequency of consumption meant that they contributed substantially to zinc intake. These findings are comparable with the findings of Kwun and Kwon (2000) who reported that, among south Koreans meat, poultry and their products were the biggest contributors to zinc intake followed by cereals and grain products (Kwun and Kwon, 2000). In contrast, the major food group contributing to zinc intake in Japanese, Chinese and American people was cereals (Ma et al., 2007, Sarukura et al., 2011, Sharma et al., 2013).

The top contributor to daily intake of phytate was cereals especially white rice, brown pitta and toast breads, whole grain corn flakes and crisp breads, and rice with tomato sauce. Whole grain corn flakes and crisp breads contains very high amount of phytate (e.g. 30 gm of whole grain corn flakes contains 447.6 mg of phytate) but are less frequently consumed in Saudi Arabia. On the other hand, white rice, rice with tomato sauce and brown breads contains lesser amounts of phytate but are more frequently consumed in this population. Similarly, cereals were found to be the major contributor to phytate intake by South Korean and British adults (Amirabdollahian and Ash, 2010, Kwun and Kwon, 2000). Amirabdollahian and Ash (2010) reported that vegetables, potatoes and savory snacks were the second contributor to phytate intake in the UK population, in agreement of our findings (Amirabdollahian and Ash, 2010).

In this study, protein intake came mainly from meats followed by vegetables and cereals. Meats contributed mostly to protein intake as they contain high amounts of protein (compared to other food groups) and they are frequently consumed in the Saudi diet. Whilst food items from vegetables and

cereals groups contain lower concentrations of protein, they were frequently consumed in relatively large amounts. In Taiwanese elderly, the main food sources of protein were cereals/roots, meat and seafood for both sexs (Wu et al., 2005).

Chapter 5. Plasma Zinc Status in Young and Older Saudi Adults and its Relationship with Diet and other Variables

5.1- Introduction:

Zinc absorbed from the intestine into the portal blood is transported loosely bound to proteins, predominantly albumin (Mahan and Escoot-Stump, 2004). Up to 60% of zinc is transported by albumin while transferrin, α-macroglobulin and immunoglobulin G (IgG) transport between 15 and 40% of zinc in the blood (Gropper et al., 2005). Circulating concentrations of metallothionein (MT), a metal storage protein, in plasma respond to changes in zinc intake, decreasing with low intakes. However, MT concentration also increases in response to stress and infection (King, 1990, Gibson et al., 2008).

Measurement of serum or plasma zinc concentration is the best available biomarker for assessing zinc status and the risk of zinc deficiency in populations. Several advisory bodies including WHO / UNICEF / IAEA and IZiNCG recommend the use of this measurement as a biomarker of zinc status because it reflects dietary zinc intake, responds consistently to zinc supplementation and reference data are available for most age and sex groups (de Benoist et al., 2007, Gibson et al., 2008). IZiNCG recommends that if more than 20% of a population (or population sub-group) has a serum zinc concentration below the relevant cutoff point, the whole population (or sub-group) should be considered to be at risk of zinc deficiency (IZiNCG, 2012a). When assessing zinc status based on plasma zinc concentration, it is important to appreciate that, in addition to nutritional factors, several non-nutritional factors including age, sex, BMI and smoking can affect zinc requirements and circulating zinc concentrations. Differences in these factors were reported to be associated with differences in plasma/serum zinc status in several countries including Tunis, Thailand, Nigeria and Mexico (Boonsiri et al., 2006, Sfar et al., 2009, Garcia et al., 2012, Northrop-Clewes and Thurnham, 2007, Ugwuja et al., 2011).

Although plasma zinc concentrations are a reasonable biomarker of zinc status at the population level, it has limitations at the individual level. This is because plasma zinc is mobilized as a result of metabolic redistribution as well as poor zinc nutrition. Plasma zinc readily decreases with metabolic redistribution induced by infection, carcinoma, steroid use, endotoxemia and after a meal. This decline represents a redistribution of zinc from the small, vulnerable pool to the tissues, not a change in zinc nutrition. Metallothionein may be the key to diagnose metabolic zinc redistribution due to infection, trauma or stress. This is because hepatic metallothionein concentrations rise with inflammation or stress, which also causes a rise in plasma metallothionein. Thus, high plasma metallothionein in association with low plasma zinc could be indicative of redistribution of tissue zinc in response to infection and stress and not to suboptimal zinc status. In contrast, low concentrations of both plasma zinc and metallothionein would

indicate a reduction in the size of the exchangeable zinc pool as a result of a decrease in zinc intake. Thus, measurement of both zinc and metallothionein in the plasma may allow plasma zinc concentrations to be better interpreted (King, 1990, King, 2011).

Thus, the aim of this study was to measure plasma zinc and metallothionein concentration, and the nutritional and non-nutritional factors that may affect these measurements in young and older Saudi adults of both sexes.

Objectives:

1. To measure plasma zinc and metallothionein concentrations in younger and older Saudi men and women.
2. To estimate the prevalence of zinc deficiency in men and women in these different age groups.
3. To investigate the effect of zinc intake and of its absorption modifiers, dietary supplements and herbal tea intake on plasma zinc and metallothionein concentration.
4. To investigate associations between non-nutritional factors, including BMI, fat distribution pattern, smoking, household annual income and education, and plasma zinc and metallothionein concentration.

5.2- Materials and Methods:

5.2.1- Participant recruitment:

Two hundred and two male and female participants aged 20-30 years (younger adults) and 55-75 years (older adults) were recruited from King AbdulAziz University students, King AbdulAziz University Hospital and King Fahad Hospital via advertisement in social media and through personal contact. Participants were divided into four groups according to sex and age (53 young males, 51 young females, 49 older males and 49 older females). Further details of recruitment are provided in section 4.2.1. Power analysis (using G power 3.1.9.2 software) indicated a 92% chance of detecting a medium effect size (calculated using the mean and SD from a previous Saudi study) between the young and older groups at the 5% level (two tailed).

5.2.2- Plasma zinc concentration, clinical chemistry and anthropometric measurements:

All participants donated blood samples and completed a questionnaire. Blood samples were collected in trace-element free, plain and EDTA anticoagulant blood collection tubes. Plasma samples separated from the trace-element free tubes were stored at -80°c for later measurement of the concentration of zinc and other metals by inductively coupled plasma atomic emission spectroscopy (see section 2.4). Hemolyzed samples (n=5) were excluded from the analysis. The prevalence of zinc deficiency was determined by calculating the percentage of participants whose plasma zinc concentration was below 10.70 or 10.09 µmol/l for morning fasting and morning non-fasting females or below 11.31 or 10.70 µmol/l for morning fasting and morning non-fasting males, respectively (IZiNCG, 2012a). Serum samples separated from the plain tubes were stored at -80°C for later clinical chemistry measurements including glucose, albumin, creatinine, ALT and CRP using Elitech clinical systems. The EDTA tube was used for a CBC test using a Beckman coulter and then for separation of plasma. This plasma was stored at -80°C for later determination of metallothionein 1 using an ELISA kit (see section 2.5). Hemolyzed samples (n=10) were excluded from the analysis. The questionnaire (see Appendix A) had been designed to collect general demographic information, medical history, smoking habits and socioeconomic status. In addition, all participants completed an FFQ (previously validated to be used in young and older Saudi adults (see chapter 3)) and data from this FFQ were used to estimate intakes of zinc, phytate and protein as described previously (see section 2.1.5). Body weight, height, waist and hip circumference were measured as described in section 2.1.1.

5.2.3- Statistical analyses:

Two-way ANOVA was used to determine differences in plasma zinc and MT1 concentrations between subjects grouped according to age and sex. The same approach was used to determine relationships between BMI, fat distribution pattern, smoking, education, household annual income, dietary supplements and herbal tea intake and plasma zinc and MT1 concentrations. Because the data were not distributed normally, correlations between intake of food components and plasma zinc and MT1 concentration were determined using Spearman's correlation test. Spearman's correlation test was also used to determine relationships between concentrations of plasma zinc and MT1 and other metals. All statistical analyses were performed using the statistical software package IBM SPSS version 19. Statistical significance was taken as $P < 0.05$.

5.3- Results:

5.3.1- Characteristics of study participants:

Two hundred and two volunteers participated in this study. All completed the questionnaire and donated blood samples. Mean (SD) of biochemical parameters (haemoglobin, albumin, ALT, creatinine, glucose and CRP) by age and sex are shown in Table 5.1. Except for glucose concentrations, which were high among older adults, all parameters were in the normal range intervals (according to the WHO and FDA normal values) for all groups.

Table 5.1 Mean (SD) of biochemical parameters of study participants

	Young Female (n=51)	Young Male (n=53)	Older Female (n=49)	Older Male (n=49)	All Participants (n=202)	Normal Ranges $
Hemoglobin (g/dl)	12.1 (2.0)	15.0 (1.4)	12.4 (3.0)	13.2 (2.2)	13.2 (2.5)	Male >13 Female >12 #
Albumin (g/dl)	4.6 (0.3)	4.7 (0.3)	4.3 (0.3)	4.3 (0.3)	4.5 (0.3)	3.5 – 5.5
ALT (U/l)	16.0 (7.3)	33.0 (20.2)	24.8 (12.8)	33.5 (15)	26.8 (16.2)	0.0 – 30
Creatinine (mg/dl)	0.64 (0.3)	0.80 (0.2)	0.57 (0.2)	0.77 (0.2)	0.70 (0.2)	1.0 – 2.0
Glucose (mg/dl)	85.2 (15)	93.0 (16.2)	122.0(61.2)	153.2(90)	113.5 (61.2)	80 – 120
CRP (mg/dl)	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0

$ FDA blood serum chemistry normal values, # WHO cutoff points

5.3.2- Associations between plasma zinc and plasma metallothionein 1 concentrations:

The relationship between plasma zinc and plasma MT1 for all subjects was determined by Spearman's correlation coefficient test. There was no statistically significant association between concentrations of zinc and MT1 in plasma (p=0.377).

5.3.3- Effects of age and sex on plasma zinc and other metal ions concentration in Saudi adults:

Figure 5.1 shows the mean plasma zinc concentration in young and older Saudi adults of both sexes measured by inductively coupled plasma atomic emission spectrometry. For all participants, mean plasma zinc (± SD) was 21.4 ± 13.1 µmol/l. In young males and females, mean plasma zinc concentrations were 15.1 and 13.4 µmol/l, respectively. However, concentrations were significantly higher in older males and females, where plasma zinc concentrations were 30.3 and 27.2 µmol/l, respectively, compared with the younger group of the same sex (P<0.001). For both age groups, mean

plasma zinc concentration was lower in females than males but did not differ significantly (P=0.126). Figure 5.2 shows the distribution of plasma zinc concentration in young and older adults.

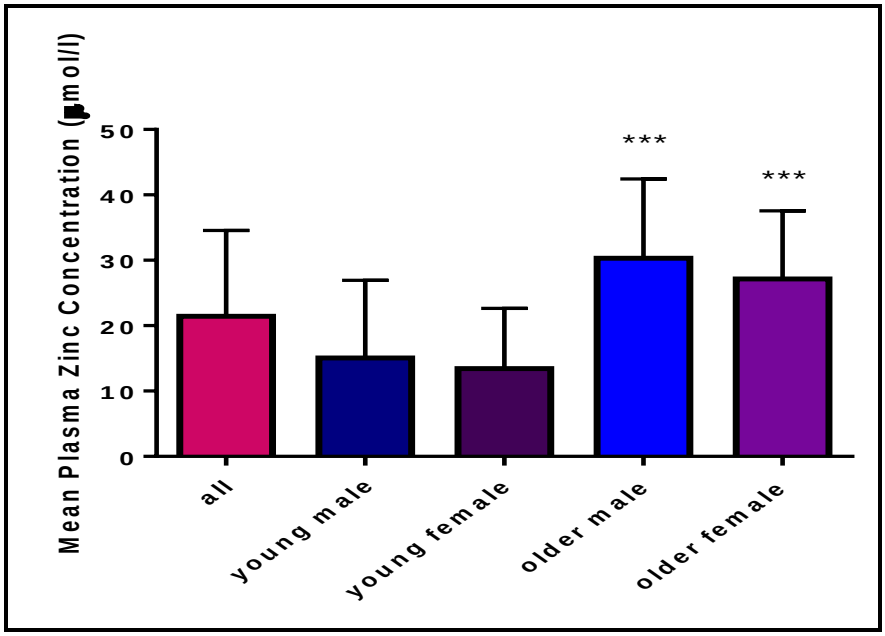


Figure 5.1 Mean plasma zinc concentrations in subjects grouped according to age and sex (n=197). Error bars show SD. *** P<0.001 compared with the corresponding younger group of the same sex

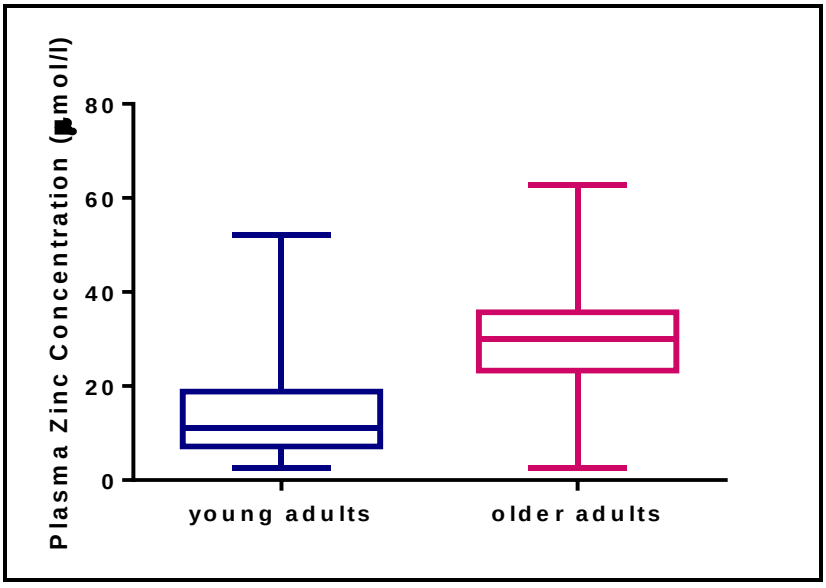


Figure 5.2 Distribution of plasma zinc concentration in young and older adults (n=197)

Concentrations of Fe, Cu, Mg, Mn and Ca were significantly higher in older adults while lead concentration was higher in younger adults (Table 5.2).

Table 5.2 Mean (± SD) concentrations of Cu, Se, Mg, Pb, Mn, As, Ca and Ni in plasma in young and older Saudi adults

Minerals	Young adults	Older adults	P
Fe (µmol/l)	73.69 ± 73.6	101.52 ± 75.9	***0.010***
Cu (µmol/l)	44.27 ± 34.2	97.31 ± 37.3	***<0.001***
Se (µmol/l)	16.9 ± 14.6	14.1 ± 12.8	0.147
Mg (mmol/l)	1.98 ± 0.9	2.7 ± 0.8	***<0.001***
Mn (µmol/l)	4.92 ± 8.0	11.21 ± 17.2	0.349
Pb (µmol/l)	0.83 ± 1.1	0.69 ± 0.9	***0.001***
As (µmol/l)	3.80 ± 8.3	4.97 ± 7.1	0.296
Ca (mmol/l)	3.99 ± 2.1	6.71 ± 2.0	***<0.001***
Ni (µmol/l)	1.19 ± 0.9	1.10 ± 1.4	0.585

5.3.4- The prevalence of zinc deficiency in young and older male and female Saudi adults:

Figure 5.3 shows the percentages of participants with plasma zinc status below the IZiNCG (IZiNCG, 2012a) cut-off values by age and sex. Almost one third of all participants had plasma zinc below the relevant cut-off values (28%). Young males showed the highest percentage of zinc deficiency (51%) followed by young females (46%) whilst deficiency was much less common in older males (6%) and older females (6%) (Figure 5.3).

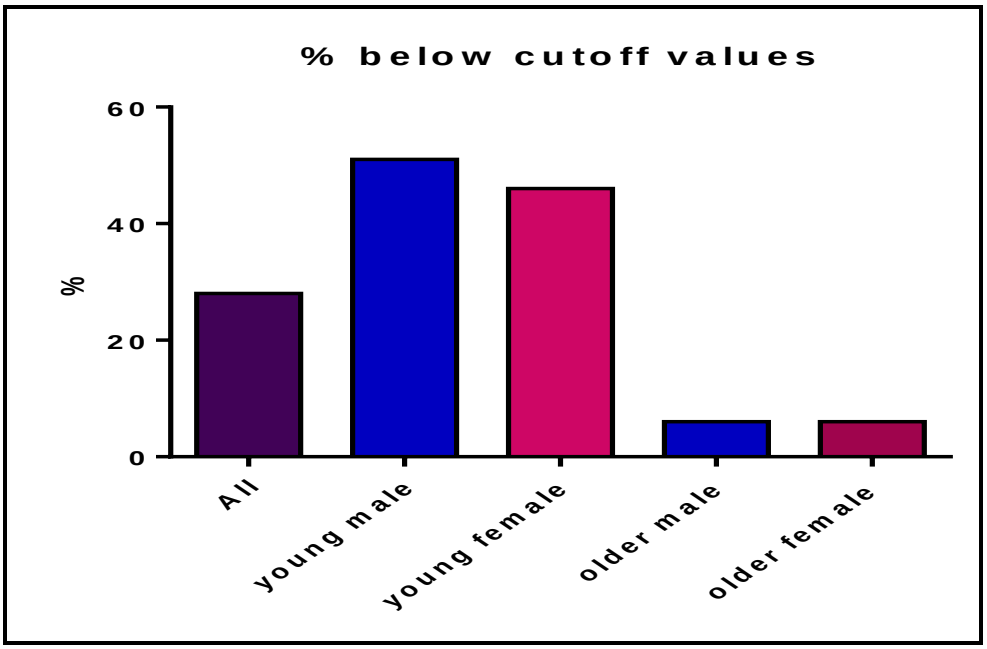


Figure 5.3 Percentage of participants with plasma zinc status below the IZiNCG (IZiNCG, 2012a) cut-off values by age and sex (n =197)

5.3.5- Relationships between dietary zinc, zinc absorption modifiers, dietary supplements and herbal tea intake on plasma zinc concentration:

The relationships between dietary zinc, zinc absorption modifiers and plasma zinc concentration were determined by calculating the correlation coefficients between intake of food components and plasma zinc concentration (Table 5.3). Significant positive correlation coefficients were found between plasma zinc concentration and intake of dietary zinc, total zinc (including supplementary) and dietary protein. In contrast, there were no significant correlations between plasma zinc concentration and intake of total protein (including supplementary), phytic acid or phytate-to-zinc molar ratio. The sequential Bonferroni test indicates that these tests are non-significant at the "table-wide" 0.05 level.

Table 5.3 Spearman correlation coefficients between intake of food components and plasma zinc concentration

Food components intake	r	p
Dietary zinc	0.153	***0.032***
Total zinc	0.174	***0.015***
Phytic acid	0.048	0.502
Phytate-to-zinc ratio	-0.116	0.106
Dietary protein	0.154	***0.031***
Total protein	0.136	0.058

Two-way ANOVA was used to investigate the effect of dietary supplements and herbal tea consumption on plasma zinc concentration in young and older adults. There was no significant difference between plasma zinc concentration and intake of herbal tea or dietary supplements (Figure 5.4). Table 5.4 shows the mean plasma zinc concentrations in consumers and non-consumers of several types of herbal tea. There were no statistically significant differences between consumers and non-consumers of any of the herbal tea. The mean plasma zinc concentration in consumers and non-consumers of dietary supplements are shown in Table 5.5. There were no statistically significant differences in plasma zinc concentration between consumers and non-consumers of any of the dietary supplements.

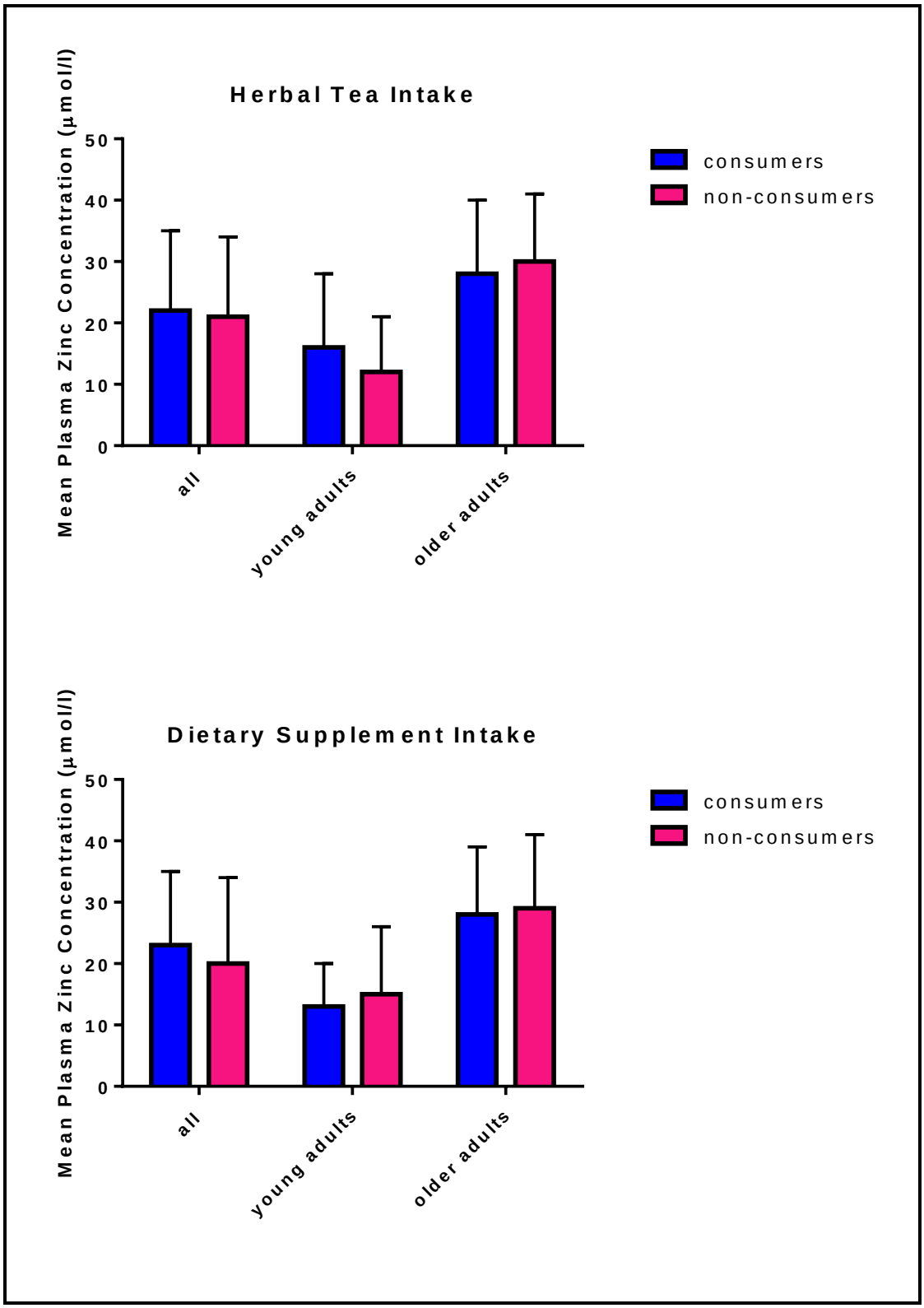


Figure 5.4 Mean plasma zinc concentration in subjects grouped according to consumption of herbal tea or dietary supplements (n= 197). Error bars show SD

Table 5.4 Mean plasma zinc concentration in consumers and non-consumers of herbal tea (n=197)

Herbal tea	Consumers		Non-consumers		Pooled SEM	Probability of effect		
	Young	Older	Young	Older		Age	Herbal tea consumption	Age* Herbal tea consumption
Mint	15.9	26.4	12.5	30.3	0.78	***<0.001***	0.887	0.020
Green tea	20.3	27.9	14.0	28.9	1.58	***<0.001***	0.404	0.249
Anise	13.9	27.6	14.3	28.8	1.40	***<0.001***	0.774	0.873
Caraway	-	23.5	14.2	28.8	2.64	***<0.001***	0.495	-
Fennel	-	22.4	14.2	28.9	2.17	***<0.001***	0.309	-
Chaff Coffee	-	27.3	14.2	28.8	1.91	***<0.001***	0.804	-
Algal leaves tea	-	28.7	14.2	28.7	2.64	***<0.001***	0.995	-
Basil	6.2	31.9	14.4	28.5	2.23	***<0.001***	0.592	0.195
Cinnamon	14.0	26.9	14.3	29.0	1.04	***<0.001***	0.576	0.663
Ginger	12.8	26.7	14.4	29.0	1.34	***<0.001***	0.480	0.898
Cumin	17.1	26.6	14.1	28.9	1.66	***<0.001***	0.918	0.431
Chamomile	3.73	30.0	14.4	28.7	3.10	***<0.001***	0.455	0.336

Table 5.5 Mean plasma zinc concentration in consumers and non-consumers of dietary supplements (n=197)

Dietary Supplements	Consumers		Non-consumers		Pooled SEM	Probability of effect		
	Young	Older	Young	Older		Age	Dietary supplement consumption	Age* Dietary supplement consumption
Calcium	5.54	26.4	14.3	29.7	2.83	***0.002***	0.284	0.631
Vitamin D	9.65	27.5	14.5	29.1	1.42	***<0.001***	0.255	0.568
Vitamin B	10.5	35.8	14.4	28.0	2.68	***<0.001***	0.617	0.133
Vitamin C	-	23.5	14.2	28.8	2.64	0.079	0.495	-
Multivitamins and minerals	17.2	31.6	14.0	28.5	1.45	***<0.001***	0.275	0.981
Omega-3	19.5	33.7	14.1	28.6	2.55	***0.006***	0.305	0.974
Iron	18.2	31.2	14.2	28.6	3.41	***0.045***	0.630	0.919
Whey Protein	10.4	-	14.5	28.8	1.72	***<0.001***	0.421	-

5.3.6- Associations between BMI, fat distribution pattern, smoking, education and household annual income and plasma zinc concentration in Saudi adults:

Mean plasma zinc concentrations in Saudi adults according to BMI category and fat distribution pattern are shown in Figure 5.5. For all participants, plasma zinc concentration tended to increase with increasing BMI but this effect was not statistically significant (P= 0.759). This trend was evident in young adults but not in older adults. Young and older adults together who had an apple-shaped fat distribution pattern had significantly higher plasma zinc concentration than participants who had a pear-shaped fat distribution pattern (P= 0.005).

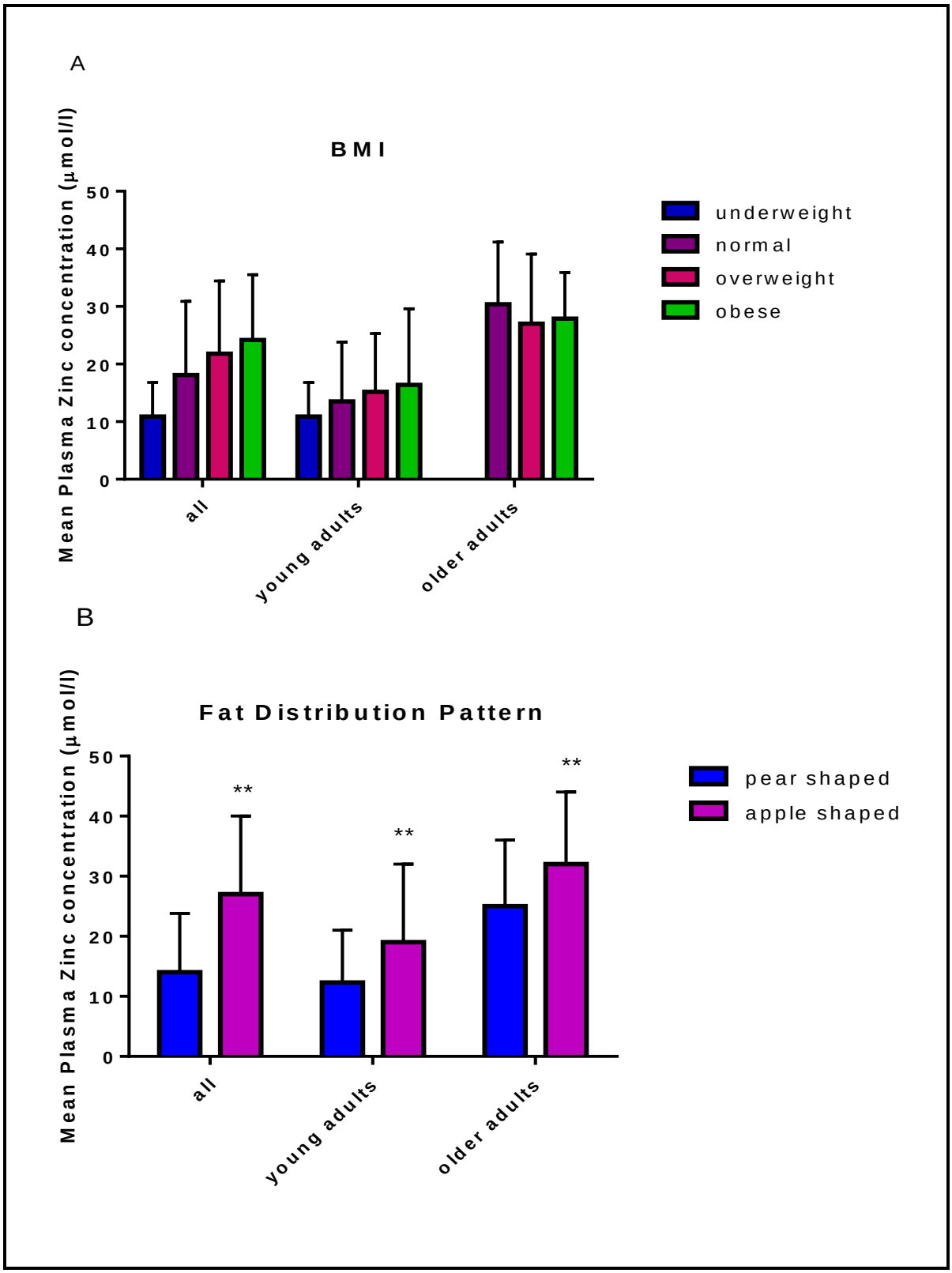


Figure 5.5 Mean plasma zinc concentration by (A) BMI category and (B) fat distribution pattern (n=197). Error bars shows SD. **P<0.01 when compared with pear shaped fat distribution pattern

Figure 5.6 and Figure 5.7 shows the mean plasma zinc concentrations in Saudi adults by smoking and socioeconomic status, respectively. There were no significant differences in plasma zinc concentration between cigarette smoking categories (P=0.810) or between shisha smokers and non-smokers (P= 0.604). Education was not related to plasma zinc concentration (P=0.682). In both young and older adults, there appeared to be a U-shaped relationship between plasma zinc concentration and household annual income but there were no statistically significant differences (P=0.245).

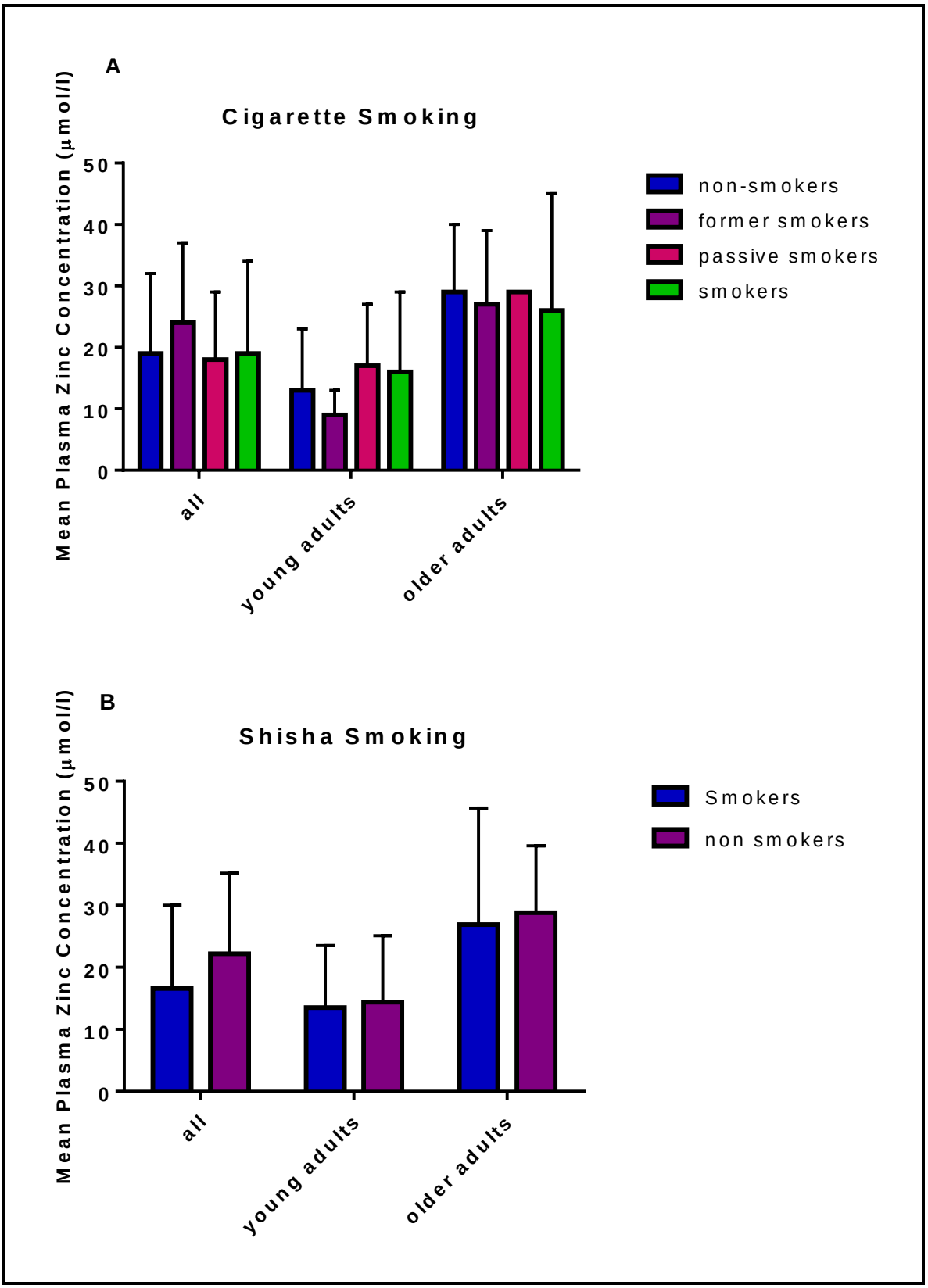


Figure 5.6 Mean plasma zinc by (A) cigarette and (B) shisha smoking (n=197). Error bars show SD

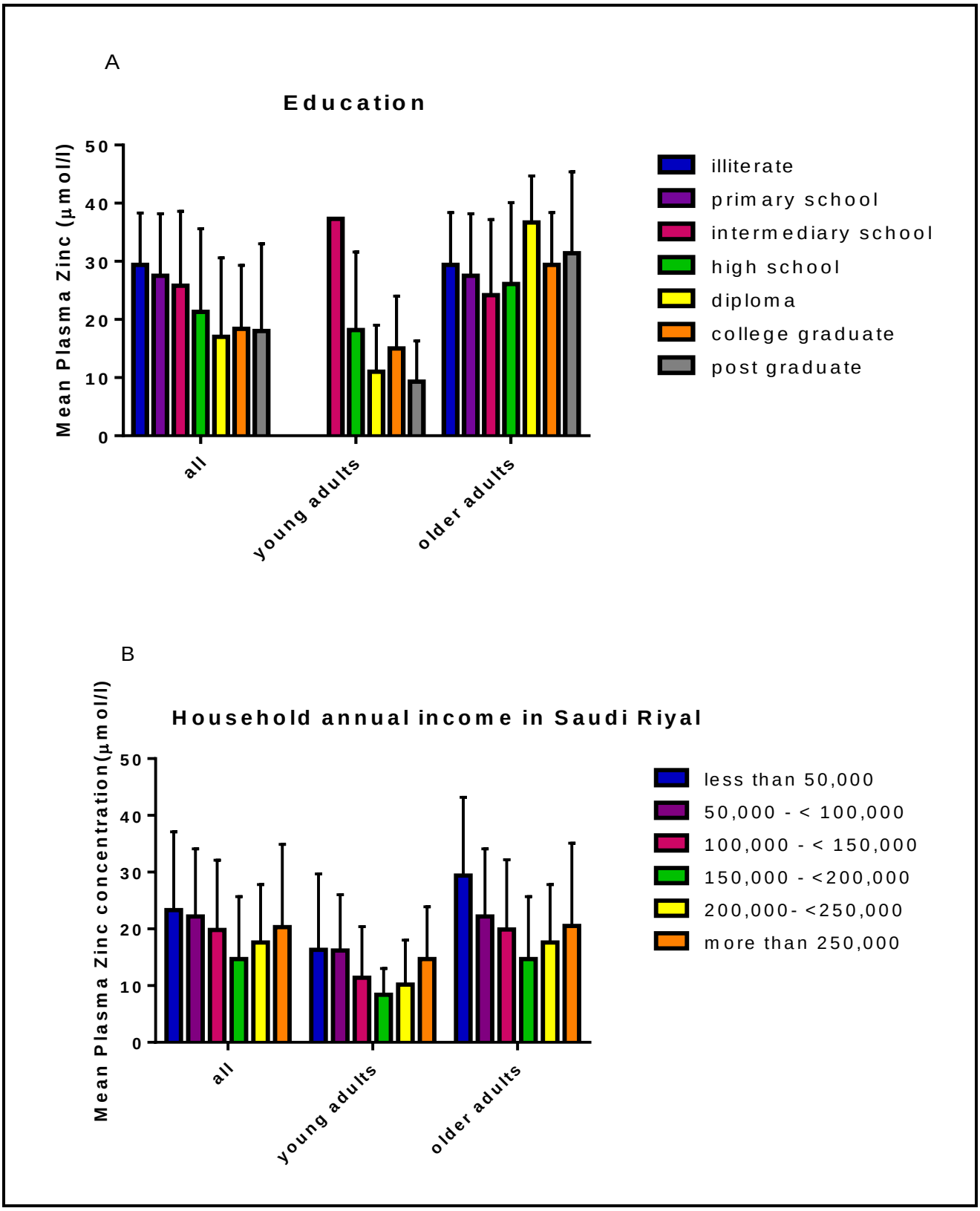


Figure 5.7 Mean plasma zinc concentration by (A) education and (B) household annual income (n=197). Error bars show SD

5.3.7- Plasma metallothionein 1 (MT1) concentrations in young and older male and female Saudi adults:

The mean plasma metallothionein 1 (MT1) concentrations in young and older male and female Saudi adults are shown in Figure 5.8. Mean (±SD) plasma MT1 in Saudi adults was 0.35 ± 0.2 ng/ml. Older adults had higher mean MT1 concentration than young adults but there was no statistically significant difference (P=0.081). Also, male participants had higher mean plasma MT1 concentration than female participants but again there was no statistically significant difference (P= 0.280).

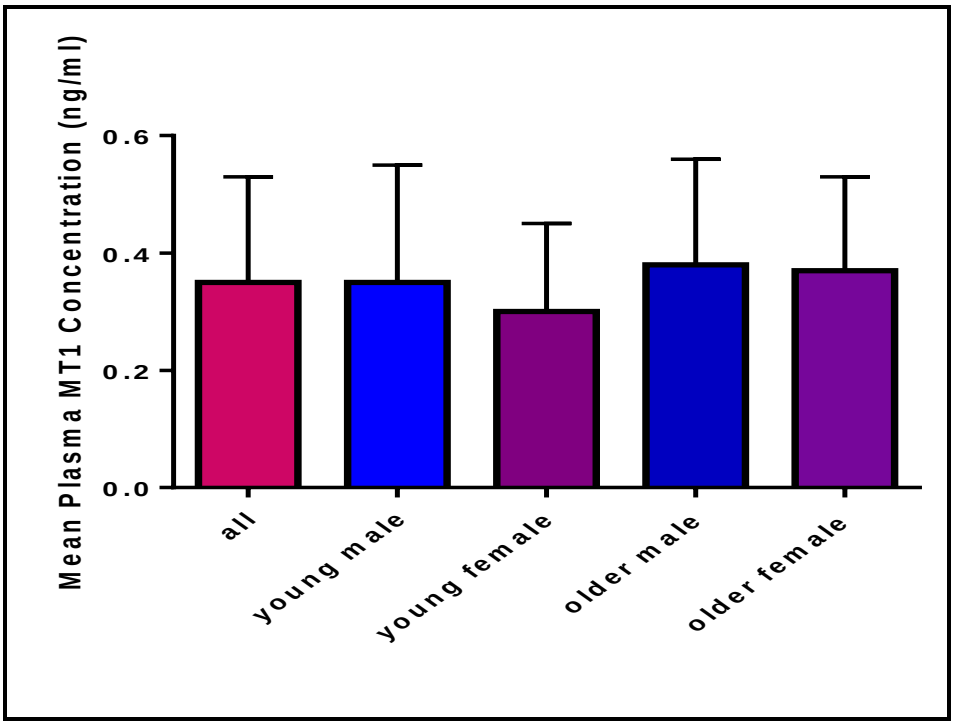


Figure 5.8 Mean Plasma metallothionein 1 concentration in subjects grouped according to sex and age (n=192). Error bars show SD

5.3.8- Relationships between dietary zinc, zinc absorption modifiers, dietary supplements and herbal tea intake on plasma MT1 concentration:

The relationships between dietary zinc, zinc absorption modifiers and plasma MT1 concentration were determined by calculating the correlation coefficients between intake of food components and plasma MT1 concentration (Table 5.6). There were no significant correlations between plasma MT1 concentration and intake of zinc, total zinc (including supplementary), protein, total protein (including supplementary), phytic acid or phytate-to-zinc molar ratio.

Table 5.6 Spearman correlation coefficients (r) between intake of food components and plasma MT1 concentration

Food components intake	r	p
Dietary zinc	0.077	0.289
Total zinc	0.113	0.119
Phytic acid	0.109	0.134
Phytate-to-zinc ratio	0.100	0.168
Dietary protein	0.095	0.192
Total protein	0.094	0.198

Two way ANOVA was used to investigate the effect of dietary supplements and herbal tea consumption on plasma MT1 concentration in young and older adults (Figure 5.9). Participants who consumed herbal tea had significantly lower mean plasma MT1 concentration compared with non-consumers. In contrast, participants who consumed dietary supplements had higher mean plasma MT1 concentration compared with non-consumers but these values did not differ significantly. Table 5.7 shows the mean plasma MT1 concentrations in consumers and non-consumers of several types of herbal tea. Green tea consumers had significantly lower plasma MT1 concentrations than non-consumers. In contrast, there were no statistically significant differences between consumers and non-consumers of other herbal tea. The mean plasma MT1 concentration in consumers and non-consumers of dietary supplements are shown in Table 5.8. There were no statistically significant differences in plasma MT1 concentration between consumers and non-consumers of any of the dietary supplements.

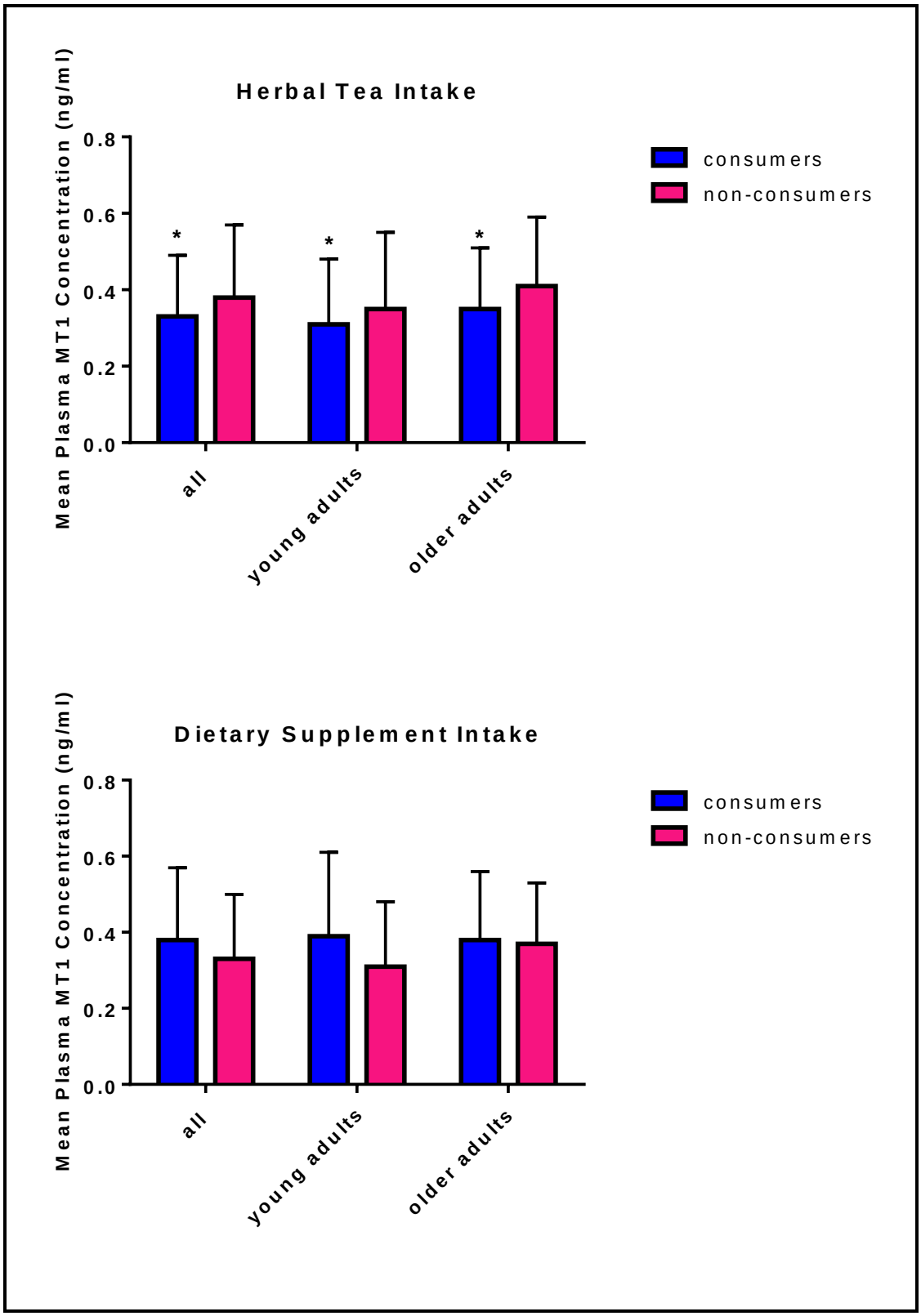


Figure 5.9 Mean plasma MT1 concentration in subjects grouped according to consumption of herbal tea or dietary supplements (n= 192). Error bars show SD.* P<0.05 compared with non-consumers

Table 5.7 Mean plasma MT1 concentrations in consumers and non-consumers of herbal teas (n=192)

Herbal tea	Consumers		Non-consumers		Pooled SEM	Probability of effect		
	Young	Older	Young	Older		Age	Herbal tea consumption	Age* Herbal tea consumption
Mint	0.31	0.37	0.35	0.37	0.01	0.092	0.352	0.446
Green tea	0.23	0.27	0.33	0.39	0.03	0.340	***0.025***	0.766
Anise	0.39	0.38	0.32	0.37	0.02	0.598	0.360	0.549
Caraway	-	0.25	0.33	0.37	0.04	0.068	0.319	-
Fennel	-	0.40	0.33	0.37	0.04	0.093	0.748	-
Chaff Coffee	-	0.29	0.33	0.38	0.03	0.063	0.316	-
Algal leaves tea	-	0.31	0.33	0.37	0.04	0.076	0.593	-
Basil	0.31	0.26	0.33	0.38	0.04	0.985	0.323	0.493
Cinnamon	0.33	0.32	0.33	0.38	0.02	0.490	0.425	0.425
Ginger	0.38	0.33	0.32	0.38	0.02	0.985	0.831	0.213
Cumin	0.34	0.38	0.33	0.37	0.03	0.437	0.840	0.980
Chamomile	0.59	0.42	0.32	0.37	0.05	0.525	0.102	0.276

Table 5.8 Mean plasma MT1 concentrations in consumers and non-consumers of dietary supplement (n=192)

Dietary Supplements	Consumers		Non-consumers		Pooled SEM	Probability of effect		
	Young	Older	Young	Older		Age	Dietary supplement consumption	Age* Dietary supplement consumption
Calcium	0.78	0.39	0.32	0.36	0.05	0.057	0.070	0.081
Vitamin D	0.38	0.40	0.32	0.36	0.02	0.460	0.324	0.901
Vitamin B	0.18	0.41	0.33	0.37	0.05	0.066	0.449	0.212
Vitamin C	0.49	0.34	0.32	0.37	0.06	0.657	0.544	0.363
Multivitamins and minerals	0.45	0.42	0.32	0.37	0.03	0.843	0.091	0.409
Omega-3	0.51	0.32	0.32	0.38	0.05	0.450	0.459	0.182
Iron	0.58	0.95	0.32	0.37		0.050	0.000	0.127
Whey Protein	0.47	-	0.32	0.37	0.04	0.048	0.163	-

5.3.9- Associations between BMI, fat distribution pattern, smoking, education and household annual income and plasma MT1 concentration in Saudi adults:

Mean plasma MT1 concentrations in Saudi adults according to BMI category and fat distribution pattern are shown in Figure 5.10. There was no difference in plasma MT1 concentration between subjects in different BMI categories (P=0.386). Participants who had an apple-shaped fat distribution pattern had significantly higher plasma MT1 concentration than participants who had a pear-shaped fat distribution pattern but this difference was not statistically significant (P=0.239).

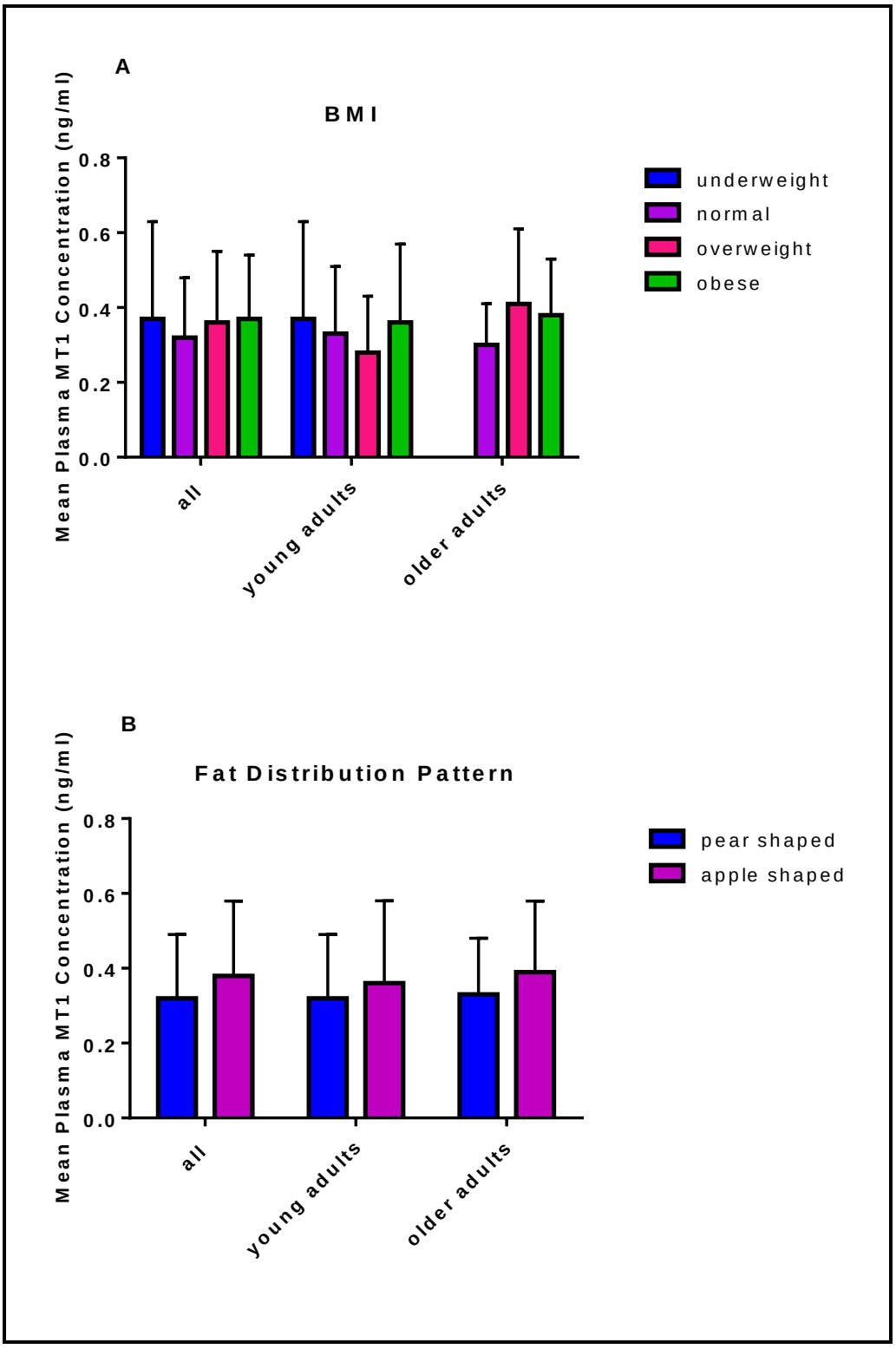


Figure 5.10 Mean plasma MT1 concentration by (A) BMI category and (B) fat distribution pattern (n=192). Error bars show SD

Figure 5.11 and Figure 5.12 shows the mean plasma MT1 concentrations in Saudi adults by smoking and socioeconomic status, respectively. There were no significant differences in plasma MT1 concentration between cigarette smoking categories (P=0.235) or between shisha smokers and non-smokers (P=0.239). Education and household annual income were not related to plasma MT1 concentration (P=0.070, P=0.736, respectively).

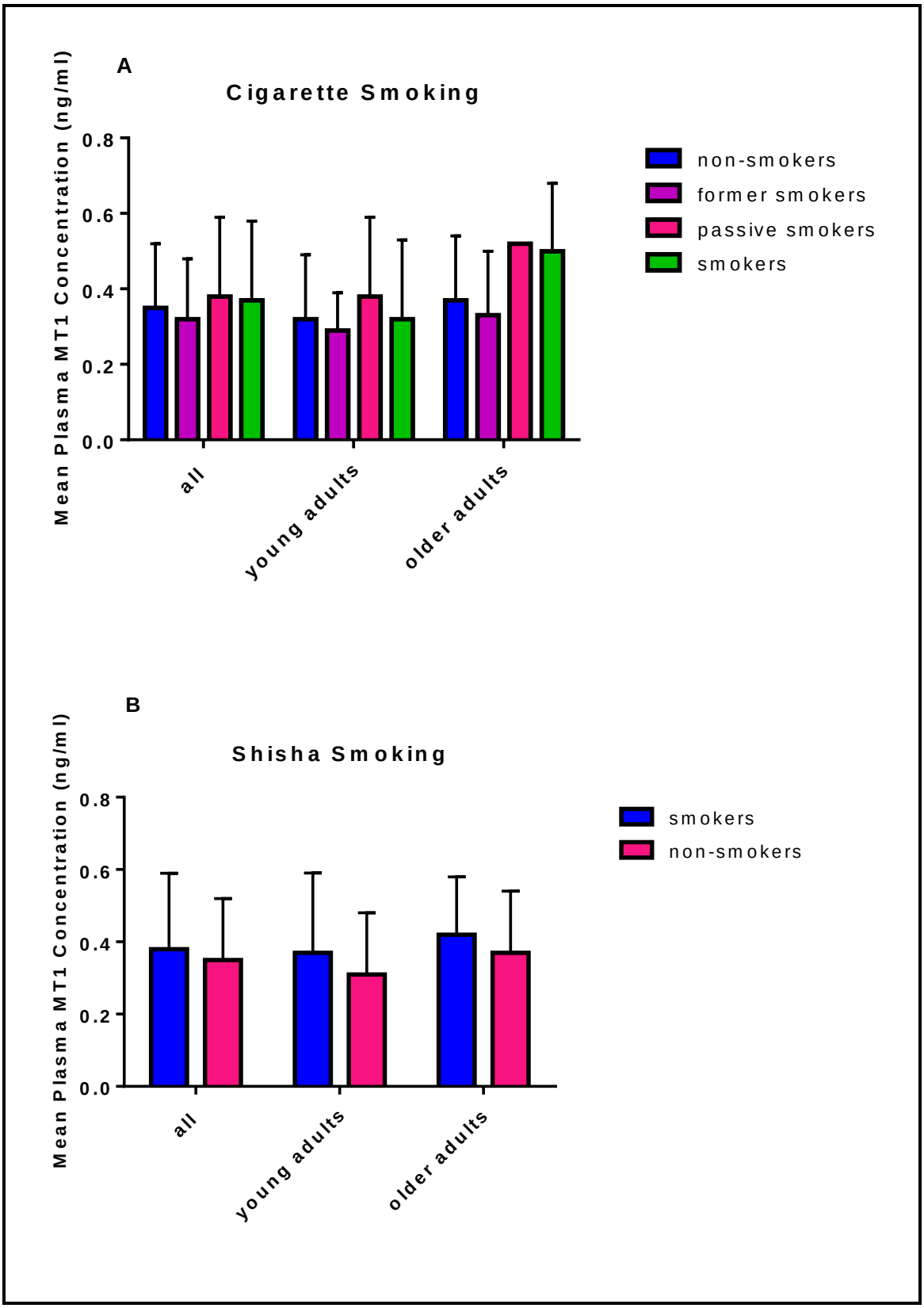


Figure 5.11 Mean plasma MT1 by (A) cigarette and (B) shisha smoking (n=192). Error bars show SD

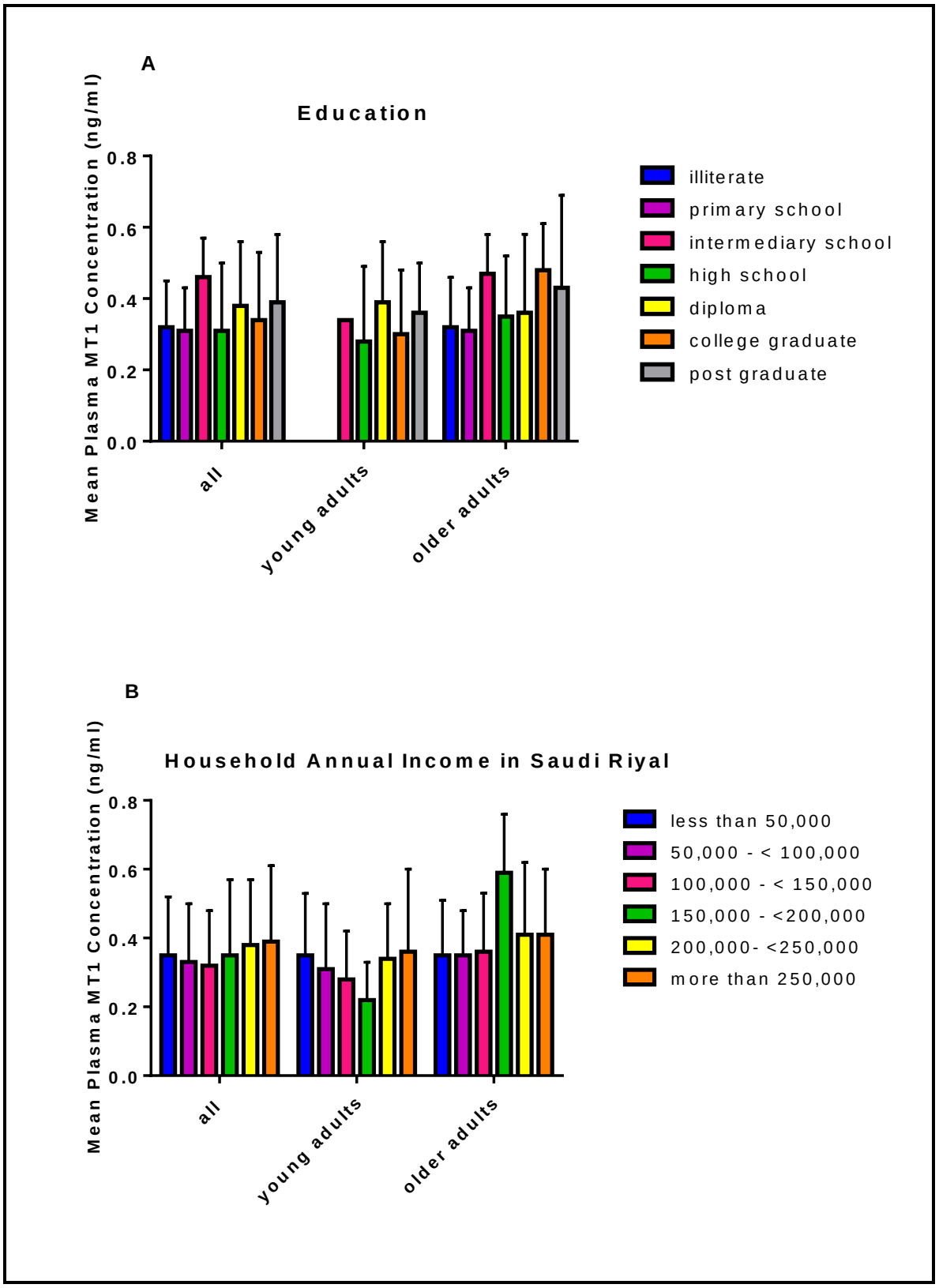


Figure 5.12 Mean plasma MT1 concentration by (A) education and (B) household annual income (n=192). Error bars show SD

5.4- Discussion:

5.4.1- Relationships between age and sex and plasma zinc and MT1 concentrations in Saudi adults:

The mean plasma zinc concentration of young and older Saudi adults of both sexes (21.4 µmol/l) was slightly higher than the mean values (18.2 µmol/l) reported in northeast Thailand (Boonsiri et al., 2006) and substantially higher than those reported in Lebanese adults (15.3 µmol/l) (Obeid et al., 2008), Kuwaiti males (17 µmol/l), Kuwaiti females (15.5 µmol/l) (Abiaka et al., 2003), Greek (11.3µmol/l), French (14.2 µmol/l), German (12.2 µmol/l), Polish (12.6 µmol/l) (Marcellini et al., 2006) and other European populations including Italian, Belgium and English (13 µmol/l) (Arnaud et al., 2012). The mean plasma zinc concentration of young females was 13.4 µmol/l, which is comparable with the results for premenopausal Australian women (12.6 µmol/l) (Lim et al., 2015) . On the other hand, the mean plasma zinc in older females (27.2 µmol/l) was much higher than the mean plasma zinc concentration in postmenopausal Turkish women (19.5 µmol/l) (Arikan et al., 2011).

In contrast with the findings from the present study, several studies have observed that the mean plasma or serum zinc concentration was lower in older adults than in young adults (Sfar et al., 2009, Al-Timimi et al., 2005, Boonsiri et al., 2006). A possible variable that could explain the higher plasma zinc concentration observed in older adults in the current study is the distribution of body fat. Participants who had an apple-shaped fat distribution pattern had significantly higher mean plasma zinc concentration than participants who had a pear-shaped fat distribution pattern. More than half of the older adults had apple-shaped fat distribution pattern while more than half the young adults had a pear-shaped distribution (see section 5.4.4). Another possible explanation for the higher plasma zinc concentration observed in older adults is dehydration (as indicated by significantly higher mean plasma magnesium concentrations in older adults compared with young adults (see Table 5.2)) (Strand et al., 2004). With aging, the proportion of water in the human body declines and, as a result, the water reservoir is reduced and the safety margin for staying hydrated become smaller (Brown et al., 2005). In Saudi Arabia, there is little information about the fluids intake by older Saudis. However, Hosa (2003) found that only 36% of females living in Riyadh drank more than 5 cups of water daily (Hosa, 2003). Moreover, fasting is another contributor to dehydration (Leiper et al., 2003). Most older Saudis fast two days per week (Monday and Thursday) and on the three middle days (13^{th}, 14^{th} and 15^{th}) of each month which may lead to hypo hydration of older adults. High blood glucose causes further dehydration in older adults as the kidneys attempt to unload glucose and ketones by producing large amounts of urine. Almost half the

older adults in the current study had diabetes and high blood glucose levels as shown in Table 5.1. In addition to the distribution of body fat and dehydration, sarcopenia could be another variable. As more than 80% of total body zinc is in muscle, bone, hair and skin, sarcopenia (age-related decline in muscle mass) could lead to the release of zinc from muscle to the blood stream (Cruz-Jentoft et al., 2010).

There is a lack of concordance in the literature concerning differences in plasma zinc concentration between men and women. Al Tamimi et al and Boonsiri el al found no differences in plasma zinc concentration between males and females while Sfar et al and Lam et al reported higher plasma zinc concentrations in males compared with females (Sfar et al., 2009, Al-Timimi et al., 2005, Boonsiri et al., 2006, Lam et al., 2008). In the present study, males had slightly higher mean plasma zinc concentration compared with females but there were no statistically significant difference.

Metallothionein is a low molecular weight cysteine-rich protein with two major isoforms comprising isoform 1 (MT1) and isoform 2 (MT2) (Milnerowicz and Bizon, 2010). In healthy Japanese subjects, the mean serum concentration of total MT was 23±4.6 ng/ml while in young Polish adults total serum MT concentrations were 0.81 and 0.76 ng/ml for females and males, respectively (Nakajima et al., 2010, Kowalska et al., 2015). In this study, the mean plasma MT1 concentration in young and older Saudi adults of both sexes combined was half the serum concentration of total MT in young Polish adults (0.35 ± 0.2 ng/ml). These differences in MT concentrations between our study and other studies may due to analytical differences in e.g. sources of the kits, antibody and type of ELISA used. There were no statistically significant differences between men and women and these results are in contrast with the findings of Kowalska et al (2015) who reported higher serum MT in females compared with males (Kowalska et al., 2015). Furthermore, the authors reported higher serum MT in prime-age adult men (mean age 36) compared to younger men (mean age 23). In current study, older adults had slightly higher mean plasma MT1 concentration compared with young adults but there was no statistically significant difference.

5.4.2- The prevalence of zinc deficiency in young and older male and female Saudi adults:

In young and older Saudi adults of both sexes, the prevalence of zinc deficiency was 28%, which is similar to the prevalence reported in Lebanese subjects (27.6%) but much higher than was observed in Kuwaiti adults (0.53%) (Obeid et al., 2008, Abiaka et al., 2003). Fifty one and forty six percent of young males and females, respectively, had low plasma zinc concentration, which indicated elevated risk of

zinc deficiency in those subpopulations (IZiNCG, 2012a). When using the same cutoff points, studies conducted in Australia and Mexico reported lower prevalence of zinc deficiency in young women compared with the findings from this study. For example, 17% of Australian women and 18% of Mexican women had zinc deficiency whereas 42% of Mexican men were zinc deficient (Garcia et al., 2012, Mejía-Rodríguez et al., 2013, Lim et al., 2015), which is less than that observed for young Saudi Arabian men (51%) in the present study. On the other hand, older Saudis of both sexes had lower prevalence of zinc deficiency (6%). Andriollo et al reported a similar prevalence (5%) of zinc deficiency in European older adults whereas Sfar et al reported higher prevalence (44%) in Tunisian older adults (Andriollo-Sanchez et al., 2005, Sfar et al., 2009).

5.4.3- Relationships between dietary zinc, zinc absorption modifiers and herbal teas intake on plasma zinc and MT1 concentration:

Inadequate zinc intake and/or decreased dietary absorption efficiency contribute to reduced zinc status and the development of zinc deficiency at the population level (Gibson, 2005, Coneyworth et al., 2009). The efficiency of absorption can be affected by the presence of dietary enhancers and inhibitors (Seal and Mathers, 1989). The amount of protein in a meal has a positive effect on zinc absorption (Sandstrom et al., 1980) while phytic acid represses it through the formation of insoluble complexes with zinc in the gastrointestinal tract (Couzy et al., 1998, Kim et al., 2007, Hunt and Beiseigel, 2009). In the present study, there were significant positive correlation coefficients between plasma zinc concentrations and intakes of dietary zinc, total zinc and dietary protein. These positive correlations agree with the findings of other studies (Andriollo-Sanchez et al., 2005, Al-Numair, 2006, Winokan et al., 2010).

Herbal teas are widely consumed by the Saudi population. In this study, green tea consumers had significantly lower mean plasma MT1 concentration than non-consumers. To date, the effect of green tea on plasma MT1 concentration has not been studied in any detail and merits further investigation. On the other hand, there was no statistically significant difference in plasma zinc concentration between green tea consumers and non-consumers. The few studies conducted in rats and humans showed that green tea consumption increased plasma zinc concentration (Suliburska et al., 2012, Meki et al., 2009, Hamdaoui et al., 2005). This effect could be due to the polyphenols in green tea, which stimulates zinc uptake and metallothionein expression as observed in Caco2 cells (Sreenivasulu et al., 2010).

5.4.4- Associations between BMI, fat distribution pattern, smoking, education and household annual income and plasma zinc and MT1 concentration in Saudi adults:

Reported changes in plasma zinc concentrations associated with obesity are not consistent. For example, obese hypertensive patients in Poland had similar serum zinc concentrations compared with normal weight healthy adults (Suliburska et al., 2011). Moreover, no differences were observed in the plasma concentrations of zinc in Mexican women according to their BMI (Garcia et al., 2012). Conversely, other studies showed either increased or decreased in plasma zinc concentrations in obese compared with lean subjects (Yakinci et al., 1997, Chen et al., 1997). In the present study, there were no significant differences in plasma zinc concentration between participants in the different BMI categories. However, there appeared to be a non-significant trend towards higher plasma zinc concentration with increasing BMI in younger adults. A similar observation was reported by Al-Timimi et al (2005) and Yakinci et al (1997), who found that obese children and adults had higher serum zinc concentrations than control groups (Yakinci et al., 1997, Al-Timimi et al., 2005). In contrast, we observed in the current study a non-significant trend in obese older adults towards lower plasma zinc concentration compared with participants from the same age group with BMIs in the normal range. Broadly consistent with this observations are the findings of Chen et al (1997), Marreiro et al (2002) and Yerlikaya et al (2013), who reported lower plasma zinc concentration in obese children and adults compared with lean controls (Chen et al., 1997, Marreiro et al., 2002, Yerlikaya et al., 2013). Alterations in zinc transporter expression could explain the differences observed in the current study in plasma zinc concentration between subjects with pear and apple-shaped fat distribution patterns. In the present study, ZnT1, ZnT5 and ZIP4 expression in participants with an apple-shaped fat distribution pattern was significantly higher than in participants with a pear-shaped fat distribution pattern, which may explain the higher plasma zinc concentration observed in the former (see section 6.3.4). Plasma MT1 concentration in participants with an apple-shaped fat distribution pattern was higher than in participants with a pear-shaped fat distribution pattern but these differences were not significant.

Smoking is a further factor that may affect plasma zinc concentration. Effects of smoking on biochemical markers of mineral status were reviewed by Northrop-Clewes and Thurnham (2007). Inflammation is a major contributory mechanism through which smoking affects mineral status, and effects were greater in heavy or long term smokers. Serum zinc concentration was depressed only in heavy smokers (Northrop-Clewes and Thurnham, 2007). In the present study, cigarette smoking was not associated with

any difference in plasma zinc concentration, which may be due to the higher zinc intake of smokers compared with non-smokers and the fact that smoking was only moderate (mean of 13 cigarettes /day). Similarly, there were no significant difference in plasma zinc concentration between smokers and non-smokers of shisha, although there was suggestion of a non-significant trend towards lower concentration in smokers, which may reflect a higher level of toxic exposure. An hour-long shisha/argela smoking session involves 200 puffs, while smoking an average cigarette involves 20 puffs (CDC, 2015). Shisha smoking was also associated with a non-significant trend towards higher plasma MT1 concentration. MT plays important roles in free radical scavenging and detoxification of metals such as cadmium in cigarette smoke (Kowalska et al., 2015, Milnerowicz and Bizon, 2010).

Difference in socioeconomic status have also been shown to be related to plasma zinc concentration (Aydemir et al., 2003). In the present study, plasma zinc concentration was not related to house hold annual income or education level. A similar result was reported by Al-Numair (Al-Numair, 2006).

Chapter 6. Associations between Zinc Intake, Zinc Status and Body Fat and Expression of ZnT1, ZnT5 and ZIP4

6.1- Introduction:

Ageing is accompanied by changes in many physiological systems including the gastrointestinal tract (Brown et al., 2005), where one effect is a decrease in dietary zinc absorption (Aamodt and Rumble, 1983). When young men (22-30 years old) and older men (65-74 years old) consumed the same purified formula diet containing 15 mg of zinc per day, mean zinc absorbance was 31% in the young men and fell significantly to 17% in the older men (Turnlund et al., 1986). August et al (1989), reported that zinc absorption from a zinc adequate diet in elderly subjects was 21% compared with 39% in young subjects (August et al., 1989). Absorption of dietary zinc in humans is predominantly in the proximal small intestine, either the distal duodenum or proximal jejunum (Lee et al., 1989, Krebs, 2000). Several studies on rat intestine and human intestinal Caco-2 cell monolayers have revealed that zinc uptake in the intestine is concentration-dependent and both carrier mediated (saturable) and non-mediated (non-saturable) pathways are involved (Yasuno et al., 2012, Steel and Cousins, 1985). A variety of zinc transporters are expressed in the enterocyte and they belong to two families – the ZnT and Zip families. The ZnT (solute-linked carrier 30 (SLC30A)) proteins decrease intracellular zinc concentration by mediating zinc efflux from cells or influx into intracellular compartments (e.g. endosomes, Golgi apparatus) whereas the Zip (Zrt- and Irt-like (SLC39A)) proteins increase cytosolic zinc concentrations (Eide, 2004, Palmiter and Huang, 2004a, Wang and Zhou, 2010). Of the zinc transporters cloned to date and expressed in the intestine, localization and transport function of ZnT1, ZnT5 and ZIP4 are consistent with a role in dietary zinc absorption (Cragg et al., 2005). Abnormalities in expression of zinc transporters in the intestinal mucosa may be associated with age-related changes in zinc absorption (Coneyworth et al., 2009). To our knowledge, the relative expression of genes involved in zinc absorption in young and older adults has not been studied in detail.

A large body of research shows that ageing is associated with extensive and widespread changes in gene expression in multiple tissues (Gonzalez et al., 2015, Jung et al., 2015, Gupta et al., 2015, Marthandan et al., 2015, Su et al., 2015). For example, ageing was associated with reduction in expression of genes in the ATP synthase, NADH dehydrogenase, cytochrome C reductase and oxidase complexes, as well as in glucose and pyruvate processing in human skeletal muscle tissues (Su et al., 2015). In cultured cerebellar granule neurons (CGNs), expression of neuropeptide Y and Slit homolog 2 (Drosophila) were reported to be increased during ageing (Gupta et al., 2015). Zinc has also a regulatory roles in gene expression. Theoretically, four fundamental mechanisms based on the binding of protein regulatory

factors to gene promoter regions may underlie transcriptional regulation by zinc, differentiated by whether the protein factor when bound exerts a positive or negative influence on transcription and whether the protein factor shows increased or reduced binding to the promoter region under conditions of increased zinc availability (Jackson et al., 2008). Based on these profound effects of ageing and zinc on gene expression, it is likely that zinc transporters expression is affected by aging and that dietary zinc intake is a modifying factor. A further variable to consider in this context is adiposity, since it has been shown in human subjects that obesity was associated with changes in zinc transporters expression (Noh et al., 2014a, Smidt et al., 2007).

Collection of peripheral blood is relatively simple in a clinical setting and blood can be a surrogate tissue to observe the status of other tissues or organs. In addition, it has been suggested that immune cells may be the first to respond to a change in zinc status, even before plasma zinc falls below the normal range. Therefore, peripheral blood mononuclear cells (PBMCs) may provide a practical and responsive measurement of zinc transporter gene expression and cellular zinc homeostasis in humans (Bogale et al., 2015, Foster et al., 2011). PBMC consist of several cellular subtypes including lymphocytes (T cells, B cells and natural killer cells) and monocytes. During aging, the cellular composition of blood has been seen to change. In particular a decrease in lymphocyte counts has been observed in older individuals (Forsey, 2009). A study by Teschendorff *et al* showed that these changes in cell composition during ageing could potentially explain the DNA hypomethylation observed with increasing age. However, this was not the case for age-related DNA hypermethylation. Specifically, the age-associated DNA hypomethylation signature observed in blood correlated with changes in blood cell-type composition in a set of cancer-related genes, while there was no relationship between the age-related DNA hypermethylation signature and the cellular composition of blood (Teschendorff et al., 2009). Therefore, it is important to ascertain whether changes in the proportions of different cell types of PBMC during aging are reflected in changes in the expression of zinc transporters genes.

The aim of the work presented in this chapter was to investigate the effect of aging on the expression of zinc transporters involved in absorption of zinc from the gut and their relationship with zinc intake, Zinc status, body fat and cellular composition of the blood.

Objectives:

1. To measure mRNAs corresponding to the zinc transporters ZnT1, ZnT5 and ZIP4 in young and older Saudi adults of both sexes.
2. To investigate associations between zinc intake and plasma zinc concentration and these mRNAs.
3. To investigate associations between BMI and fat distribution pattern and these mRNAs.
4. To determine the leucocyte composition of blood in younger and older Saudi adults of both sexes and its possible association with zinc transporter mRNAs and plasma zinc concentration.

6.2- Materials and Methods:

6.2.1-Participant recruitment:

Two hundred and two male and female participants aged 20-30 years (younger adults) and 55-75 years (older adults) were recruited from King Abdulaziz University students, King Abdulaziz University Hospital, King Fahad Hospital, via advertisement in social media and via personal contact. Participants were divided into four groups according to sex and age (53 young males, 51 young females, 49 older males and 49 older females). Further details of recruitment are given in section 4.2.1.

6.2.2- Measurement of plasma zinc concentration, anthropometric parameters and dietary zinc intake:

All participants donated blood samples and completed a questionnaire. Blood samples were collected in trace-element free (one sample) and EDTA (2 samples) anticoagulant blood collection tubes. Plasma samples separated from the trace-element free tubes were stored at -80°c for later measurement of zinc concentration by inductively coupled plasma atomic emission spectroscopy (see section 2.4). Hemolyzed samples (n=5) were excluded from the analysis. The two EDTA tubes (each containing 3 ml) were used for the separation of buffy coat for RNA extraction and analysis of cells by flow cytometery (see sections 2.6 and 2.11, respectively). All buffy coat samples were stored immediately at -80°c for later use. RNA was converted to cDNA by reverse transcription then ZnT1, ZnT5 and ZIP4 were measured by qPCR (see sections 2.9 and 2.10). Percentages of T cells, B cells, natural killer cells and monocytes were measured by flow cytometery (see section 2.11). In addition, all participants completed an FFQ (previously validated to be used in Saudi young and older adults (see chapter 3)) and data from this FFQ were used to estimate intakes of zinc as described previously (see section 2.1.5). Body weight, height, waist and hip circumference were measured as described in section 2.1.1.

6.2.3- Statistical analysis:

Two-way ANOVA was used to determine differences between subjects grouped according to age and sex in mRNA for ZnT1, ZnT5 and ZIP4 relative to GAPDH. The same approach was used to determine differences between older and younger subjects grouped according to BMI and fat distribution pattern in the same mRNAs. An independent t-test was used to determine differences between subject groups with respect to the percentage of T cells, B cells, natural killer cells and monocytes. Correlations between dietary zinc intake, total zinc intake, plasma zinc concentration, plasma MT1 concentration and proportions of leucocyte subtypes and the zinc transporter mRNAs measured were investigated using Pearson and/or Spearman correlation (r). All statistical analyses were performed using the statistical software package IBM SPSS version 19. Statistical significance was taken as $P<0.05$.

6.3- Results:

6.3.1- Relative mRNA level of the zinc transporters ZnT1, ZnT5 and ZIP4 in young and older male and female Saudi adults:

Figure 6.1 shows ZnT1, ZnT5 and ZIP4 mRNAs relative to GAPDH measured by qPCR (detailed CT values are summarized in Appendix F). For all participants, relative to GAPDH mean *ZIP4* mRNA was higher than ZnT1 and ZnT5. Older males had significantly higher ZIP4 mRNA compared with young male adults ($p= 0.042$). Older males had higher mean ZnT1 and ZnT5 mRNAs than young males but there were no significant differences ($P= 0.270$ and $P= 0.224$, respectively). There were no differences in expression of any of the mRNAs measured between males and females ($P >0.05$).

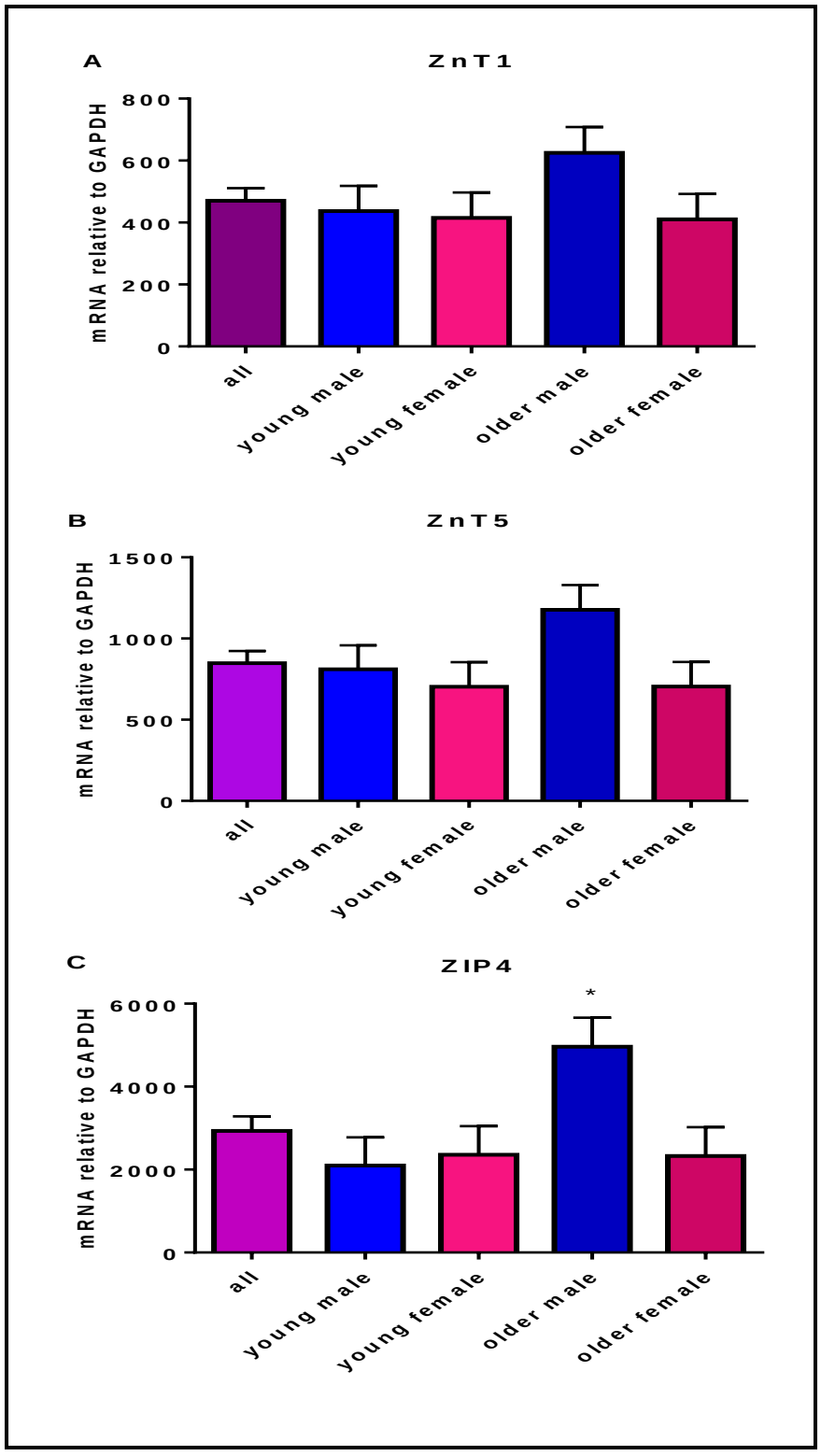


Figure 6.1 Zinc transporter mRNAs (A) ZnT1 (B) ZnT5 (C) ZIP4 for subjects grouped by age and sex (n=201). Data are means ± SEM relative to GAPDH mRNA. * P< 0.05 older males compared with young males

6.3.2- Associations between zinc intake and plasma zinc and MT1 concentrations and zinc transporter mRNAs:

Analysis of the relationship between dietary zinc intake, total zinc intake (including supplementary), plasma zinc concentration and plasma MT1 concentration and the zinc transporter mRNAs measured are shown in Table 6.1. A strong and significant correlation was found between ZIP4, ZnT1 and ZnT5 mRNAs in all participants and also when subjects were grouped according to age and sex. Sequential Bonferroni test also indicates a significant correlation at the "table-wide" 0.05 level. In young males, there was a significant inverse correlation between dietary zinc intake and ZnT5 mRNA. There was also a significant inverse correlation between plasma zinc concentration and MT1 in this group. There was a positive relationship between plasma MT1 concentration and ZnT1 and ZnT5 mRNAs in young females and an inverse relationship between MT1 and dietary zinc intake in the same group. In addition, plasma zinc concentration was correlated inversely with ZnT5 mRNA in this group. In older males but not older females, plasma zinc concentration was significantly correlated with ZIP4 mRNA. Sequential Bonferroni test indicates that these tests are non-significant at the "table-wide" 0.05 level.

Table 6.1 Pearson and/or Spearman correlation coefficients (r) between dietary zinc intake, total zinc intake, plasma zinc and MT1 concentrations and zinc transporters mRNAs

	ZnT5	ZIP4	Dietary zinc intake	Total zinc intake	Plasma Zinc Conc.	Sqrt Plasma Zinc Conc.	Plasma MT1
ZnT1							
all	**0.811****	**0.655****	-0.044	-0.044	0.006	0.047	0.031
Young male	**0.786****	**0.568****	-0.266	-0.243	-0.012	-0.012	0.001
Young female	**0.824****	**0.612****	0.050	0.114	-0.119	0.124	**0.430***
Older male	**0.802****	**0.775****	-0.107	-0.143	0.090	0.030	-0.019
Older female	**0.834****	**0.619****	-0.002	0.017	-0.002	-0.014	-0.238
ZnT5							
all		**0.716****	-0.068	-0.067	-0.006	0.041	0.050
Young male		**0.690****	**-0.284***	-0.267	-0.077	-0.077	0.066
Young female		**0.714****	0.027	0.093	**-0.292***	-0.064	**0.442***
Older male		**0.834****	-0.122	-0.168	-0.187	0.116	-0.057
Older female		**0.655****	-0.121	-0.098	0.003	-0.038	-0.269
ZIP4							
All			0.049	0.032	0.086	**0.143***	0.074
Young male			-0.113	-0.099	-0.156	-0.156	0.018
Young female			0.010	0.030	-0.257	0.014	0.268
Older male			-0.046	-0.074	**0.305***	0.195	-0.050
Older female			0.102	0.020	-0.014	-0.058	-0.005
Dietary zinc intake							
all							0.054
Young male							0.107
Young female							**-0.298***
Older male							0.199
Older female							0.065

	ZNT5	*ZIP4*	Dietary zinc intake	Total zinc intake	Plasma Zinc Conc.	Sqrt Plasma Zinc Conc.	Plasma MT1
Total zinc intake							
all							0.104
Young male							0.124
Young female							-0.094
Older male							0.204
Older female							0.035
Plasma zinc conc.							
all							-0.034
Young male							**-0.377****
Young female							-0.178
Older male							-0.042
Older female							0.137

Significance of the correlation: * P <0.05, ** P <0.01. All the data are log-transformed. Sqrt, square root; MT1, metallothionein 1

6.3.3- Associations between BMI, fat distribution pattern and the zinc transporters mRNAs:

Measurement of ZnT1, ZnT5 and ZIP4 mRNAs according to BMI category and fat distribution pattern are shown in Figure 6.2 and Figure 6.3, respectively. In young adults, there was a non-significant apparent trend towards higher ZnT1, ZnT5 and ZIP4 mRNAs with increasing BMI. In contrast, there appeared to be a non-significant trend towards lower levels of these mRNAs with increasing BMI in older adults. Young and older adults who had an apple-shaped fat distribution pattern had significantly higher ZnT1, ZnT5 and ZIP4 mRNAs compared with participants who had a pear-shaped fat distribution pattern ($P=0.016$, $P=0.02$ and $P=0.027$, respectively).

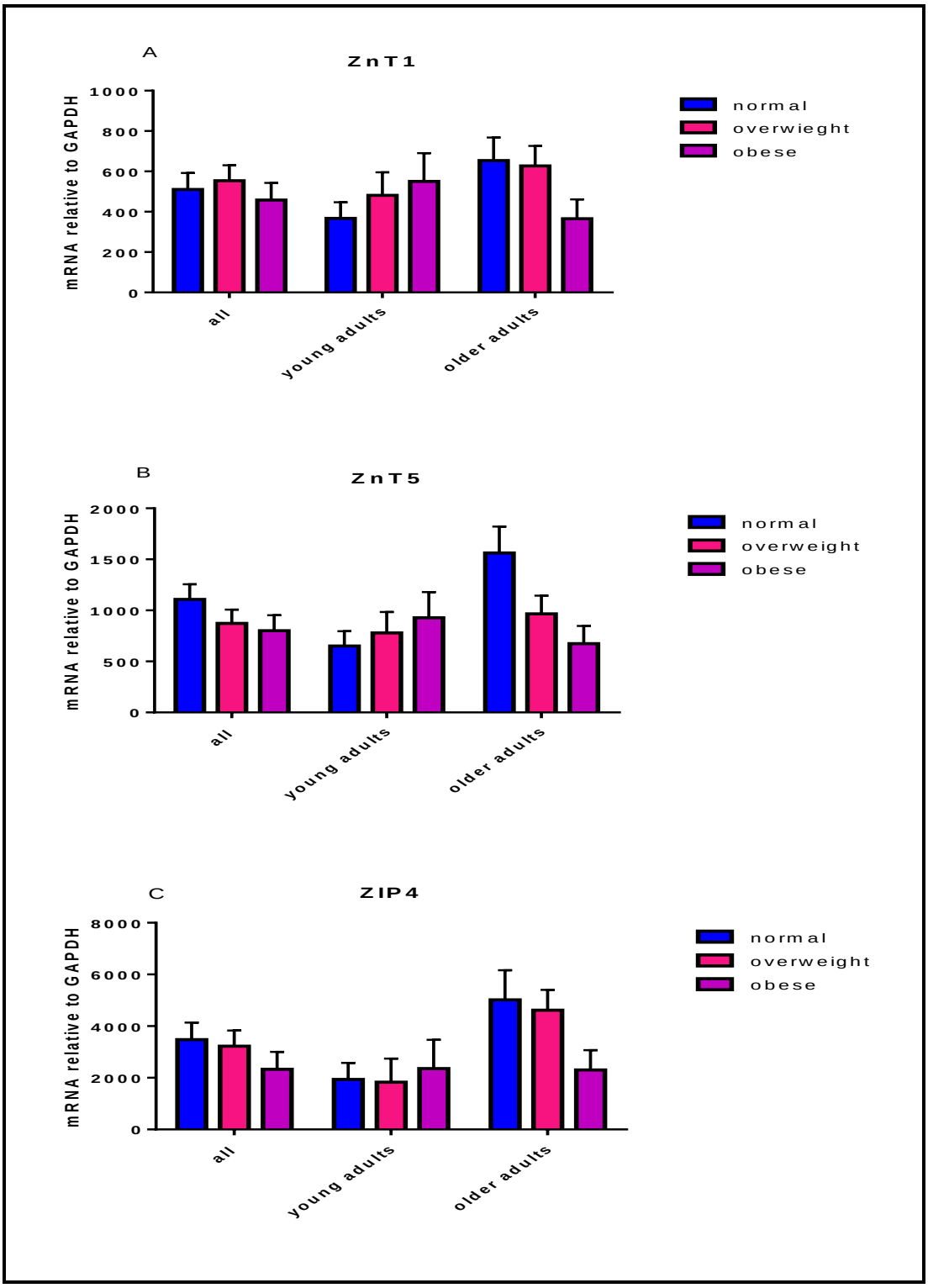


Figure 6.2 Zinc transporter mRNAs (A) ZnT1 (B) ZnT5 (C) ZIP4 in subjects grouped according to BMI category (n=201). Data are expressed as means ± SEM relative to GAPDH mRNA

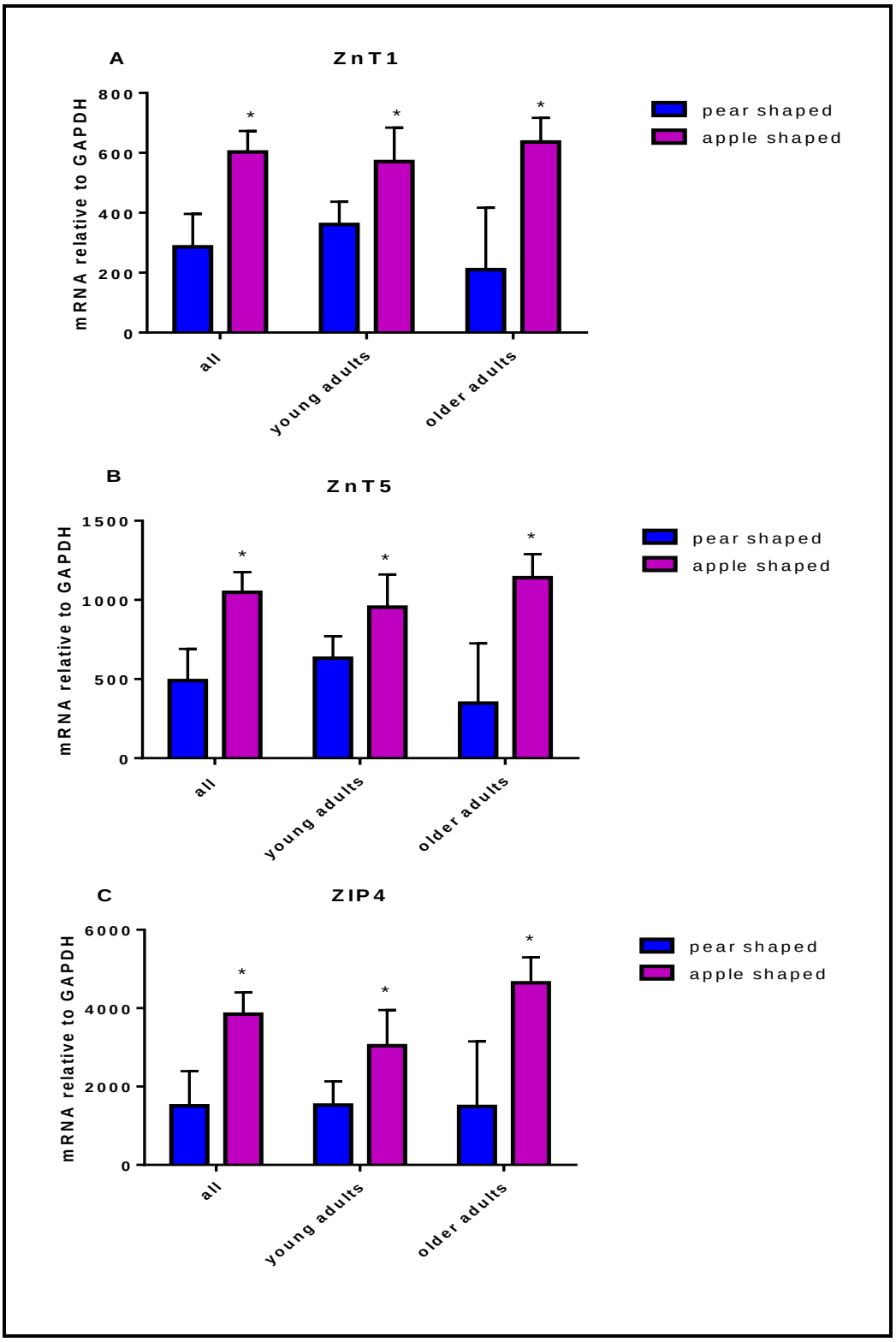


Figure 6.3 Zinc transporters mRNAs in subjects grouped according to fat distribution pattern (n=201) (A) ZnT1 (B) ZnT5 (C) ZIP4. Data are expressed as means ± SEM relative to *GAPDH* mRNA. * Apple shaped compared with pear shaped fat distribution pattern ($P<0.05$)

6.3.4- The leucocytes composition of blood in young and older adults and its possible association with zinc transporters mRNAs and plasma zinc concentration:

Figure 6.4 shows the proportions of T cells, B cells, natural killer cells and monocytes in young and older Saudi adults measured by flow cytometry. Mean (±SD) percentage of T cells for all participants was 52.4 ± 11.5 %. Older adults had a significantly lower proportion of T cells compared with young adults ($P < 0.001$). Proportions of B cells, natural killer cells and monocytes were 11%, 13% and 6%, respectively. There were no significant differences in the proportions of these cell subtypes between young and older adults ($P > 0.05$). Spearman correlation coefficients between the proportion of cell subtypes and zinc transporter mRNAs and plasma zinc concentration are shown in Table 6.2. There was a significant positive correlation between plasma zinc concentration and proportion of monocytes and an inverse correlation between plasma zinc concentration and proportion of T cells. There were no significant correlations between proportions of cell subtypes and any of the zinc transporters mRNAs measured.

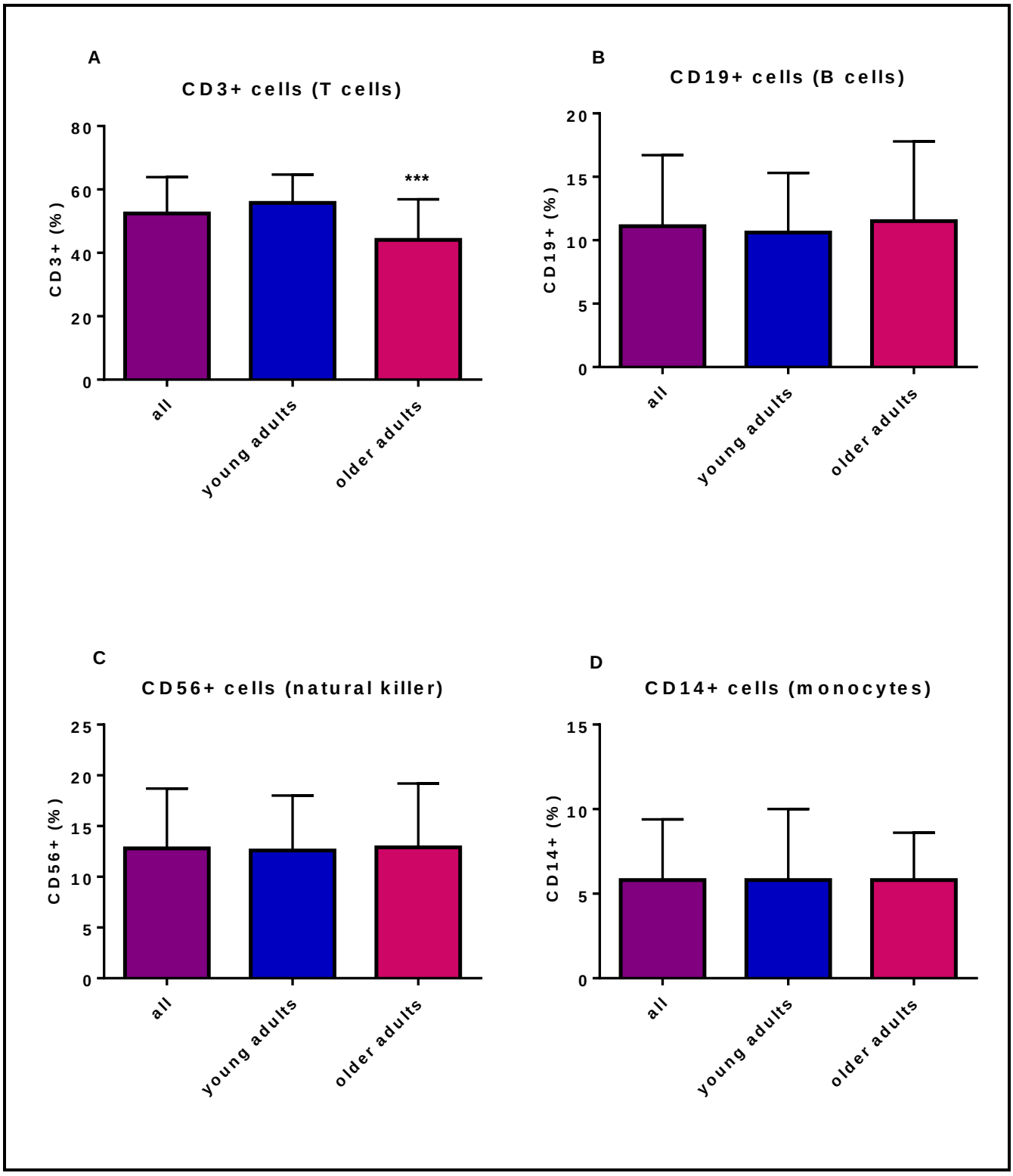


Figure 6.4 The percentage of (A) T cells (B) B cells (C) natural killer cells and (D) monocytes in young and older Saudi adults (n=166). Data are shown as mean ± SD. *** P<0.001 compared with young adults

Table 6.2 Spearman correlation coefficients (r) between the proportion of cell subtype and zinc transporters mRNAs and plasma zinc concentration

	ZnT1	ZnT5	ZIP4	Plasma zinc concentration
CD3+	0.100	0.142	0.071	**-0.239****
CD19+	0.008	-0.024	-0.002	-0.049
CD14+	-0.040	-0.080	-0.138	**0.204****
CD56+	-0.076	-0.033	0.036	0.004

** P<0.01

6.4- Discussion:

6.4.1- Relationships between ZnT1, ZnT5 and ZIP4 mRNAs and zinc intake, plasma zinc concentration and plasma MT1 concentration in young and older male and female Saudi adults:

Several studies in animals and humans report regulatory effects of zinc on ZIP4 expression in the intestine and PBMCs. For example, Cragg et al (2005) found a reduction in ZIP4 protein in ileal mucosa of human subjects after zinc supplementation (Cragg et al., 2005). Similarly, Weaver et al (2007) found that administration of oral zinc caused degradation of ZIP4 in enterocytes while zinc deficiency increased ZIP4 protein level (Weaver et al., 2007). In young women, PBMC ZIP4 expression was decreased following zinc supplementation (Bogale et al., 2015). At the mechanistic level, ubiquitination and degradation of ZIP4 was observed in response to high zinc concentration (10-20μM) in tissue culture (Mao et al., 2007) and Kruppel-like factor (KLF4) was found to bind to the mouse ZIP4 promoter under zinc-limiting conditions to increase expression (Liuzzi et al., 2009). The positive association between ZIP4 expression and dietary zinc intake observed in older males in the current study appears discordant with these published findings. In a recent study conducted on Australian elderly subjects, ZIP1 expression was increased significantly in blood lymphocytes in a zinc supplemented group compared with a placebo group (Sharif et al., 2015) which concurs with this positive relationship between a zinc transporter of the ZIP family and zinc intake in older adults.

The strong positive association between expression of ZIP4, ZnT1 and ZnT5 shown in this study indicates that the expression of these 3 zinc transporters is highly coordinated and may indicate an interrelationship between zinc importers and exporters to maintain cellular zinc homeostasis. Such a model is consistent with a non-significant trend toward higher expression of ZnT1 and ZnT5 in older adults than young adults which could be a consequence of increased ZIP4 expression. In addition, a molecular mechanism involving the activity of metal responsive transcription factor (MTF-1) could be responsible for the induction of ZnT1 and ZnT5 transporters as a result of high zinc intake. MTF-1 is a transcription factor that enhances the transcription of target genes by binding to metal responsive elements (MRE) located in the promoter regions. In previous studies, several MREs were identified in the promoter regions of both ZnT1 and ZnT5 genes (Langmade et al., 2000; Cragg et al., 2002). However, deletion of an MRE from an *SLC30A5* (ZnT5) promoter-reporter construct did not affect the response to zinc (Jackson et al., 2007); hence it cannot be assumed that these MREs are active and that MTF-1

regulates this gene. Higher expression of the zinc transporter genes we measured could explain the higher plasma zinc concentration observed in the older group.

Unlike older male adults, older female adults did not show a differences in ZIP4 expression according to zinc intake. A possible reason is the lower zinc intake of older females compared with older males (see section 4.3.2) and/or the hormonal differences between sexes. To date, the regulation of zinc transporters by hormones has not been studied in any detail and merits further investigations.

An inverse correlation between dietary zinc intake and expression of ZnT5 was observed in young males. ZnT5B is a bidirectional zinc transporter and mediates zinc uptake, as well as zinc efflux. Thus, inadequate zinc intake observed in this age group (see section 4.3.3) may result in a homeostatic response of ZnT5 to increased zinc uptake. Somewhat discordant with these results, Ryu et al (2011) reported a significant decrease in ZnT5 expression with dietary zinc depletion for 10 days in male subjects (Ryu et al., 2011). In the present study, zinc intake was assessed over the year previous to recruitment, which possibly reflects that short-term and long-term effects of inadequate zinc intake result in different responses of ZnT5. The strong positive correlation between expression of ZnT5, ZnT1 and ZIP4 may possibly reflect an increase in ZIP4 and ZnT1 expression with decreased zinc intake. A similar observation was reported by Burke et al (2014), who report an increase in ZnT1 expression during a depletion phase in healthy men (Burke et al., 2014).

In young females, several correlations between plasma MT1 protein, zinc transporter mRNAs and dietary zinc intake were observed. For example, plasma MT1 was correlated significantly with expression of ZnT1 and ZnT5 and correlated inversely with dietary zinc intake. Previous studies have investigated the relationship between zinc intake and/or plasma zinc concentration with either MT1 or MT2 expression in PBMC. For instance, Chu et al (2015) showed that changes in MT2A expression were correlated with changes in the expression of zinc transporters genes, especially ZIP1, in response to zinc supplementation but no significant changes in plasma zinc concentration were observed (Chu et al., 2015). On the other hand, expression of MT1 did not change significantly following zinc supplementation (Bogale et al., 2015).

6.4.2- Associations between BMI, fat distribution pattern and zinc transporters mRNA:

Previous published work has revealed a relationship between obesity and expression of zinc transporters associated with alterations in zinc metabolism (Noh et al., 2014b). In the present study, there was a trend

towards higher ZnT1, ZNnT5 and ZIP4 mRNAs in younger adults with higher BMI but there were no statistically significant relationships. On the other hand, zinc transporters mRNAs tended to decrease with increasing BMI in older adults, but again there were no statistically significant relationship.

Both age groups showed a significant increase in expression of all zinc transporter mRNAs measured with abdominal fatness. Higher levels of inflammatory markers such as hs-CRP and IL-6 were observed in abdominally obese individuals (Faam et al., 2014) and these inflammatory markers were reported to be related to changes in expression of several zinc transporters in obese women (Noh et al., 2014a). In the present study, CRP measurements were normal for all participants. Thus, differences in inflammation are unlikely to be an explanation for the differences in zinc transporter mRNAs observed in our study. Expression of zinc transporter genes may also change between different types of adipose tissue. When comparing expression of zinc transporters in subcutaneous fat tissue with expression in visceral fat from lean and obese subjects, Smidt et al (2007) observed that ZnT6 and ZnT8 were more highly expressed in subcutaneous fat from lean subjects compared to subcutaneous fat from obese subjects and also that expression was higher in visceral compared with abdominal fat tissue from both lean and obese subject. However, ZnT1 and ZnT5 were expressed at similar levels irrespective of leanness/obesity and the type of adipose tissue (Smidt et al., 2007). Interpretation of our own data in the context of these published findings is complicated by the fact we measured the zinc transporter mRNAs in leukocytes, not in adipose tissue.

6.4.3- The leucocytes composition of blood in young and older adults and its possible relationship with zinc transporter mRNAs and plasma zinc concentration:

The mean percentages of CD3+ cells (Tcells) in young and older Saudi adults measured in the current study (52%) was lower than the mean values (72%) reported in Saudi male adults living in Riyadh city (Al Qouzi et al., 2002), Chinese young adults (69%), Chinese older adults (64%) (Jiao et al., 2009), Brazilian young adults (75%), and Brazilian older adults (67%) (Tavares et al., 2014). The mean proportions of CD19+ (B cell) (11%) and CD56+ (natural killer cells) (13%) is comparable with the findings of these other studies (Tavares et al., 2014, Jiao et al., 2009, Al Qouzi et al., 2002). Consistent with findings of the present study, Tavares et al (2014) found a statistically significant reduction in the number of T cell in older compared with young adults (Tavares et al., 2014). In an investigation of T cell subsets, Stervbo et al. found a significant decrease in γδTCR+T cells in elderly compared with younger subjects (Stervbo et al., 2015). A possible cause of an age-related decrease in Tcell counts is thymic

involution (a prominent feature of immunosenescence) which leads to a decrease in the generation of new T cells, finally resulting in a lower number of naïve (CD45RA+) and a higher number of memory (CD45R0+) T cells. Zinc deficiency can also cause thymic involution and thus decrease T cell counts (Haase and Rink, 2009, Mitchell et al., 2006), but no such effect is indicated by the findings of the current study because plasma zinc concentration and proportion of T cells correlated inversely. Consistent with this inverse correlation, Ibs and Rink (2003) reported a decrease in T cells function (measured by suppression of alloreactivity) with high zinc dosage (> 30µmol/l) in the mixed lymphocyte culture (Ibs and Rink, 2003).

We predicted an age-related relationship between leucocytes subtype proportion and zinc transporter gene expression on the basis of the reported correlation between the age-associated changes in blood cell-type composition and DNA hypomethylation in blood (Teschendorff et al., 2009). However, we observed no relationship between the proportion of T cells, B cells, natural killer cells or monocytes with any of the mRNAs measured. Therefore, age-related changes in the leucocytes composition of blood are unlikely to underlie the differences between the groups we observed with respect to zinc transporter mRNAs.

Chapter 7. General Discussion

7.1- Introduction and Hypothesis:

Around the world, the number of people aged 60 years and over is on the rise (World Health Organization, 2011). For example, there were 784 million older people in 2011 which represents 11% of the total population. This is expected to reach 30% in 2100 (World Health Organization, 2011, United Nations, 2011). In Saudi Arabia, the number of older people (>60 years) in 2009 was 907,529 which represents 5.2 % of the Saudi population (Ministry of Economy and Planning, 2009a). Ageing is accompanied by changes in all physiological systems including the gastrointestinal tract (Brown et al., 2005), where one effect is a decrease in dietary zinc absorption (Aamodt and Rumble, 1983). Several zinc transporters belonging to the ZnT and Zip families are expressed in the enterocyte. Of the zinc transporters cloned to date and expressed in the intestine, localization and transport function of ZnT1, ZnT5 and ZIP4 are consistent with a role in dietary zinc absorption (Cragg et al., 2005). Aberrant expression of zinc transporters in the intestinal mucosa may associated with age-related changes in zinc absorption (Coneyworth et al., 2009).

Dietary zinc intake may also change with age. NHANES III data showed that 60.7% of U.S adults aged 19-50 years had adequate zinc intake but this fell to 42.5 % in people aged 71 and older (Briefel et al., 2000). Inadequate zinc intake and/or decreased dietary absorption efficiency are contributing factors to age-related decline in zinc status (Gibson et al., 2008, Coneyworth et al., 2009). In Saudi Arabia, the mean serum zinc concentration of healthy older adults (> 50 years old) was approximately 40% lower than that in young adults (18-22 years old) (6.1 and 10.2 µmol/L, respectively) (Kumosani et al., 1997). In another study conducted on healthy northeast Thais aged between 23 and 75 years old, serum zinc concentrations declined as age increased (Boonsiri et al., 2006). A decline in zinc status may contribute to the development of frailty in older people because of its role in impaired immune response (Haase et al., 2006) and to other age-related degenerative diseases (Coneyworth et al., 2009). The limited number of studies of zinc intake and zinc status in the Saudi population, especially in the light of recent changes in patterns of eating in Saudi Arabia, motivated me to do this research. In addition, studying the effect of aging on expression of zinc transporters involved in absorption of zinc from the gut and their relationship with zinc status may help to reveal the mechanisms underlying age-related changes in zinc status. Based on this my hypothesis were:

1) Zinc intake and zinc status in Saudi elderly are lower than in young Saudi adults.

2) Ageing changes the expression of zinc transporter genes including *SLC30A1, SLC30A5* and *SLC39A4*.

3) Age-related changes in expression of these zinc transporter genes may be associated with changes in zinc intake and zinc status in older people.

7.2- Summary of main findings:

7.2.1 Zinc intake and zinc status in young and older Saudi adults:

To assess zinc status and estimate the risk of zinc deficiency in young and older Saudi adults, I measured dietary intake and plasma zinc concentration (de Benoist et al., 2007). Since there was no suitable tool designed specifically for quantifying intakes of zinc and its absorption modifiers by adults of all ages and both genders in the Saudi population, this project started by developing and validating a food frequency questionnaire (FFQ) suitable for quantifying intakes of zinc and of zinc absorption modifiers. Following piloting, this instrument was then tested in young and older Saudi adults of both genders living in Jeddah city, the western region of Saudi Arabia. The FFQ demonstrated reasonable relative validity and high repeatability in estimating and ranking intakes of zinc, phytic acid and protein. Moreover, its use demonstrated clear gender and age-related differences in intakes of these food components. Then, the FFQ was used to estimate the intake of zinc and its absorption modifiers in two hundreds and two male and female participants aged 20-30 years (younger adults) and 55-75 years (older adults). In addition, all participants donated blood samples which were used to estimate plasma zinc concentration by inductively coupled plasma atomic emission spectroscopy. The findings of the study showed that there were no difference in intakes of zinc and its absorption modifiers between young and older adults either from diet alone or from diet plus dietary supplements. On the other hand, plasma zinc concentrations were significantly higher in older males and females compared with younger groups from both genders. These results are discordant with previous published findings (Alsufiani et al., 2015, Sfar et al., 2009). The current study demonstrated that males had significantly higher intakes of zinc and protein than females. Plasma zinc concentration was also higher in males compared with females but these differences were not significant. These observations agree with findings from other studies (Madej et al., 2013), Mcdaid et al (McDaid et al., 2007), Coulibaly et al (Coulibaly et al., 2009) and Kim and Choi (Kim and Choi, 2013) and Boonsiri et al (Boonsiri et al., 2006).

Human zinc deficiency was first described in the Middle East (Sandstead et al., 1967) and in the Arab region, low zinc intake still persists in different age groups. Twelve percent of the Saudi adults who participated in this study had inadequate intake of zinc (below UK LRNI) either from diet alone or from diet plus dietary supplements. These results indicates elevated risk of zinc deficiency among Saudi adults and the risk was appeared to be greatest for young males followed by young females. Similarly, young males and young females had low plasma zinc concentrations (less than the age/sex/time of day-specific cutoffs shown in Table 1.10) which indicated greater risk of zinc deficiency in younger compared with older adults.

The findings of this study did not agree with my hypothesis that older adults in Saudi Arabia have lower zinc intake and lower plasma zinc concentrations than their younger counter-parts. This motivated me to further investigate changes in expression of zinc transporters involved in absorption of zinc from the gut and their relationship with zinc intake and zinc status in young and older Saudi adults.

7.2.2- Age-related changes in expression of zinc transporters and relationships with zinc intake and zinc status:

I used qPCR to quantify expression of the zinc transporters ZnT1, ZnT5 and ZIP4 at the mRNA level in buffy coat. The results of current study demonstrated for the first time differences in expression of zinc transporters between young and older Saudi adults of both sexes. Older males had significantly higher mean ZIP4 mRNA compared with young males. Older adults had also a non-significant trend toward higher ZnT1 and ZnT5 mRNAs than in young males. Strong and significant correlations were found between ZnT1, ZnT5 and ZIP4 mRNA abundance in all participants and also when subjects were grouped according to age and sex. Moreover, several age and sex-specific correlations between zinc intake, plasma zinc concentration and expression of zinc transporters were found. For instance, in older males but not females, plasma zinc concentration was correlated significantly with ZIP4 mRNA. The positive relationship between ZIP4 expression and dietary zinc intake observed in older males appears discordant with published findings (Cragg et al., 2005, Bogale et al., 2015) but is in line with findings from a recent study conducted on Australian elderly subjects in which ZIP1 expression in PBMCs was increased with zinc supplementation (Sharif et al., 2015). The strong positive associations between expressions of each of the 3 zinc transporters investigated in this study suggests that regulation of expression of the corresponding genes is coordinated. Increased expression of these zinc transporters could then explain the increased plasma zinc concentration in this age group and also explain the positive

relationship between ZIP4 expression and plasma zinc concentration. However, in young males, there was an inverse correlation between dietary zinc intake and expression of ZnT5. Inadequate zinc intake observed in this age group may result in a homeostatic response of ZnT5 to increased zinc uptake because ZnT5B is a bidirectional zinc transporter and mediates zinc uptake, as well as zinc efflux. In young females, several relationships between dietary zinc intakes, zinc transporter mRNAs and plasma MT1 concentrations were observed. For example, plasma MT1 was correlated significantly with expression of ZnT1 and ZnT5 and correlated inversely with dietary zinc intake. All these results supports my hypotheses.

This is the first study, to my knowledge, to examine the potential influence of body fat distribution (dichotomised as apple-shaped or pear-shaped fat distribution patterns) on the plasma zinc concentration and expression of zinc transporters. Young and older adults who had an apple-shaped fat distribution pattern, had significantly higher expression of ZnT1, ZnT5 and ZIP4 and also higher plasma zinc concentration compared with participants who had a pear-shaped fat distribution pattern. Higher levels of inflammatory markers such as hs-CRP and IL-6 were observed in abdominally obese individuals (Faam et al., 2014) and these inflammatory markers were reported to be related to changes in expression of several zinc transporters in obese women (Noh et al., 2014a). In the present study, CRP measurements were normal for all participants. Thus, differences in inflammation are unlikely to explain the differences in zinc transporter mRNAs observed in our study.

7.3- Strengths and Limitations of the Study:

One of the strengths of the current study is the use of an FFQ which was designed and validated specifically for assessing intakes of zinc and of zinc absorption modifiers by Saudi adults. The use of a FFQ rather than e.g. a food diary reduces the burden associated with dietary intake estimation for both study participants and researchers. Such an approach may be particularly useful in some cultural settings because it lowers the cognitive complexity and difficulty for participants not involved in cooking. Since including too few items has been shown to lead to underestimation of intake using an FFQ (Cade et al., 2002) the present study used a relatively large number of food items (64) to assess intake of zinc and its absorption modifiers. Additionally, we used a relatively large number of frequency categories to reduce participants' frustration if unable to find the correct response (Cade et al., 2002). However, in common with all conventional approaches to estimating dietary intake, FFQs have potential limitations due to

subjectivity in intake recording (Penn et al., 2010). Newer developments, including the use of metabolomics approaches, may provide more objective estimates of dietary exposure (Fave et al., 2011).

Another strength of this study is the success in recruiting the full number (200) of participants to the main study. In addition these were divided equally between younger and older participants and with similar numbers of females and males within each age group. The younger and older cohorts were well separated in age (20-30 and 55-75 years for younger and older groups, respectively). These participant features strengthened my ability to detect effects of age and gender and also possible age*gender interactions. My recruits were also highly compliant in completing the dietary and health questionnaires (100% compliance) and in providing blood samples (100% compliance).

Further, the use of qPCR is a robust method for quantifying mRNAs corresponding to the zinc transporters ZnT1, ZnT5 and ZIP4. qPCR provides a highly sensitive and reproducible method for measuring changes in expression of specific genes through transcript sequence detection. Analytical sensitivity of qPCR rests with the fluorometric basis of this technology, which markedly reduces sample size requirements (Aydemir et al., 2006). Zinc transporters were not determined at the protein level so I cannot be confident that changes in zinc transporter expression observed at the mRNA level resulted in changes in the abundance of the transporter proteins and, therefore, in capacity to transport zinc. It is possible that there are difference in post-transcriptional and translational regulation of these zinc transporters in response to age and other factors and this should be investigated in future studies. In addition, my measurements were made in buffy coat and not in small bowel epithelial cells (enterocytes) which are the key cells regulating zinc absorption from the gut into the body. Finally, because of its cross-sectional nature, this study cannot reveal casual relationships and longitudinal studies would be helpful in clarifying the direction of causality for effects of age. Evidence of causality for effects of zinc intake on expression of zinc transporters would require intervention studies.

7.4- Implications for Public Health in Saudi Arabia:

The present study provides useful information about the dietary intake of zinc and its absorption modifiers by young and older Saudi adults of both sexes. This includes intakes from the diet *per se* and also from supplements. Given the rapid changes in socioeconomic circumstances within Saudi Arabia coupled with changes in eating patterns, contemporary information on nutrient intakes and the food sources of those nutrients is important in understanding links between diet and health. This is especially

important for older Saudi adults since life expectancy is increasing steadily in Saudi Arabia as it is in most other countries worldwide. Despite growing prosperity, the traditionally high prevalence of zinc deficiency (assessed by comparison of dietary intakes with the UK LRNI for zinc), appears to continue in young Saudi adults of both genders. Importantly, in this population, intakes of phytate were relatively low leading to low PZR which suggests that any inhibitory effect of phytate on zinc absorption in these Saudi adults will be relatively modest. In addition, the prevalence of low plasma zinc concentrations was greater in younger than in older Saudi adults. Since younger Saudi adults are more likely to have adopted Western-style eating patterns, this observation suggests that secular changes in eating patterns are not advantageous, at least from the perspective of zinc nutrition. These data could help in the planning and development of a nutrition education programs oriented specifically for young adults of both sexes to improve their zinc intake and, therefore, zinc status as assessed by plasma zinc concentration. The detailed information on food contribution to zinc intake provided by this study will be useful for the development of food-based dietary guidelines and to make culturally appropriate recommendations to improve dietary quality.

7.5- Future Work:

In the short term, further research aimed to examine the effect of ageing on the methylation status of the promoter regions of the zinc transporters ZnT1, ZnT5 and ZIP4 would be of interest. This would help to determine whether the observed changes in expression of these zinc transporter genes occurred via epigenetic mechanisms. I have collected DNA samples from participants in the current study and, in future work, I intend to determine the association between DNA methylation and expression of these zinc transporters. In addition, this work could be extended to examine the effects of other epigenetic mechanisms including the roles of histone modifications and microRNA (Mathers et al., 2010).

In the longer term, it will be important to extend these investigations of DNA methylation and expression of zinc transporters in a more relevant cell type such as enterocyte using animal models or intestinal mucosal biopsies from humans. The latter studies could be conducted using participants with ileostomies as used by (Cragg et al., 2005). In addition, investigating the regulation of zinc transporters by hormones is of similar importance. For example, determination of sex hormones such as testosterone and estrogen in young and older adults from both sexes could help in explaining the sex-specific changes in gene

expression during ageing. Further studies are required to investigate the mechanisms through which fat distribution pattern appear to affect plasma zinc concentration and expression of zinc transporters genes.

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Appendix A

Serial number

QUESTIONNAIRE

Time of blood draw	
Time of last meal	

section 1:

- Demographic data

1.1) Gender: ☐ Male ☐ Female

1.2) Age:…….

1.3) Weight:……....Kg

1.4) Height:……cm=……m

1.5) BMI=……..Kg/m^2

1.6) Waist circumference:……

1.7) Hip circumference:……

1.8) waist-to-hip ratio:……

section 2:

- Medical history

2.1) Have you ever been diagnosed by a medical doctor or have any of the Following conditions: (please mark with a check) and if you currently Taking any medications, please specify?

	Disease	**Mark**	**medication**
2.1.1-	Cancer		
2.1.2-	High blood pressure		
2.1.3-	Heart disease		
2.1.4-	Liver disease		
2.1.5-	Kidney disease		
2.1.6-	Arthritis		
2.1.7-	Osteoporosis		
2.1.8-	Diabetes		
2.1.9-	Lung disease		
2.1.10-	Allergies		
2.1.11-	Thyroid disease		
2.1.12-	Sickle cell anemia		
2.1.13-	Blood disorders		
2.1.14-	Depression		
2.1.15-	Any gastrointestinal disorders		

2.2) are you currently taking any herbs?

☐ Yes ☐ no

2.3) if yes, please specify?

……

2.4) are you currently taking dietary supplements?

☐ Yes ☐ no

2.5) if yes, please specify the amount and the duration?

……

2.7) are you currently (or during the past year) on a diet?

☐ Yes ☐ no

2.8) if yes, please specify the type of the diet?

……..

section 3:

- Smoking habits

3.1) Are you currently

☐ Smoker (go to question 3.2)

☐ Passive smoker (go to section 4)

☐ Former smoker (go to section 4)

☐ Non-smoker (go to section 4)

3.2) How many cigarettes do you usually smoke per day? ……

3.3) Do you smoke shisha/argella?

☐ Yes ☐ no

3.4) If yes, how many shisha/ argella do you usually smoke? .....

section 4:

- socioeconomic status

4.1) What is the highest grade you completed in school?

- ☐ Illiterate
- ☐ Primary school or less
- ☐ Intermediary school
- ☐ High school graduate
- ☐ Diploma degree
- ☐ College graduate
- ☐ Post graduate

4.2) What is your household annual income?

- ☐ Less than 50,000 Saudi riyals
- ☐ 50,000 - < 100,000 Saudi riyals
- ☐ 100,000 - < 150,000 Saudi riyals
- ☐ 150,000 - < 200,000 Saudi riyals
- ☐ 200,000 - < 250,000 Saudi riyals
- ☐ More than 250,000 Saudi riyals

4.3) What is your current occupation?

- ☐ Student
- ☐ Unemployed
- ☐ Employee
- ☐ Businessman/ businesswoman
- ☐ Retired

section 5:

- Food frequancy qustionnaire

How often **on average** you had consumed each food item over the past year and please specify if the usual serving size differed from the standard serve:

		Average										
Food item	**Quantity**	**S**	**M**	**L**	**never**	**Less than 1/month**	**1-3 times/ month**	**Once / week**	**2-4 times/ week**	**5-6 times/ week**	**Once/ day**	**2 or more/ day**
1-Eggs:	1egg											
1.1-Eggs												
2-Meat:	120 gm											
2.1-Beef												
2.2-Lamb	2 chops, 100 gm											
2.3-Veal	1 chop,											
	90 gm											
2.4-Diced meat (kabab or hamburger)	150 gm											
2.5-Meat shawerma	1 medium sandwich											
2.6-Chicken shawerma	1 medium sandwich											
2.7-Chicken	1 breast or thigh,											
	110 gm											
2.8-Liver	3/4 cup, 150 gm											
3-Seafood:	whole; 1 fillet, 120 gm											
3.1-Fish												
3.2-Oysters	smoked; 1; 5 gm											

		Average										
Food item	**quantity**	**S**	**M**	**L**	**never**	**Less than 1/month**	**1-3 times/ month**	**Once / week**	**2-4 times/ week**	**5-6 times/ week**	**Once/ day**	**2 or more/ day**
3.3- Crab/ scallops	1/2 cup, 90 gm											
3.4-Lobster/ prawn/ squid	1/2 cup, 90 gm											
4-Dairy: 4.1-Cheese	1 slice, 20 gm											
4.2- Yogurt	1 cartoon, 200 g)											
4.3- Cow's Milk	1 glass, 200 ml											
4.4- Cow's Milk (with cereal)	½ cup, 125 ml											
4.5- Cow's Milk (in tea/coffee)	2 tbsp, 40 ml											
4.6- Fermented milk (laban)	1 glass, 200 ml											
4.7- Ice cream	2 scoops, 60 gm											
5- Vegetables:												
5.1-Potato	1 medium, 150 gm											
5.2- Broccoli	1 cup, 80 gm											

		Average										
Food item	**quantity**	**S**	**M**	**L**	**never**	**Less than 1/month**	**1-3 times/ month**	**Once / week**	**2-4 times/ week**	**5-6 times/ week**	**Once/ day**	**2 or more/ day**
5.3- Spinach, silver beet	1/3 cup, 60 gm											
5.4- Tabouli	1 cup, 200 gm											
5.5- Okra with tomato sauce	1 cup											
5.6- Vegetables, stuffed	1 piece											
5.7- Peas	1/3 cup, 60 gm											
5.8- Chickpeas	1/2 cup, 100 gm											
5.9-Broad bean	1/2 cup, 100 gm											
6- fruits:												
Citrus fruits	1 peace											
7- Beverages:												
Cocoa/												
7.1- drinking chocolate	1 tbsp,9gm											
8-Seeds and nuts:												
8.1-Sesame butter	1 teaspoon											
8.2-Nuts	15 gm											

		Average										
Food item	**quantity**	**S**	**M**	**L**	**never**	**Less than 1/month**	**1-3 times/month**	**Once / week**	**2-4 times/ week**	**5-6 times/week**	**Once/ day**	**2 or more/ day**
9-Cereals:												
9.1- Whole grain corn flakes	1 cup, 30gm											
9.2- Corn flakes	1 cup, 30 gm											
9.3- brown Beta Bread	1 slice, 25 gm											
9.4- white Beta Bread	1 slice, 25 gm											
9.5- brown Loaf bread	1 medium size											
9.6- White Loaf bread	1 medium size											
9.7- brown Toast	1 slice, 25 g											
9.8- whiteToast	1 slice, 25 g											
9.9- white Pasta	1 cup, 100gm											
9.10- white Rice	1 cup,75 gm											
9.11- Rice with milk (saleeq)	1 cup											
9.12- Rice with tomato sauce (kabsa)	1 cup											

		Average										
Food item	**quantity**	**S**	**M**	**L**	**never**	**Less than 1/month**	**1-3 times/ month**	**Once / week**	**2-4 times/ week**	**5-6 times/ week**	**Once/ day**	**2 or more/ day**
9.13- Rice with lentil	1 cup											
9.14- whole meal Crisp bread	1; 5 gm											
9.15- white Crisp bread	1; 5 gm											
9.16- Rolled oats (boiled)	1 cup, 230 g											
9.17- Fruit cake/ loaf	1 slice, 75 gm											
9.18- Muffin	1; 55 g											
9.19-Biscuits (bran, whole meal)	1; 20 gm											
10- Miscellaneous:												
10.1- Meat pie (sambosak)	1 piece											
10.2-Cheese pie (sambosak)	1 piece											
10.3- Meet pie (fatayer)	160 gm											
10.4-Cheese pie (fatayer)	160 gm											
10.5- Pizza	150 gm											
10.6-Tameya	3 pieces											
10.7- Pop corn	1 cup											
10.8- Chips/ corn chips/ twisties	50 gm											
10.9-Chocolate	1 bar,50 gm											
10.10-Custard	1/2 cup											
10.11- Rice with milk and sugar (mohalabeya)	1/2 cup											
10.12-Cream caramel	1/2 cup											

5.1) are there any other foods not mentioned above that you usually eat?

☐ Yes ☐ no

5.2) if yes, what is it? And how often on average you had consumed it?

…….

section 6:

- Food record

(Day 1- week day)
Date:
Day of the week:

Meal	Food type and description (give brand name if applicable)	quantity
Breakfast		
Snack		
Lunch		
Snack		
Diner		
snack		

(Day 2- week day)
Date:
Day of the week:

Meal	Food type and description (give brand name if applicable)	quantity
Breakfast		
Snack		
Lunch		
Snack		
Diner		
snack		

(Day 3- weekend day)
Date:
Day of the week:

Meal	Food type and description	quantity
Breakfast		
Snack		
Lunch		
Snack		
Diner		
snack		

Appendix B

Example of dietary analysis

participant ID	zinc content/1 serving of egg (A)	size of egg consumed (B)	frequency of egg consumption (C)	amount of zinc consumed from egg (A*B*C)	zinc content/1 serving of beef (A)	size of beef consumed (B)	frequency of beef consumption (C)	amount of zinc consumed from beef (A*B*C)	…	…..	total zinc intake from all food items (mg/d)
1	0.462	1.5	0.0667	0.046	5.826	1	0.1429	0.83			10.80
2	0.462	1.5	0.4286	0.297	5.826	0	0	0			30.259
3	0.462	0.5	0.1429	0.033	5.826	0.5	0.0667	0.194			9.096
4	0.462	0.5	0.4286	0.099	5.826	0	0	0			16.238
5	0.462	0.5	1	0.231	5.826	0.5	0.0167	0.048			6.594

Appendix C

Raw data for intake of zinc, phytic acid and protein for participants of validation study

participant ID	zinc intake (mg/d)	phytic acid intake (mg/d)	protein intake (g/d)
1	9.25864821	524.0480533	65.6837537
2	11.42890631	503.3335291	76.54543795
3	5.72516596	261.0743761	43.30056171
4	8.56373091	421.7385172	61.6120977
5	14.12612917	617.9800984	94.4832198
6	9.43956872	1073.383721	59.66802206
7	6.69800706	431.220008	43.94686865
8	10.3870993	643.0866585	92.03641646
9	9.85950215	617.1965855	73.26523428
10	7.40459957	353.6541104	48.32384582
11	17.96186722	1216.465104	124.5798296
12	8.04954921	557.593961	69.72522793
13	8.48711767	490.4663948	64.1846757
14	6.2982817	295.6046549	41.40348078
15	7.43273912	221.7488404	56.68901248
16	14.55595367	441.0880422	98.90557641
17	7.73922367	558.4070134	70.55494209
18	5.89847651	323.7368581	45.67170137
19	15.74631971	709.4125655	103.1477231
20	17.39414696	401.0496301	122.9424064
21	4.22407637	269.1067541	29.5527208
22	8.33003056	643.9765086	61.05490924
23	26.391	1312.73	194.4184
24	12.20258277	607.8738236	80.17612557
25	11.3275361	606.0223701	81.04179277
26	14.18548237	740.9741693	105.9486937
27	12.29983215	407.5859075	93.65469164
28	5.96962581	472.376765	41.54915516
29	9.39159936	409.1374672	60.28974647
30	6.41362131	228.5585847	46.1623856
31	7.93147828	366.3598712	63.06354011
32	11.61747402	462.8995693	70.01424705
33	9.0519732	575.5555967	65.16856595
34	5.46835812	255.5150298	43.81290665
35	9.29992742	553.0388488	55.48007978

36	9.6601506	489.5515351	75.78224728
37	3.9832854	155.9438859	25.09845218
38	14.47	685.4739	99.9275
39	4.2903868	132.6983283	31.23645533
40	4.409121	133.9633375	24.00339723
41	6.3094675	319.126054	59.09385586
42	8.55318026	413.4792021	64.82602106
43	9.47463636	452.9870293	84.86946803
44	7.40804117	346.8431862	55.03055937
45	11.15531886	756.1812448	99.45003073
46	19.22069521	1137.033772	140.6537971
47	13.61293628	552.0007621	99.04647304
48	13.14441202	678.9908091	98.77169196
49	8.40770096	415.2190393	57.99735332
50	15.25489836	668.6601615	116.2046283
51	12.94641942	522.781052	90.09180166
52	10.00893942	444.5891179	69.71911805
53	8.98639782	354.842259	61.78089128
54	8.54371552	407.606724	74.71563336
55	19.8303423	1117.107492	149.5247001
56	7.8927437	385.249731	57.95591663
57	11.58090512	604.6198428	96.89727921
58	10.8965025	423.7031509	99.04816246
59	12.86312696	640.9616951	94.05489011
60	5.5539003	251.5214756	50.42079606
61	13.44328011	393.1801181	83.68011902
62	12.28567835	443.6224261	80.69904261
63	14.27682	758.9512	91.5549
64	8.13643625	224.4291993	51.66945179
65	17.24400667	363.5002774	103.8783211
66	18.0560083	388.3026812	106.6905229
67	17.70757775	1291.653792	133.2709228
68	13.44851532	362.5450936	88.85671703
69	8.99347912	445.7832505	50.11659994
70	10.28484235	555.3207381	77.75686143
71	7.91617545	397.5149293	62.51030786
72	11.86270676	766.1542199	87.07074623
73	14.23282163	636.2020606	110.1397319
74	5.18116823	354.7527527	33.46700318
75	10.70228694	385.1149496	74.38885941

76	7.54852587	370.5185734	61.14191365
77	13.52756772	400.0056381	88.46412571
78	13.50618099	817.2955003	90.84864994
79	7.46166863	423.0604098	59.05549025
80	9.20620045	562.1675946	68.2606347
81	14.61884738	863.8769677	104.8390999
82	9.63864325	525.9677437	64.6185024
83	7.71907972	441.4528549	55.9453729
84	5.73095395	349.2057349	45.26927738
85	11.77948739	573.0805713	103.3503504
86	8.9691429	506.6951143	69.7311385
87	14.04003514	789.436863	101.8797578
88	10.9812854	751.9635524	73.04690678
89	16.09628156	891.8174448	117.0006241
90	21.34618074	685.8230768	132.9052193
91	9.37268391	713.9797556	65.53322704
92	6.61417857	315.9937145	46.50632368
93	10.6829985	479.9399932	70.36785495
94	16.82871577	965.2812698	126.4616956
95	7.9788354	327.8255676	84.29212203
96	4.56909687	264.5654711	33.52182113
97	11.97647964	472.6640161	81.4442452
98	8.61046886	345.3999396	66.71442896
99	5.21345907	292.4645685	35.064079
100	4.78397693	170.4568158	33.6929127

Appendix D

Raw data for zinc intake of participants of the main study

participant ID	zinc intake (mg/d)
1	10.80298
2	30.25996
3	9.096576
4	16.23887
5	6.594625
6	6.45074
7	7.773274
8	7.566641
9	3.847592
10	25.33129
11	16.89895
12	8.48454
13	11.64969
15	4.649884
16	4.102952
17	11.15229
18	16.97242
19	4.874043
20	6.908182
21	14.2806
23	25.28748
24	39.77378
25	3.567685
26	9.859602
27	7.202284
28	17.21102
29	9.904947
30	9.284128
31	9.556275
32	6.272592
33	12.12372
34	4.224291
35	11.50598
36	6.955037
37	6.254953
38	3.301857
39	7.443996
40	7.697296
41	9.163277

42	2.42842
43	8.040598
44	32.30861
45	8.307898
46	10.40254
47	5.772159
48	19.4406
49	34.35042
50	9.733813
51	10.04801
52	16.87125
53	7.195335
54	10.15215
55	12.0032
56	19.15833
57	4.428718
58	9.77238
59	3.30553
60	13.7074
61	13.10154
62	7.200376
63	7.405242
64	6.190081
65	6.598013
66	6.873601
67	7.400145
68	7.86702
69	3.09983
70	6.683962
71	22.4795
72	10.56144
73	13.54657
74	14.09758
75	4.456412
76	9.703492
77	3.929793
78	14.28881
79	11.70587
80	18.06818
81	18.67103
82	11.82378

83	5.728675
84	8.331228
85	5.65616
86	7.606377
87	6.612202
88	7.634465
89	19.66739
90	24.31117
91	5.799805
92	10.87504
93	9.152188
94	6.073008
95	6.905123
96	4.799942
97	7.336238
98	12.80155
99	14.19721
100	4.683997
101	7.33562
102	20.48949
103	35.36006
104	7.995299
105	7.238709
106	6.813947
107	11.10644
108	10.6373
109	11.73492
110	13.03866
111	9.787564
112	6.380718
113	4.905188
114	10.35702
115	5.290186
116	5.984145
117	11.38142
118	7.595044
119	13.8753
120	20.11827
121	7.480049
122	8.227486
124	6.500306

125	15.91242
126	10.48426
127	4.772961
128	5.269564
129	11.61615
130	6.944205
131	4.821005
132	19.34347
133	7.996156
134	8.987689
135	8.491692
136	5.447627
137	10.12384
138	6.587354
139	10.91655
140	7.664847
141	13.85371
142	9.727458
143	5.871782
144	12.24471
145	11.8933
146	6.658255
147	10.10051
148	5.931104
149	6.629374
150	11.73738
151	11.43126
152	27.87895
153	36.58801
154	13.00869
155	37.5872
156	20.60777
157	14.66097
158	5.011547
159	19.27911
160	8.938238
161	7.066104
162	13.49623
163	13.26847
164	8.478604

165	20.20768
166	11.75054
167	9.231413
168	11.37443
169	23.86972
170	9.89127
171	6.011448
172	11.03012
173	4.081334
174	14.05431
175	9.510537
176	6.74149
177	14.82102
178	10.16138
179	9.606388
180	3.207858
181	8.471256
182	11.44424
183	11.2147
184	14.55491
185	8.367536
186	14.0082
187	8.783955
188	9.37234
189	8.763071
190	8.937041
191	12.17081
192	17.23748
193	13.74477
194	17.52057
195	8.943402
196	9.486558
197	24.56024
198	8.393264
199	9.851438
200	10.72525
201	7.216202
202	7.673033
203	6.267477
204	10.60237

Appendix E

Percent contribution of food items and food groups to intake of zinc and its absorption modifiers in Saudi adults

Food item	Zinc	Phytic acid	Protein	Food item	Zinc	Phytic acid	Protein	Food item	Zinc	Phytic acid	Protein
Eggs	**1.5**	**0**	**2.5**	**Vegetables**	**8.8**	**25.8**	**23**	white toast	0.3	0.4	0.4
Eggs	1.5	0	2.5	potato	2	6.6	1.7	white pasta	1.8	3.1	1.5
Meat	**36.9**	**3.1**	**35.5**	broccoli	0.1	0.1	0.1	white rice	4.2	9.5	3.1
Beef	3.8	0	2.9	spinach, silver beet	0.3	0.1	0.1	rice with milk (saleeq)	1	1.3	1
lamb	10.6	0	6.3	tabouli	0.5	3.2	0.4	rice with tomato sauce (kabsa)	1.9	3.8	1.5
veal	0.8	0	0.9	okra with tomato sauce	0.5	0.3	0.4	rice with lentil	0.6	2.1	0.5
diced meat	5.5	0.2	3.2	vegetables, stuffed	2.4	0.7	1.6	whole meal crisp bread	0.7	3.8	0.4
meat shawerma	0.6	0.5	0.1	chickpeas	1.7	6.8	1.2	rolled oats (boiled)	0.7	1.1	0.5
chicken shawerma	0.9	1.2	0.7	broad bean	1.8	8	2	fruit cake/loaf	0.3	0.5	0.3
chicken	10.5	1.2	18.4	**Fruits**	**0.4**	**0.4**	**0.7**	muffin	0.1	0.2	0.1
liver	3.8	0	3	citrus fruits	0.4	0.4	0.7	biscuits (bran, whole meal)	0.4	2.3	0.4
Seafood	**1.6**	**0.2**	**7.4**	**Beverages: Cocoa**	**0.1**	**0.2**	**0.03**	**Miscellaneous**	**13.1**	**17.6**	**12.6**
Fish	0.1	0.1	6.2	drinking chocolate	0.1	0.2	0.03	meat pie (sambosak)	0.8	0.2	0.7
oysters	**0.3**	**0**	**0**	**Seeds and nuts**	**1.2**	**4.7**	**0.7**	cheese pie (sambosak)	0.2	0.3	0.5
crab/scallops	0.3	0	0.2	sesame butter	0.3	2.2	0.1	meat pie (fatayer)	1	0.3	0.8
lobster/prawn/squid	0.9	0.1	1	nuts	0.9	2.5	0.6	cheese pie (fatayer)	0.6	0.9	1.4
Dairy	**15.8**	**0**	**15.5**	**Cereals**	**18.9**	**46.5**	**17**	pizza	1.9	1.2	2.5
Cheese	4.5	0	4.5	whole grain corn flakes	0.6	3.8	0.2	Beans and vegetables patty (tameya, flafel)	3.8	7.1	3.6
yogurt	4.4	0	3.5	corn flakes	0.03	0.01	0.2	popcorn	0.2	0.6	0.1
cow's milk	2.8	0	3.2	brown pita bread	2.5	5.8	2.2	chips/ corn chips/ twisties	0.5	2.3	0.6
cow's milk (with cereal)	0	0	0.5	white pita bread	1	2.9	1.5	chocolate	3.5	4.2	1.6
cow's milk (in tea/coffee)	0.6	0	0.7	brown loaf bread	0.7	2.9	0.7	custard	0.2	0	0.3
fermented milk (laban)	2.6	0	2.8	white loaf bread	1.2	0.6	1.7	rice with milk and sugar (mohalabeya)	0.2	0.1	0.2
ice cream	0.4	0	0.3	brown toast	0.8	3.2	0.8	cream caramel	0.2	0.4	0.3

Appendix F

Raw Ct values

Sample Number	Gender	Age Group	average GAPDH CT values	average ZnT1 CT values	average ZnT5 CT values	average ZIP4 CT values
sample1	female	young	18.649	26.1669	27.88335	30.1727
sample 2	female	young	20.0326	28.098	29.6682	31.2217
sample 3	female	young	20.23095	28.3245	31.79085	34.9044
sample 4	female	young	19.15395	26.7374	28.57955	30.23075
sample 5	female	young	19.22195	26.8598	27.242	28.4573
sample 6	female	older	19.55555	27.2377	27.40055	28.8871
sample 7	female	young	20.16365	27.2806	27.77115	28.212
sample 8	female	young	18.50905	27.34355	28.03305	29.9085
sample 9	male	young	19.57845	27.51155	27.9602	28.6219
sample 10	male	young	19.1167	25.9179	27.06555	29.6253
sample 11	male	young	19.2531	27.70025	28.67455	28.7274
sample 12	female	young	18.3021	26.6019	27.66485	28.9482
sample 13	female	young	17.90425	27.33335	28.10215	28.7738
sample 15	female	young	18.02415	27.44505	28.3546	29.29045
sample 16	female	young	18.2482	27.32975	28.1349	28.6775
sample 17	male	young	16.0368	27.3752	28.3134	29.761
sample 18	male	young	15.3224	26.59435	27.77995	29.14515
sample 19	female	young	15.78205	26.29595	26.9878	28.528
sample 20	male	young	17.927	27.3869	27.66905	28.7971
sample 21	male	young	17.3494	26.18915	26.865	27.90665
sample 23	female	young	17.2738	27.28015	27.85455	28.9634
sample 24	female	young	17.85775	27.6021	27.46855	29.3454
sample 25	female	young	18.1638	26.9614	27.6162	29.2222
sample 26	female	young	18.863	27.3989	27.76335	29.2963
sample 27	female	young	18.20305	26.7441	27.98995	29.2944
sample 28	male	young	19.7692	26.5698	26.96695	28.42015

sample 29	male	young	19.2699	26.36895	26.8764	29.0076
sample 30	male	young	19.9738	26.997	27.03595	29.33905
sample 31	male	young	17.57245	27.1207	27.2113	28.63415
sample 32	male	young	16.7731	26.30445	27.03385	28.5112
sample 33	female	young	18.03075	28.5253	29.0546	30.64045
sample 34	female	young	17.21175	26.74685	27.512	28.6899
sample 35	male	young	18.1587	25.5477	29.2977	30.49655
sample 36	male	young	17.35235	24.4066	27.83065	29.80025
sample 37	male	young	17.88355	25.02045	27.9063	29.12665
sample 38	male	young	18.15765	27.30755	27.8101	29.95455
sample 39	male	young	17.67745	26.58295	27.17435	28.8196
sample 40	male	young	18.2488	26.97275	27.4521	29.53805
sample 41	female	young	17.79525	26.454	27.001	28.41755
sample 42	male	young	17.9228	26.8915	27.2892	28.96855
sample 43	female	older	18.111	26.91295	27.63655	29.2851
sample 44	male	young	16.9678	25.97395	27.1946	28.68135
sample 45	male	young	17.6801	26.96675	27.7954	28.9037
sample 46	male	young	17.5852	26.8323	27.4136	28.9052
sample 47	male	young	20.63675	29.3629	30.3201	31.47135
sample 48	male	young	19.3855	27.10775	27.8239	28.98925
sample 49	male	young	19.95885	27.6284	28.80665	27.88185
sample 50	male	young	20.48355	27.5519	29.0989	29.32835
sample 51	male	young	19.74815	26.3551	27.5299	28.2911
sample 52	male	young	19.9169	26.7378	28.0045	28.23955
sample 53	female	young	20.3418	27.1112	27.74835	28.925
sample 54	female	young	18.7644	26.98305	27.84825	28.8479
sample 55	male	young	18.7611	26.84365	27.885	29.3799
sample 56	male	young	18.62615	27.06915	27.6017	28.70665
sample 57	male	young	17.97365	26.6155	27.65815	25.4041
sample 58	male	young	18.12585	26.73275	26.95345	25.30355

sample 59	male	young	18.0839	27.6572	28.5447	25.78465
sample 60	female	young	18.78445	27.3159	28.62565	26.63205
sample 61	female	young	18.9918	26.72895	27.06535	28.2821
sample 63	female	young	18.2832	27.01625	27.71475	28.4999
sample 64	female	young	18.32395	27.11075	27.2982	28.3297
sample 65	female	young	18.2685	27.30945	27.56345	28.2819
sample 66	female	young	19.34085	27.2049	28.0253	29.3323
sample 67	female	young	19.3865	26.7087	27.78425	28.7612
sample 68	male	young	19.3216	27.83945	27.8934	30.18575
sample 69	female	young	17.92525	27.10045	27.84625	28.91925
sample 70	female	young	18.13115	26.74265	27.4158	28.56905
sample 71	male	young	17.39275	26.5395	26.9635	28.1618
sample 72	male	young	17.54535	26.0706	26.8527	29.00115
sample 73	female	young	19.1823	26.64475	28.0811	28.2231
sample 74	male	young	18.6731	26.3078	27.0026	29.2644
sample 75	male	young	19.1052	27.0013	28.122	29.5731
sample 76	male	young	18.7795	26.89335	27.6347	28.79935
sample 77	male	young	17.22205	26.484	27.1775	28.7155
sample 78	male	young	18.3788	27.03875	27.76925	29.42985
sample 79	female	young	18.1022	27.03925	27.2912	28.82155
sample 80	male	young	19.82695	26.9567	25.22935	29.47365
sample 81	female	young	20.43045	27.2297	25.2573	28.9683
sample 82	male	young	19.9685	26.78045	25.5027	29.18145
sample 83	male	young	20.1616	26.34785	25.3192	28.8346
sample 84	male	young	19.9827	26.89365	27.55465	28.20505
sample 85	female	young	19.8631	27.13605	26.7441	28.94625
sample 86	female	young	20.2025	26.1339	26.63855	28.6847
sample 87	female	young	19.6538	26.8072	27.55255	28.897
sample 88	female	young	21.023	27.79615	28.72335	29.494
sample 89	male	young	19.96065	26.80085	27.37115	28.27475

sample 90	male	young	20.3021	26.78165	27.47845	28.22595
sample 91	female	young	17.977	26.41015	27.1343	28.5834
sample 92	female	older	18.1226	27.79955	27.94135	28.91605
sample 93	male	young	18.2414	27.41005	28.08285	29.51555
sample 94	female	young	18.05145	27.5022	27.7542	28.54645
sample 95	female	young	20.78355	27.06385	27.89095	28.7199
sample 96	female	young	20.1448	26.84465	27.88085	28.33395
sample 97	female	young	20.29315	27.81325	27.99705	30.28495
sample 98	male	young	18.4087	27.3395	27.91685	29.454
sample 99	male	young	19.11825	27.89985	28.3395	28.7676
sample 100	male	young	18.46575	27.8401	28.248	28.3823
sample 101	female	young	18.03105	27.2729	27.6396	28.35375
sample 102	male	young	18.3151	27.47605	27.77915	30.2596
sample 103	male	older	18.29605	26.9778	27.60245	29.5706
sample 104	male	older	17.29615	26.39765	27.4746	29.0024
sample 105	male	older	19.7654	26.7898	27.62865	28.1684
sample 106	female	older	20.47115	27.7083	28.5491	30.52575
sample 107	male	older	18.78165	27.2792	27.6286	30.119
sample 108	female	older	18.9404	26.7121	27.6978	29.95755
sample 109	male	older	19.54215	27.67155	27.708	30.56275
sample 110	female	older	18.7238	26.723	26.93155	28.59915
sample 111	male	older	19.631	26.6834	27.75915	28.43795
sample 112	female	older	18.2956	27.8742	27.92385	29.6877
sample 113	female	older	17.71385	26.5703	27.3225	29.1302
sample 114	female	older	17.9188	26.9427	28.3988	29.328
sample 115	female	older	19.37145	27.04325	28.4834	29.1332
sample 116	female	older	19.76085	28.5861	29.81275	31.74915
sample 117	female	older	19.49695	27.0535	28.1282	28.3597
sample 118	female	older	19.2303	26.88785	27.5903	28.92135
sample 119	male	older	20.81625	28.0731	27.1187	28.6223

sample 120	male	older	20.6622	27.92155	27.63775	30.2346
sample 121	female	older	19.6928	26.9748	28.416	28.8824
sample 122	female	older	19.6363	26.72755	27.60665	28.8956
sample 123	female	older	20.2921	26.8958	27.251	28.21155
sample 124	female	older	19.87635	26.39685	27.81175	29.66095
sample 125	female	older	18.14325	26.3509	26.80585	29.3449
sample 126	female	older	19.1789	27.19545	28.0286	31.19
sample 127	female	young	18.5413	27.5281	27.32665	29.15065
sample 128	female	young	18.1531	27.02355	27.6654	32.3045
sample 129	female	young	18.6121	27.5492	28.3494	26.0485
sample 130	female	older	17.7444	25.6964	26.8743	24.04855
sample 131	female	older	18.2357	26.82625	27.9147	26.4972
sample 132	male	older	17.7356	27.26915	28.25805	28.17145
sample 133	female	older	17.92675	27.0489	27.6495	28.59835
sample 134	female	older	17.9664	27.51535	28.0242	28.19995
sample 135	female	older	17.7462	26.46395	27.0263	29.93265
sample 136	female	older	19.86135	27.4598	28.58385	29.14055
sample 137	female	older	19.77665	27.70025	27.76395	29.29205
sample 138	male	older	19.07305	27.56925	27.6851	29.9336
sample 139	male	older	19.50755	27.6949	28.38155	29.6083
sample 140	male	older	18.83025	26.83745	27.8879	30.54105
sample 141	male	older	18.86875	27.65735	27.8424	27.63215
sample 142	female	young	19.195	27.8981	27.9561	27.8655
sample 143	female	older	19.095	26.98645	28.17035	28.54775
sample 144	male	older	18.7479	27.03435	27.39675	28.3662
sample 145	male	older	18.71895	26.9725	27.3144	28.70145
sample 146	female	older	17.71975	27.8523	28.75425	30.7473
sample 147	female	older	19.2054	26.9584	27.57675	29.9437
sample 148	female	older	18.9572	26.8727	27.451	28.2637
sample 149	male	older	19.09295	27.42875	27.514	29.4283

Sample 150	male	older	18.09135	27.14685	27.40775	30.18385
Sample 151	female	older	18.13755	27.42685	28.10965	29.8179
sample 152	male	older	17.8844	26.3085	27.99655	28.95785
Sample 153	female	young	18.1445	27.5167	27.89515	29.2397
Sample 154	female	older	20.5608	27.43905	25.8706	29.77545
Sample 155	male	older	19.80245	26.6904	26.00275	29.47255
Sample 156	male	older	20.1966	27.26125	25.97205	30.84415
Sample 157	female	older	19.52485	26.7311	27.61385	28.9839
Sample 158	female	older	20.55605	27.74645	27.46855	30.30065
Sample 159	female	older	20.1663	26.92975	28.2811	29.71695
Sample 160	female	young	19.3281	26.7008	27.19985	27.8989
Sample 161	male	older	19.50185	27.0209	27.92685	28.97455
Sample 162	female	older	20.27985	27.73575	28.902	31.3651
Sample 163	female	older	19.5328	26.09005	26.31195	28.6405
Sample 164	male	older	20.46835	26.97995	27.1614	29.9061
Sample 165	male	older	19.3583	26.37695	27.0513	29.3073
Sample 166	male	older	17.4405	26.97595	27.455	29.6712
Sample 167	female	older	17.29075	26.59235	27.74545	29.75315
Sample 168	male	older	17.18245	26.0221	27.337	29.46155
Sample 169	male	older	17.7875	24.2699	27.3647	28.9189
Sample 170	male	older	17.77305	23.4355	26.7366	29.84685
Sample 171	female	older	17.76625	23.92135	27.41075	28.61125
Sample 172	male	older	19.6055	27.73635	32.5714	32.3818
Sample 173	male	older	17.2439	26.29435	26.9823	28.6559
Sample 174	male	older	17.8295	26.6388	27.05395	28.8107
Sample 175	female	older	17.33375	26.98245	27.5587	28.5379
Sample 176	male	older	17.38105	26.14735	26.92505	28.6039
Sample 177	male	older	17.73985	27.76605	28.7081	30.7247
Sample 178	male	older	18.87095	27.594	28.21335	29.825
Sample 179	male	older	17.16145	26.8944	27.8435	29.5682

Sample 180	female	older	17.25105	27.0849	27.73095	29.55325
Sample 181	male	older	17.63755	27.10915	27.7796	29.64335
Sample 182	female	older	20.52645	29.9981	30.74685	31.0038
Sample 183	female	older	19.8143	27.19915	27.50995	27.89575
Sample 184	male	older	19.3178	26.6509	27.6136	29.2484
Sample 185	female	older	19.51645	26.61865	27.35585	28.4934
Sample 186	male	older	18.93065	26.23155	27.3849	29.26632
Sample 187	male	older	19.67085	27.0157	27.89955	29.4848
Sample 188	male	older	20.04685	27.91415	28.27275	30.82385
Sample 189	male	older	17.6749	27.08845	28.3532	30.8423
Sample 190	male	older	18.6408	28.4131	29.25515	30.91645
Sample 191	male	older	18.48165	27.5874	28.58915	31.7746
Sample 192	female	older	15.88255	27.9218	28.5501	30.61545
Sample 193	male	older	15.6954	27.57735	28.00915	30.6304
Sample 194	male	older	16.20545	27.31505	28.13335	31.23435
Sample 195	male	older	16.09155	28.1501	28.4258	30.82725
Sample 196	male	older	17.25645	28.0078	28.75825	31.43055
Sample 197	male	older	17.2288	27.44635	28.6097	30.24195
Sample 198	male	older	16.91605	27.4858	28.26855	29.8584
Sample 199	female	older	18.4232	26.54455	26.9785	29.13125
Sample 200	male	older	18.45905	26.76065	27.11195	29.6687
Sample 201	female	older	18.6189	26.30165	26.87965	28.68455
Sample 202	female	older	18.0768	25.756	26.73745	28.7652
Sample 203	female	older	18.08985	25.17715	27.17	28.7808
Sample 204	female	older	18.8004	25.2799	27.165	30.92155