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[Signatures]

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<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAS</td>
<td>Atomic absorption spectroscopy</td>
</tr>
<tr>
<td>ABS</td>
<td>Australian Bureau of Statistics</td>
</tr>
<tr>
<td>AEC</td>
<td>Australian Electoral Commission</td>
</tr>
<tr>
<td>AI</td>
<td>Adequate intake</td>
</tr>
<tr>
<td>ANZFA</td>
<td>Australia New Zealand Food Authority</td>
</tr>
<tr>
<td>ARMS</td>
<td>Amplification refractory mutation system</td>
</tr>
<tr>
<td>ATDS</td>
<td>Australian Total Diet Survey</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>COX</td>
<td>Cytochrome oxidase</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
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<tr>
<td>Cys</td>
<td>Cysteine</td>
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<tr>
<td>DMT1</td>
<td>Divalent metal transporter 1</td>
</tr>
<tr>
<td>EAR</td>
<td>Estimated average requirement</td>
</tr>
<tr>
<td>FAAS</td>
<td>Flame atomic absorption spectroscopy</td>
</tr>
<tr>
<td>FFQ</td>
<td>Food frequency questionnaire</td>
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<tr>
<td>FSANZ</td>
<td>Food Standards Australia New Zealand</td>
</tr>
<tr>
<td>GFAAS</td>
<td>Graphite furnace atomic absorption spectroscopy</td>
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<td>GLM</td>
<td>General linear modelling</td>
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<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
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<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HGAAS</td>
<td>Hydride generation atomic absorption spectroscopy</td>
</tr>
<tr>
<td>ICPMS</td>
<td>Inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>ID</td>
<td>Iodothyronine deiodinase</td>
</tr>
<tr>
<td>IZiNCG</td>
<td>International Zinc Nutrition Consultative Group</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>Met</td>
<td>Methionine</td>
</tr>
<tr>
<td>NATA</td>
<td>National Association of Testing Authorities</td>
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<tr>
<td>NHMRC</td>
<td>National Health and Medical Research Council</td>
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<tr>
<td>NNS</td>
<td>National Nutrition Survey</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>QAP</td>
<td>Quality assurance program</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RCPA</td>
<td>Royal College of Pathologists of Australia</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended daily allowance</td>
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<tr>
<td>RDI</td>
<td>Recommended dietary intake</td>
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<tr>
<td>Se</td>
<td>Selenium</td>
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<td>Sec</td>
<td>Selenocysteine</td>
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<td>SEIFA</td>
<td>Socioeconomic indexes for areas</td>
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<tr>
<td>SelP</td>
<td>Selenoprotein P</td>
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<tr>
<td>SelW</td>
<td>Selenoprotein W</td>
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<tr>
<td>Sem</td>
<td>Selenomethionine</td>
</tr>
<tr>
<td>Sep15</td>
<td>15 kDa selenoprotein</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TAS</td>
<td>Total antioxidant status</td>
</tr>
<tr>
<td>TPN</td>
<td>Total parenteral nutrition</td>
</tr>
<tr>
<td>TR</td>
<td>Thioredoxin reductase</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc</td>
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Journal articles and conference presentations to learned societies arising from work in this thesis

**Journal articles**

Presentations to learned societies


Abstract

Micronutrient deficiency is a public health problem thought to affect a third of the world's population. In Tasmania, selenium deficiency occurred in livestock, and it has been hypothesised that the human population may be at risk of inadequate intakes. There are few Australian studies of trace element status, and previous studies in Tasmania have been very limited and have provided conflicting results.

The primary aim of this thesis was:

- To assess the selenium status of people in northern Tasmania;
- To identify factors that may influence selenium status in these people;
- To determine groups in this population that may be at increased risk of low selenium status.

A secondary aim was to conduct an opportunistic study to assess the copper and zinc status in the same population, and determine some of the factors which may be associated with copper and zinc status in this population.

The main study was a cross sectional population study of approximately 500 subjects randomly selected from the electoral roll in the northern Tasmania; this was preceded by a preliminary study which used a convenience sample from this same geographical region, and some technical work on the assessment of copper and zinc status.

Indices of trace element status measured included dietary intake, serum levels and functional markers of status, such as glutathione peroxidase activity for selenium status. This was linked with data on lifestyle habits, anthropometric measurements,
dietary analysis for other nutrients, and the measurement of total antioxidant status and lipid profiles.

Results from the preliminary research (n = 198) suggested that marginal selenium status may be reasonably widespread in this population, and that certain gender/age groups may also consume inadequate zinc. Hereditary haemochromatosis was not observed to have a major effect on trace element status.

The population study on 498 subjects from the electoral rolls of north, north west and north eastern Tasmania, suggested a high prevalence of marginal selenium status. Northern Tasmanians had mean selenium intakes of 77.4 and 65.1 µg/d for men and women respectively; with 27% of all subjects consuming inadequate amounts of selenium as indicated by NH&MRC guidelines on dietary intakes.

Mean serum selenium was 1.13 µmol/L; and hence a large proportion of the population (80%) was estimated to have serum selenium concentrations below threshold levels associated with selenoprotein requirements. The majority of subjects also had serum selenium concentrations below the level suggested to offer chemopreventative benefits for some cancers. Associations with a common selenoprotein SNP were not found.

In investigations of zinc status, men in particular appeared at risk of inadequacy. Zinc intakes were 12.6 and 10.9 mg/d for men and women respectively. Fifty two percent of men consumed inadequate zinc compared to only 9% of women. Mean serum zinc concentration was 13.0 µmol/L and when compared to the WHO cut-off, 15% of all men had low serum zinc; the prevalence of which rose in older age ranges.

Investigations of copper status suggested that copper deficiency was unlikely in this population. Mean serum copper concentrations were 15.5 and 18.9 µmol/L for men and women respectively; well above the lower clinical reference range.
These findings indicate that many Tasmanians may have marginal selenium status, and that particular population sub-groups may additionally be susceptible to inadequate zinc status. Further research is required. However, with our increasing understanding of the importance of these essential trace elements in maintaining health and for reducing susceptibility to some chronic diseases, the findings are important. There is potential, to be tested, that increased intakes could possibly benefit an aging Tasmanian population, which leads the country in chronic disease rates.
Chapter 1

General Introduction

1.1 Introduction

The last 25 years have seen our understanding of the importance of trace element nutrition increase many-fold. Selenium, in particular, has emerged from a time early last century where its reputation was one of a toxic nuisance, to now be considered an essential trace element and likely chemopreventative agent. Despite the developing knowledge of selenium requirements and potential significant benefits from improving selenium status, little is known in Australia about the status of most of the population.

For much of the country, and especially Tasmania, there have been few concerted efforts to investigate trace element status in soils or the human populations which depend on them for their food supply. Due to the relatively old age of much of Australia in geological terms, large areas of the country are considered likely to be deficient in many nutrients due to constant weathering processes. Much of our understanding with regard to areas of high or low soil levels of trace elements comes from the agriculture industry where crops may have historically failed due to particular deficiencies, or where toxicity or deficiency have been observed in grazing animals.

This is the case in Tasmania, where actual soil content data for most trace elements is non-existent or at best very limited. Problems relating to deficiencies in soils are recognised when they impact on crops, livestock or even the human population, as is the case with iodine (Guttikonda et al. 2002).
1.2 History of trace element deficiency in Tasmania

Since the early 20th Century, Tasmania has been recognised as a region of endemic iodine deficiency. In 1950 an iodine supplementation program was established (in Tasmanian school children) (Richards 2007b) and to more effectively increase overall population intakes of iodine, this program was eventually replaced by iodine fortification of bread in 1966-7. Fortuitous use of iodophors in disinfection procedures in the dairy industry in the 1970's contributed to iodine intakes (Richards 2007a).

Attempts to improve iodine status have been reasonably successful, but iodine deficiency in Tasmania remains a recurring problem whenever such programs are halted or reduced; the fundamental cause of deficiency in the population lying in the soils upon which the state grows its crops and grazes its livestock. Leeching, due to the states' high rainfall and often steep terrain, combines with the effects of past glaciation to render the soils across much of Tasmania deficient in iodine. The environmental conditions that have lead to problems with iodine nutriture are known to also affect other elements of nutritional importance.

Significant areas of Tasmania are thought to be affected by soil deficiencies of a number of other trace nutrients. Data is particularly scarce, but selenium (Judson and Reuter 1999), copper and cobalt (Green 1956) and zinc and molybdenum (Donald and Prescott 1975) have all been identified at various times as being present in lower than required amounts in many soils across Tasmania.
1.3 Selenium in Tasmania

With recent findings regarding its importance to human health, selenium status is of particular interest for the maintenance of optimal health of a population. Selenium in soil is susceptible to removal by leeching and glaciation; as well as farming practices which lead to the acidification of soils, further reducing the metals' bioavailability. As with iodine, a deficiency of selenium in the soil does not manifest in plant life, as it appears to only be required by animals; it may therefore be difficult to detect prior to its effects being felt by animal or human populations.

Although actual soil analysis data for Tasmania appears to be unavailable, evidence from the agricultural industry shows that deficiency problems have been encountered throughout the state (Mason 2007), particularly in sheep (Figure 1.1). Almost all areas of agriculturally active land in Tasmania have recorded incidences of low selenium in sheep, such that the use of livestock selenium supplements is now widespread; taking the form of fortified drenches, slow release rumen pellets, or fortified pasture fertilisers.

Of course, animals such as these are generally entirely dependent on feed that is grown on the properties on which they reside and as such are more susceptible to potential deficiencies than humans who may consume food from local, interstate and international sources. The Tasmanian population however, being proportionally more rurally based than other Australian states (A.B.S. 2003) and an island state, may conceivably be more likely to be eat local foods than their interstate counterparts, and hence the impact of food importation may be lessened.
Since the discovery of its essentiality, our knowledge regarding selenium and its importance in human health has increased greatly, yet much remains to be discovered regarding the influence of this mineral. Apart from newly discovered proteins, selenium research has also brought a new understanding of the molecular mechanisms involved in the creation of proteins; the translational incorporation of selenium into
peptide chains utilises unique molecular mechanisms to form selenoproteins (Hatfield and Gladyshev 2002).

The known selenoproteins, though mostly poorly understood, generally possess a powerful reduction-oxidation (red-ox) capacity. With this ability, they function in wide ranging roles such as antioxidant defence and thyroid metabolism. It is in their role as powerful antioxidants that selenoproteins appear likely to assist in the prevention of cancer and cardiovascular disease, where the aetiology may be linked with oxidative stress and free radical damage. In addition to the influence of selenoproteins, many studies in the last two decades have suggested that low molecular weight selenium metabolites also possess potent chemopreventative abilities (Redman et al. 1997; Ip et al. 2000; Zhong and Oberley 2001; Jiang et al. 2002). Selenium nutrition has garnered great interest worldwide, due to the possible promise of an achievable reduction in disease through nutritional intervention. At least one large study has been conducted in the US, the recently concluded SELECT trial (Lippman et al. 2009), which aimed to confirm the protective effect of supra-supplemental selenium in cancer, while another (PRECISE trial) is planned for the UK and Europe, however only pilot studies have been completed as yet (Ravn-Haren et al. 2008; Rayman et al. 2008).

1.4 Copper and zinc

Copper and zinc are also involved in numerous processes in humans, including antioxidant defence. Indeed copper, zinc and selenium enzymes work in conjunction in the front line of antioxidant defence - as the primary antioxidants Cu/Zn superoxide dismutase and glutathione peroxidase, respectively.

Copper is an essential constituent of numerous metalloproteins, functioning in hormone metabolism, biological membranes, cellular respiration and connective
tissue metabolism. Though severe deficiency is rare in humans, mild deficiency is thought to impair immune function and increase the risk of cardiovascular disease (Percival 1998; Klevay 2000).

Zinc, in addition to antioxidant defence, plays wide ranging roles as a component of >300 enzymes; active in DNA and RNA metabolism, signalling, hormone receptors and biological membranes (Coleman 1992). After iron, zinc is the second most abundant trace metal in the human body, and even mild deficiency can affect growth and development (MacDonald 2000) and impair immune system function (IBS and Rink 2003).

There has been no data relating to Tasmanian copper or zinc status published to date.

1.5 Rationale

There is great interest in selenium nutrition and a growing concern over copper and particularly zinc status in many populations. Nutritional problems that have generally been considered a concern for developing countries may indeed affect some developed countries as well, as has been the experience with iodine in Tasmania. In a state with a rapidly aging population, the second highest cancer incidence nationally, the highest rate of cardiovascular disease and overall chronic disease (A.B.S. 2006a), as well as the second highest rate of smoking in Australia (A.B.S. 2006b), the identification of factors that may assist in the reduction of chronic disease rates would be an important finding.

With increasing evidence that selenium may provide protection against some chronic diseases, and with further results expected from larger studies in the future, it is an opportune time to determine whether Tasmania does indeed have a problem with marginal deficiency of selenium.
Given that these three trace metals, selenium, copper and zinc, may be assessed in the population in a similar manner and share roles in antioxidant protection, a combined study to assess the three nutrients was considered the most judicious use of the time and resources committed to a single study. The potential benefit of such a study is the identification of a population or a subset of the population that might subsequently be responsive to an increased trace element intake, which might improve general health and perhaps decrease the susceptibility to chronic disease.

The approach to meet these aims involved a cross-sectional population study to provide a representative sample from northern Tasmania.

1.6 Aims of thesis

The primary aim of this thesis was:

- To assess the selenium status of people in northern Tasmania;
- To identify factors that may influence selenium status in these people;
- To determine groups in this population that may be at particular risk of low selenium status.

A secondary aim was to conduct an opportunistic study to assess the copper and zinc status in the same population, and determine some of the factors which may be associated with copper and zinc status in this population.
Chapter 2

Literature review

2.1 General Introduction

In humans, there are at least nine trace minerals for which there is a nutritional requirement: chromium, copper, iodine, iron, manganese, molybdenum, selenium and zinc (Solomons and Ruz 1998). Due to more overt effects of deficiency, the majority of interest has previously focussed on iron and iodine; however zinc and particularly selenium have seen much interest more recently.

As these minerals enter the human food chain mostly through the soil, the mineral content and availability in soils used for food production plays a critical role in human nutriture. This is especially the case for a mineral such as selenium where the content of most foods is quite low normally; meaning compensation for lowered content in some foods is not easily achieved with other foods.

In Australia, due to the geological age of much of the land, and natural weathering processes that strip away nutrients, there are many instances of trace element deficiency. Although much of the arid interior of the continent sees little agricultural activity, the more temperate regions of south Western Australia, South Australia, Victoria, Tasmania and New South Wales are cropped and grazed extensively. Here, trace element deficiencies have caused white muscle disease in sheep (selenium), and coastal disease (copper and cobalt) and goitre (iodine) in ruminants (Judson and McFarlane 1998). Crops grown in these regions have also suffered from soil deficiencies of iron, molybdenum, zinc, manganese, cobalt and copper (Peverill et al. 1999).
While nutrient deficiencies in humans have traditionally been considered to be confined mostly to developing countries, it is thought now that mild deficiencies in zinc (Brown et al. 2001) and selenium (Combs 2001) at least, may be widespread, affecting many millions worldwide.

2.2 Selenium: an introduction

The mineral selenium is an essential trace element in animal biology. In the form of what is now considered the twenty first aminoacid, selenocysteine, and incorporated using a specialised mechanism, selenium is an integral component of numerous selenoproteins, of which there are more than 20 known.

Selenium (Se) was first discovered in 1817 by the Swedish chemist Jons Jakob Berzelius. With an atomic weight of 78.96, selenium belongs to group 16 of the periodic table; other members of this group include oxygen, sulphur and tellurium. Selenium and sulphur in particular share many chemical properties and it is as selenomethionine (Sem) and selenocysteine (Sec), analogues of the sulphur containing amino acids methionine (Met) and cysteine (Cys), that selenium is found in tissues in mammals (Terry et al. 2000).

For many years after its discovery, selenium was considered a toxic element due to the effects of excessive consumption, seen very rarely in certain parts of the world in both livestock and humans, where soil and hence food crops have very high selenium content (Yang et al. 1983). A change in this perception came with the discovery that selenium could help prevent specific necrotic conditions in mice and rats (Schwarz and Foltz 1957), and the subsequent observation of selenium deficiency syndromes in pigs, sheep, cattle and poultry (Oldfield 2003). The first specific biological function was finally elucidated when the research groups of Rotruck (1973) and Flohe (1973) demonstrated the essentiality of a selenium atom in each subunit of the enzyme
glutathione peroxidase (GPx), and then by the end of the decade, human selenium deficiency diseases, known as Keshan disease and Kashin-Beck disease, were described in China (Lancet Editorial 1979).

As research efforts have expanded further, details regarding its important and in some ways unique place in animal biology have begun to emerge.

2.3 Selenium and selenoproteins: structure and function

2.3.1 Selenocysteine: the 21st amino acid

Selenium may be incorporated into proteins as either Sem or Sec. Sem is included into a protein non-specifically as a substitute for methionine. The term selenoprotein however is reserved for proteins in which Sec is specifically encoded for in protein mRNA. For a number of years the mammalian glutathione peroxidases, along with a small number of bacterial enzymes, remained the only proteins known to incorporate selenium as an essential component (Stadtman 1977). The process of Sem incorporation into proteins is a case of simple substitution during 'charging' of the Met transfer RNA (tRNA^{met}). As the methionyl-tRNA synthetase does not differentiate between Sem and Met, the amount incorporated during protein synthesis is related to the ratio of Sem and Met available for attachment to the tRNA^{met}. Such non-specific incorporation can also occur with Sec (Birringer et al. 2002).

The specific incorporation of Sec is coded for in RNA by the normal stop codon UGA. A number of specialized molecular components are required to ensure successful co-translational incorporation of Sec (Hatfield and Gladyshev 2002).
2.3.2 Selenoproteins

Early work utilised $^{75}$Se labelling in rats to identify nearly 20 mammalian selenoproteins (Hawkes et al. 1985; Evenson and Sunde 1988). Subsequent computer based screening of sequenced genomes has identified a total of 25 selenoproteins (Kryukov et al. 2003). A number of these have well defined enzymatic functions but most remain poorly described. The inclusion of Sec at the active centre of these enzymes is a definite advantage, as the catalytic activity has been shown to be decreased markedly when replaced with a Cys residue (Lee et al. 2000). A selenol ($pK_a = 5.2$), in contrast to a normal thiol ($pK_a = 8.3$), is fully ionised at physiological pH and therefore fully active (Stadtman 1996). The first selenoprotein discovered, cellular glutathione peroxidase, is a member of a family of at least five glutathione peroxidase enzymes that, in addition to various specific functions, metabolise hydrogen peroxide and other organic peroxides. Other selenoproteins with known functions belong to the iodothyronine deiodinase and thioredoxin reductase families, while those with lesser defined functions include selenoprotein P, selenoprotein W and 15 kDa selenoprotein.

2.3.3 Glutathione peroxidases

The glutathione peroxidase family consists of five known enzymes; cellular glutathione peroxidase (GPx-1), gastrointestinal glutathione peroxidase (GPx-2), extracellular glutathione peroxidase (GPx-3), phospholipid hydroperoxide glutathione peroxidase (GPx-4) and olfactory glutathione peroxidase (GPx-6). With the exception of GPx-4 all are similar structurally, consisting of tetramers of approximately 20-22 kDa each. In contrast GPx-4 is a monomeric protein of around 19 kDa. Functionally GPx-1, GPx-2, GPx-3 and GPx-6 are again similar, efficiently catalysing the
reduction of hydrogen peroxides, other organic peroxides (Allan et al. 1999) and peroxynitrite (Arteel et al. 1999; Klotz et al. 2003), while GPx-4 functions primarily in the catalysis of the reduction of fatty acid hydroperoxides, cholesterol hydroperoxides and other lipoprotein hydroperoxides (Thomas et al. 1990; Sattler et al. 1994). All enzyme forms utilise glutathione as a reducing substrate yet both GPx-3 and GPx-4 may also use a range of other substrates (Birringer et al. 2002).

The tissue distribution of GPx-1 is broad but uneven. It is primarily measured in the red blood cell fraction as a useful functional index for the assessment of those with low selenium intakes. It is a major antioxidant enzyme due to its ability to metabolise various peroxides to water or alcohol, however Se-deficient and GPx-knockout animal models reveal no obvious pathological phenotypes in the absence of oxidative challenge (Arthur 2000; Behne and Kyriakopoulos 2001). In contrast, mice over expressing GPx-1 have been observed to spontaneously develop hyperglycaemia, hyperinsulinaemia, insulin resistance and obesity (McClung et al. 2004), while strong associations have been observed between increased GPx-1 activity and insulin resistance in gestational diabetes (Chen et al. 2003).

The major site of expression of GPx-2 is the gastrointestinal epithelium. Studies of mRNA stability under Se-depletion and the rate of biosynthesis after repletion suggest that GPx-2, along with GPx-4, is the highest ranked of the glutathione peroxidases in the selenoprotein hierarchy (Wingler et al. 1999; Muller et al. 2003). Due to tissue distribution and substrate specificity, it is theorised that GPx-2 is important in the defence against ingested lipid hydroperoxides and possibly the early stages of tumourogenesis in the colon (Brown and Arthur 2001).

Synthesis of GPx-3, found in plasma, primarily occurs in the renal proximal tubular cells (Avissar et al. 1994). Extracellular levels of glutathione appear insufficient to be
a suitable substrate *in vivo* and hence others such as thioredoxin and glutaredoxin may be utilised (Behne and Kyriakopoulos 2001).

Early studies showed that GPx-3 may be active in regulating the bioavailability of nitric oxide (NO) from platelets and vascular tissue (Freedman *et al.* 1995), and found associations between GPx-3 deficiency and platelet hyperactivity and subsequent thrombosis (Freedman *et al.* 1996). More recent studies have observed an association between GPx-3 gene polymorphism and an increased risk of thrombosis and stroke (Voetsch *et al.* 2007; Voetsch *et al.* 2008), these findings though, have not been replicated in all studies (Grond-Ginsbach *et al.* 2009).

The monomeric GPx-4 has the lowest specificity for substrates of all glutathione peroxidases (Birringer *et al.* 2002), although its main substrate appears to be phospholipid hydroperoxides in membranes (Conrad *et al.* 2007). It is clearly an important component of the defence of biological membranes from oxidative attack due to its unique ability to directly reduce complex peroxides such as phospholipid and cholesterol hydrogen peroxides (Thomas *et al.* 1990) and lipoprotein-associated cholesterylesters (Sattler *et al.* 1994). A recent study has shown over expression of GPx-4 delays the development of atherosclerosis in mouse models by protecting against lipid peroxidation and limiting the sensitivity of vascular cells to oxidised lipids (Guo *et al.* 2008). While knockout models of other forms of GPx have to date not provided overt pathological phenotypes, embryonic lethality is observed in null GPx-4 (-/-) mice (Imai *et al.* 2003), with embryos dying between 7½ to 8½ days of gestation, GPx-4 appearing to be essential for embryological brain development (Ufer *et al.* 2008).
Gpx-4 also plays important roles in male fertility. Functioning as an active peroxidase in immature spermatozoa before converting to an important structural protein during sperm maturation (Ursini et al. 1999). Another form of GPx-4 is known as sperm-nuclei glutathione peroxidase (snGPx). Localised in the sperm nuclei, snGPx acts to protect the germ line from peroxidative damage and also assist the stabilisation of condensed chromatin (Pfeifer et al. 2001).

In addition to protection against general lipid peroxidative damage and its role in fertility, GPx-4 also functions in the modulation of inflammation via regulation of eicosanoid metabolism. It appears to achieve this through the control of peroxide tone (Weitzel and Wendel 1993). By controlling the levels of hydrogen peroxide, GPx-4 regulates the activity of cyclooxygenase and lipoxygenase (Weitzel and Wendel 1993; Schnurr et al. 1996; Sakamoto et al. 2000), enzymes directly responsible for the synthesis of prostaglandins and thromboxanes, and leukotrienes respectively.

GPx-6 is the most recently discovered isoform and shares a close homology with GPx-3 but understanding of its function is limited. At this time it is thought to be expressed only in the embryo and in adult olfactory epithelium (Kryukov et al. 2003).

2.3.4 Iodothyronine deiodinases

The iodothyronine deiodinase group of enzymes plays a major metabolic role in vertebrates. The family consists of three distinct membrane bound deiodinases approximately 30 kDa in size, differing in tissue distribution and responsible for the catalysis of the activation and inactivation of thyroid pro-hormones.

Type I iodothyronine deiodinase (ID1) is found primarily in the thyroid, kidney, liver and pituitary (Berry et al. 1991). Able to catalyse the deiodination of both phenolic and tyrosyl rings, it preferentially catalyses the 5'-deiodination of thyroxine (T4) to
form the active hormone 3,3',5-triiodothyronine (T3) but may also catalyse the 5-deiodination to form the inactive 3,3',5'-triiodothyronine (reverse T3). The conversion of T3 and reverse T3 to the inactive T2 is also catalysed by the 5-deiodination and 5'-deiodination of these iodothyronines respectively (Larsen and Berry 1995).

Type II iodothyronine deiodinase (ID2) shares similar catalytic functions with ID1 but exhibits differing tissue distribution, being found predominantly in brain, cardiac, pituitary and placental tissues (Croteau et al. 1996; Salvatore et al. 1996). Unlike ID1, the catalytic activity of ID2 is restricted to the 5'-deiodination of the phenolic ring but is able to convert T4 to T3 and reverse T3 to T2.

Type III iodothyronine deiodinase (ID3) is mostly expressed in the central nervous system, lung and placenta. In contrast to the dual activation/inactivation functions of ID1 and ID2, ID3 acts only to catalyse the deiodination of the tyrosyl rings of T3 and T4 producing inactive T2 and reverse T3 respectively (Salvatore et al. 1995).

The iodothyronine deiodinases major biological functions are therefore the supply of active T3 in plasma and the inactivation of T4, T3 and reverse T3 (ID1), intracellular conversion of T4 to active T3 (ID2) and the inactivation of T4 and T3 (ID3). Despite the importance of the thyroid hormones for growth and development, ID1 and ID2 knockout studies in mice have described relatively mild phenotypes (Schneider et al. 2001; Schneider et al. 2006); the major deficits observed being decreases in regulation of thyroid stimulating hormone (TSH) (Schneider et al. 2001), thermogenesis (de Jesus et al. 2001), and auditory function (Ng et al. 2004). The ID1/ID2 knockout
model has demonstrated that although important, the enzymes may not be essential for T3 production.

The iodothyronine deiodinases appear to be relatively high in the hierarchy of selenoproteins with regard to selenium supply during selenium restriction and as a result are reasonably resistant to selenium deficiency (Croteau et al. 1996; Bates et al. 2000). As such their usefulness for assessing selenium status appears limited.

2.3.5 Thioredoxin reductases

The thioredoxin reductases were only recognised as selenoproteins in 1996 (Gladyshev et al. 1996); the TGA codon coding for the Sec positioned as the penultimate C-terminal residue was initially thought to be a stop codon. The three member family of thioredoxin reductases (TR1, TR2 and TR3) are homodimeric enzymes, consisting of 55 kDa subunits (Gasdaska et al. 1995), whose primary function is the catalysis of the NADPH-dependent reduction of thioredoxin (Sun et al. 1999). The enzymes also efficiently reduce the antioxidant lipoic acid (Arner et al. 1996), dehydroascorbic acid (May et al. 1997), hydrogen peroxides (Bjornstedt et al. 1995; Sun et al. 1999) and other proteins (Lundstrom and Holmgren 1990; Lundstrom-Ljung et al. 1995).

The thioredoxin reductases play a major regulatory role in the process of cell growth and proliferation by providing reduced thioredoxin for various redox-dependent reactions of cellular signalling (Sun et al. 1999), DNA synthesis and inhibition of apoptosis (Mustacich and Powis 2000). Work with mice has demonstrated the essentiality of TR1 and TR3 for life, with knockout models resulting in embryonic death (Conrad et al. 2004; Jakupoglu et al. 2005).

As well as playing vital roles in normal cells, the thioredoxin reductases are also important in many cancers where they support the growth and progression of tumours.
Perhaps not surprisingly, thioredoxin reductases are over expressed in many cancers cells (Rundlof and Arner 2004; Biaglow and Miller 2005; Arner and Holmgren 2006; Fujino et al. 2006). Reduction in tumour growth resulting from the inhibition of TR activity has been observed in recent studies (Yoo et al. 2006; Yoo et al. 2007; Wang et al. 2008; Honegger et al. 2009) and hence the TR’s have emerged as a potential cancer therapy target of numerous inhibitors, including nitrosoareas and gold, platinum and phosphole complexes (Urig and Becker 2006).
### Table 2.1: Summary of tissue expression and known functions of selenoproteins (modified from Reeves and Hoffman (2009)).

<table>
<thead>
<tr>
<th>Selenoprotein</th>
<th>Abbreviation</th>
<th>Important insights into function and significance</th>
<th>Dietary selenium effects</th>
<th>Sub-cellular localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosolic glutathione peroxidase</td>
<td>GPx-1</td>
<td>GPX1 knockout is more susceptible to oxidative challenge. Over-expression of GPX1 increases risk of diabetes.</td>
<td>Very sensitive to Se status, following insufficient Se, or oxidative stress rapid GPx1 recovery. Se deficiency leads to nonsense mediated decay of GPx1 mRNA</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Gastrointestinal glutathione peroxidase</td>
<td>GPx-2</td>
<td>GPX1/GPX2 double knockout mice progressively develop intestinal cancer, one allele of GPX2 added back confers protection</td>
<td>Relatively resistant to dietary Se changes</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Plasma glutathione peroxidase</td>
<td>GPx-3</td>
<td>Important for cardiovascular protection, perhaps through modulation of Nitrous Oxide levels; antioxidant in thyroid gland</td>
<td>Sensitive to Se status</td>
<td>Secreted</td>
</tr>
<tr>
<td>Phospholipid hydroperoxide glutathione peroxidase</td>
<td>GPx-4</td>
<td>Genetic deletion is embryonic lethal; GPX4 acts as crucial antioxidant, structural protein in sperm, and sensor of oxidative stress and pro-apoptotic signals</td>
<td>Relatively resistant to dietary Se changes</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Olfactory glutathione peroxidase</td>
<td>GPx-6</td>
<td>Importance unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thioredoxin reductase Type I</td>
<td>TR1, TrxR1</td>
<td>Localized to cytoplasm and nucleus. Genetic deletion is embryonic lethal</td>
<td>Increased Se increases activity. Se deficiency decreases activity, but does not change mRNA levels</td>
<td>Cytoplasmic, nuclear</td>
</tr>
<tr>
<td>Thioredoxin reductase Type II</td>
<td>TR3, TrxR2</td>
<td>Localized to mitochondria. Genetic deletion is embryonic lethal</td>
<td>Subject to dietary Se changes (i.e. increased Se increases expression)</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Thioredoxin reductase Type III</td>
<td>TR2, TrxR3</td>
<td>Testes-specific expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iodothyronine deiodinase Type I</td>
<td>ID1, DIO1</td>
<td>Important for systemic active thyroid hormone levels</td>
<td></td>
<td>Membrane-associated</td>
</tr>
<tr>
<td>Iodothyronine deiodinase Type II</td>
<td>ID2, DIO2</td>
<td>Important for local active thyroid hormone levels</td>
<td>Expression levels are stable under low Se conditions</td>
<td>Membrane-associated</td>
</tr>
<tr>
<td>Iodothyronine deiodinase Type III</td>
<td>ID3, DIO3</td>
<td>Inactivates thyroid hormone</td>
<td></td>
<td>Membrane-associated</td>
</tr>
<tr>
<td>Selenoprotein H</td>
<td>Sel H</td>
<td>Nuclear localization, involved in transcription. Essential for viability and antioxidant defence in Drosophila</td>
<td>Highly dependent on adequate dietary Se Levels</td>
<td>Nuclear</td>
</tr>
<tr>
<td>----------------</td>
<td>-------</td>
<td>----------------------------------------------------------------------------------------------------------</td>
<td>---------------------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>Selenoprotein I</td>
<td>Sel I, HEPT1</td>
<td>Possibly involved in phospholipid biosynthesis</td>
<td>Transmembrane</td>
<td></td>
</tr>
<tr>
<td>Selenoprotein K</td>
<td>Sel K</td>
<td>Transmembrane protein localized to endoplasmic reticulum</td>
<td>ER, plasma membrane</td>
<td></td>
</tr>
<tr>
<td>Selenoprotein M, Selenoprotein 15</td>
<td>Sel M, Sep15</td>
<td>Thiol-disulfide oxidoreductases localized to endoplasmic reticulum</td>
<td>ER</td>
<td></td>
</tr>
<tr>
<td>Selenoprotein N</td>
<td>Sel N, SEPNI, SepN</td>
<td>Potential role in early muscle formation; involved in RyR-related calcium mobilization from ER; mutations lead to multiminicore disease and other myopathies</td>
<td>ER</td>
<td></td>
</tr>
<tr>
<td>Selenoprotein O</td>
<td>Sel O</td>
<td>Contains a Cys-X-X-Sec motif suggestive of redox function, but importance remains unknown</td>
<td>Secreted, cytoplasmic</td>
<td></td>
</tr>
<tr>
<td>Selenoprotein P</td>
<td>Sel P</td>
<td>Selenium transport to brain and testes; Sel P knockout leads to neurological problems and male sterility. Sel P also functions as intracellular antioxidant in phagocytes</td>
<td>Serves as a biomarker for Se status, and is moderately sensitive to Se status</td>
<td></td>
</tr>
<tr>
<td>Selenoprotein R</td>
<td>Sel R, MrBI</td>
<td>Functions as a methionine sulfoxide reductase and Sel R knockouts show mild damage to oxidative insult</td>
<td>Cytoplasmic</td>
<td></td>
</tr>
<tr>
<td>Selenoprotein S</td>
<td>Sel S, SEPS1, SELENOS, VIMP</td>
<td>Transmembrane protein found in plasma membrane and endoplasmic reticulum. May be involved in ER stress</td>
<td>ER</td>
<td></td>
</tr>
<tr>
<td>Selenoprotein T</td>
<td>Sel T</td>
<td>Endoplasmic reticulum protein involved in calcium mobilization</td>
<td>ER</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.1 continued.

<table>
<thead>
<tr>
<th>Selenoprotein V</th>
<th>Sel V</th>
<th>Testes-specific expression</th>
<th>Highly dependent on adequate dietary Se levels as well as levels of Sel P</th>
<th>Cytoplasmic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenoprotein W</td>
<td>Sel W, SEPW1</td>
<td>Putative antioxidant role, perhaps important in muscle growth</td>
<td>Highly dependent on adequate dietary Se levels as well as levels of Sel P</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Selenophosphate synthetase</td>
<td>SPS2</td>
<td>Involved in synthesis of all selenoproteins, including itself</td>
<td>Highly dependent on adequate dietary Se levels as well as levels of Sel P</td>
<td>Cytoplasmic</td>
</tr>
</tbody>
</table>
2.3.6 Selenoproteins with poorly understood functions

The remaining known selenoproteins are generally less well described and while some basic functions may be known, further research is needed to determine definitive biological functions.

A protein of much interest, the 57 kDa selenoprotein known as Selenoprotein P (SelP), accounts for almost 50-60% of selenium in plasma (Himeno et al. 1996; Mostert 2000). It is a secreted protein predominantly expressed in the liver and is the only known selenoprotein with more than a single Sec residue per peptide chain (Himeno et al. 1996). The incorporation of between 7 to 10 Sec residues in the sequence of the protein (Read et al. 1990; Himeno et al. 1996) led to the hypothesis that the main function of SelP was that of a selenium storage and supply protein; a number of recent studies have supported this (Hill et al. 2003; Schomburg et al. 2003; Schweizer et al. 2005). Evidence exists to demonstrate the ability of SelP to restore selenoenzyme activity in selenium depleted cells (Saito and Takahashi 2002) and it appears to be an important, but not exclusive, supplier of selenium to the brain (Burk et al. 2003; Hill et al. 2004). SelP may also possess antioxidant abilities (Read et al. 1990) as suggested by its reduction of lipid hydroperoxides (Saito et al. 1999) and peroxynitrite (Sies et al. 1997) although an efficient in vivo reductant is yet to be identified.

Due to recent advances in our understanding of the role of SelP, its expression and its regulation, it has emerged as a useful index for the assessment of selenium status. A greater selenium intake requirement for maximal expression compared to GPx has led to the suggestion that it may be a better biomarker for selenium status than GPx (Xia
et al. 2005) and its use as such has been reported in some recent studies (Koyama et al. 2009; Rasmussen et al. 2009).

Selenoprotein W (SelW) is a small intracellular protein of 9.5 kDa; so named because it is one of the proteins absent in lambs with the selenium deficiency condition white muscle disease (Whanger 2002). The Sec residue is incorporated in a likely catalytic centre suggesting a possible redox role (Vendeland et al. 1993). The tissue distribution of SelW is broad with highest levels found in skeletal and cardiac muscle and the kidneys (Yeh et al. 1997; Gu et al. 2000; Whanger 2002). Due to this, SelW likely functions in protecting muscle cells from oxidative stress, however no definitive role has been determined for this protein.

The 15 kDa selenoprotein (Sep15) is another small selenoprotein (Gladyshev et al. 1998) predominantly expressed in the prostate, liver, brain and kidney (Kumaraswamy et al. 2000). While its function remains unknown, the isolation of Sep15 in association with UDP-glucose: glycoprotein glucosyltransferase reveals a possible protein-folding role (Korotkov et al. 2001; Ferguson et al. 2006). Despite uncertainty over its function, Sep15 has been associated with various tumour types, including prostate (Hu et al. 2001) and lung (Apostolou et al. 2004).

The remaining selenoproteins include selenoprotein I, K, M, N, O, R, S, T and V, and are briefly summarised in Table 2.1; a number of these were only identified as a result of in silico screening of likely nucleotide sequences for SECIS-like structures or in-frame TGA codons encoding Sec (Kryukov et al. 1999).
2.4 Selenium in the diet

2.4.1 Food selenium content dependency on soils

Selenium enters the food cycle from the soil via passive uptake into plants, which appear to have no requirement for the element. Thus the selenium content of soils in cropping and grazing areas is critical in its influence on selenium intakes in humans. While the majority of soils contain sufficient levels of selenium (0.1-2.0 μg Se/kg) (Combs 2001) there are a number of areas throughout the world including New Zealand, Finland, Zaire and parts of China in which the soil selenium content is very low and as a result populations are at risk of sub-optimum selenium intakes. Conversely there are areas of seleniferous soils in North America, Venezuela, Colombia and China where soil content may be as high as 90 mg Se/kg (Combs 2001). Many seleniferous soils originate from sedimentary shale type rocks, while soils with low selenium content are often associated with igneous parent material such as granite and basalt.

The availability of soil selenium to plants is affected by soil type, rainfall and farming practices. Poorly aerated and acidic soils result in insoluble forms of selenium, as do soils with high iron content. Soils may be naturally acidic or result from the application of agricultural fertilisers. Selenium is also removed from the soil due to leaching in areas of high rainfall or irrigation. Australia as a whole is generally not thought to suffer from severely deficient soils, but many coastal areas have such terrain and climate that leads to trace element removal and significant areas of selenium deficient soil have been identified (Figure 2.2).
The form of selenium with the greatest bioavailability for plants in the soil is selenate, however plants may also absorb selenite as well as Sem and Sec. Selenate is assimilated into plants via the sulphate pathway and incorporated non-specifically into proteins, primarily as Sem (Terry et al. 2000). Plants therefore, especially grain crops, are a major source of Sem in the human diet.

Figure 2.2: Distribution of seleniferous and selenium depleted soils in Australia (Judson and Reuter 1999).
Animal tissues contain both Sem and Sec, forming the major source of Sec in human diets. Organ meats such as kidney and liver generally contain high concentrations. Dietary supplements consist mostly of inorganic forms such as selenite and selenate but also Sem, as found in selenized yeast (Sunde 2001).

Dietary analysis reveals that a large proportion of the selenium consumed in the normal diet is generally provided by a relatively small group of foods (Duffield and Thomson 1999; Combs 2001). Major contributors to dietary selenium intake are animal products (meat, seafood, dairy products and eggs) and grain-based products (bakery goods, cereals). In comparison fruits and vegetables are relatively small contributors to dietary selenium (Duffield and Thomson 1999). A wide geographical variation of food sources is possible in modern diets due to importation of food products. The importation of significant selenium sources (such as wheat) can therefore result in an increase in selenium intakes in areas of low soil selenium status (De Jong et al. 2001), as a consequence the estimation of dietary intakes is complicated and without food analysis calculated intakes may lack accuracy.

The homeostasis of selenium in humans is not regulated by absorption, unlike other minerals such as iron. Indeed both inorganic and organic forms of selenium when ingested exhibit high rates of absorption (Thomson 2004a). The immediate fate of the absorbed selenium depends on its chemical form. Sem, as an analogue of Met, enters the general aminoacid protein pool for non-specific incorporation during protein synthesis. Despite being the aminoacid specifically incorporated into selenoproteins, Sec, and the inorganic forms selenite and selenate are not immediately available for incorporation into protein. All three are converted to the key intermediate hydrogen
selenide (H$_2$Se) which undergoes conversion to selenophosphate (Figure 2.3), the immediate donor of selenium to selenocysteyl-tRNA$^{[ser]_{sec}}$. This catabolism of Sec is catalysed by selenocysteine lyase (Mihara et al. 2000). Once in the form of H$_2$Se it may be utilised in selenoprotein synthesis. At normal intake levels, selenium is excreted by the kidneys primarily as selenosugar 1 (Se-methyl-N-acetylgalactosamine) (Gammelgaard and Bendahl 2004; Kuehnelt et al. 2005). In cases of very high selenium intake, trimethylselenonium (TMSe) ((CH$_3$)$_3$Se$^+$) may also be excreted in the urine (Kuehnelt et al. 2005), and dimethyl selenide ((CH$_3$)$_2$Se$^+$) may be excreted on the breath, causing the characteristic garlic odour that is experienced in selenosis (Allan et al. 1999). The methylation of selenium compounds is therefore a critical step in selenium metabolism, enabling the body to excrete the element easily but also synthesise metabolites with purported anticarcinogenic abilities.

The form of selenium consumed in the diet therefore has some influence on its fate within the body. Sem is a relatively poor provider of selenium for selenoprotein synthesis. With lower levels of Met intake, Sem is more likely to be incorporated non-specifically in place of Met and is therefore adept at increasing the level of selenium in the body but not necessarily at increasing the functional activity of selenoproteins. Sec, selenite and selenate however, contribute better to the intermediate pool that contributes to the synthesis of selenoproteins. Sem can still enter this pool but it must first undergo catabolism through Sec to H$_2$Se (Combs 2001).
Figure 2.3: Metabolism of selenium at adequate and excessive intake levels (Gammelgaard and Bendahl 2004).
2.4.2 Minimum requirements

There is growing interest in selenium nutriture worldwide, with much work focusing on resolving the 'adequate' versus 'optimal' intake debate. Currently, recommended dietary intake values throughout the world are proposed on the basis of avoiding selenium deficiency diseases and/or to meet the requirement, or a portion of the requirement, for the selenoenzyme GPx. The rationale for this latter approach is founded in the belief that maximizing selenoenzyme activity is sufficient to ensure selenium related health requirements are met (GPx is low in the selenoenzyme hierarchy and therefore most other selenoenzyme activity is maximized when GPx requirements are met). The potential flaw in this approach is that numerous studies have indicated supranutritional amounts of selenium - substantially more than is required for enzyme expression - may provide significant protection from a number of cancers (Clark et al. 1996), the mode of which may not be due to the action of selenoenzymes (Clark et al. 1996; Ip 1998).

The minimum dietary intake required to avoid Keshan disease was established by Chinese scientists to be around 19.4 and 14.1 μg/day for males and females respectively (Yang et al. 1988). This group also concluded that the physiological requirement for glutathione peroxidase was approximately 41 μg/day. More recently, a New Zealand study by Duffield and co-workers (1999) assessed the response of a number of selenium biomarkers to supplemental selenomethionine (10-40 μg/day), and initially determined that a total intake of 90 μg/day was required for the saturation of subjects' plasma and whole blood glutathione peroxidase activity. This study data was subsequently reanalysed (Institute of Medicine 2000) and, due to an inability to statistically differentiate the GPx activity increases between supplementation levels,
the physiological requirement was revised down by this group to a conservative value of 38 µg/day. The Chinese and New Zealand findings have both been referred to when recommended dietary intakes have been formulated in many countries around the world.

2.4.3 Recommended intakes

Using the Chinese value as a base, the U.S. National Academy of Sciences’ Food and Nutrition Board (Neve 2000) calculated their original recommended daily allowance (RDA) of 55 and 70 µg/day for women and men respectively. Since then, and considering the New Zealand data, the same authority revised the RDA down to 55 µg/day for both men and women (Food and Nutrition Board 2000b). Other countries have adopted recommended daily intakes that vary above and below these values. The German, Austrian and Swiss recommendation is a range of 30-70 µg/day for persons >10 years of age, while British values are 75 and 60 µg/day for adult men and women respectively (Thomson 2004a). The German/Austrian/Switzerland lower limit of 30 µg/day may suffice for children, but for adults may prove insufficient considering the Chinese and New Zealand experimental data.

In Australia, the National Health and Medical Research Council (NHMRC) in conjunction with the New Zealand Ministry of Health, recently released updated recommended dietary intake (RDI) and estimated average requirement (EAR) values (NHMRC 2006). The NHMRC working party determined the EAR, a value considered to meet the requirements of 50% of healthy individuals in a particular age group or gender, based on New Zealand (Duffield et al. 1999) and Chinese (Xia et al. 2005) supplementation data, to be 60 µg/day and 50 µg/day for adult males and
females respectively. The new RDI values were derived from EAR values using the formula \( \text{RDI} = \text{EAR} + 2\text{SDEAR} \) (assuming an EAR CV of 10%) resulting in recommended daily adult intakes of 70 \( \mu \text{g/day} \) and 60 \( \mu \text{g/day} \) for males and females. New recommendations were also produced for infants, adolescents and pregnant and lactating women (Table 2.2).
Table 2.2: Australian and New Zealand nutrient reference values for selenium (NHMRC 2006).

<table>
<thead>
<tr>
<th>Infants</th>
<th>AI</th>
<th>Upper level of intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-6 months</td>
<td>12</td>
<td>45</td>
</tr>
<tr>
<td>7-12 months</td>
<td>15</td>
<td>60</td>
</tr>
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<table>
<thead>
<tr>
<th>Children and Adolescents</th>
<th>EAR</th>
<th>RDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-3 yrs</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>4-8 yrs</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>Boys</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-13 yrs</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>14-18 yrs</td>
<td>60</td>
<td>70</td>
</tr>
<tr>
<td>Girls</td>
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<td></td>
</tr>
<tr>
<td>9-13 yrs</td>
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<td>50</td>
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<tr>
<td>14-18 yrs</td>
<td>50</td>
<td>60</td>
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<table>
<thead>
<tr>
<th>Adults</th>
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<tbody>
<tr>
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<tr>
<td>19-30 yrs</td>
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<td>70</td>
</tr>
<tr>
<td>31-50 yrs</td>
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<tr>
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<td>51-70 yrs</td>
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<td>60</td>
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<tr>
<td>&gt;70 yrs</td>
<td>50</td>
<td>60</td>
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<table>
<thead>
<tr>
<th>Pregnancy</th>
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<tbody>
<tr>
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<td>65</td>
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<tr>
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<td>65</td>
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<tr>
<td>31-50 yrs</td>
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<th>Lactating</th>
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<tbody>
<tr>
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<td>75</td>
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<td>75</td>
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<tr>
<td>31-50 yrs</td>
<td>65</td>
<td>75</td>
</tr>
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</table>

AI — Adequate intake; EAR — Estimated average intake; RDI — Recommended dietary intake; all intakes μg/d
Intakes in the upper part of these recommended ranges may indeed be adequate for normal selenoenzyme function, but recent research indicates that they could fall well short of providing the maximal health benefits associated with selenium. Results from a number of studies so far suggest that intakes of >150 µg/day are likely to be effective in the reduction of cancer incidence (Schrauzer 2000). Beyond the action of antioxidant selenoenzymes, the exact mechanism for this protective effect remains unclear, but it appears methylselenol is the key metabolite. Numerous studies have shown that methylselenol, which can be derived most effectively from monomethylated forms of selenium such as Se-methylselenocysteine (Se-MCys), is capable of modulating a number of molecular mechanisms involved in controlling cellular division and cell death (Redman et al. 1997; Ip et al. 2000; Zhong and Oberley 2001; Jiang et al. 2002), even at low concentrations.

While Se-MCys is the most effective precursor for methylselenol, it is, unfortunately, not consumed in significant amounts in normal diets. The major dietary forms of selenium are Sem and Sec, and while quite effective for induction of selenoenzyme activity (Finley and Davis 2001), they are poor precursors of methylselenol. This may explain why a much higher dietary intake is required to provide the chemopreventative effects against cancer than is needed to maximize selenoenzyme expression.
2.4.4 Toxicity and safe upper limits of intakes

The symptoms of toxicity include hair and nail loss and in more severe cases skin and nervous system lesions. The symptoms of selenosis are very rare in humans and ironically, given the country's association with selenium deficiency, the most widespread example occurred in China, specifically Enshi County in Hubei Province (1983). The severity of selenium overload resulted in a morbidity rate of almost 50% of the 248 inhabitants of the most heavily affected villages. The average estimated daily intake in these cases was 4.99 mg/day. Nearby villages without symptoms of selenosis had estimated average intakes of 750 μg/day. The recent study by Reid et al (2004) assessed the response of 24 men with prostate cancer to supra-nutritional doses of either 1600 or 3200 μg/day for between 6 months and 2 years with up to four years follow up. Despite the limitations of this study, the lack of reports of widespread overt symptoms of chronic toxicity at such high levels suggests perhaps that the safe maximum intake is likely to be significantly higher than early estimates.

There is a lack of official agreement on the safe upper level of intake. The European Commission – Scientific Committee on Food (2000) determined a rather conservative tolerable upper intake level of 300 μg/day. In contrast, the NHMRC (Table 2.2) recommends a safe upper limit for adults of 400 μg/day which considering previous findings (Yang et al. 1983; Reid et al. 2004) may also be deemed conservative.

Despite data indicating possible benefits of dietary selenium intakes in the range of 150-300 μg/day for some people, national dietary intake recommendations are likely to remain at current levels for the time being. This is primarily because of the
potential for toxicity due to the relatively narrow therapeutic range for selenium. Also of concern are recent findings from some randomised controlled trials that have suggested that higher selenium status may actually be associated with increased risk of diabetes and hypercholesterolaemia (Bleys et al. 2007; Stranges et al. 2007; Lippman et al. 2009; Stranges et al. 2009). Such findings indicate intakes at the higher end of the normal range may actually increase risk of some chronic diseases; however causality and/or potential mechanisms are still to be determined.
2.5 Associations with disease

In the 30 years subsequent to the discovery of the requirement for selenium in glutathione peroxidase, widespread research has indicated that selenium is likely to play a greater biological role than previously understood. The findings that have prompted the most interest are those that have indicated selenium has apparent anti-cancer properties, which appear to be corroborated by findings in large scale epidemiological and intervention studies. Selenium is postulated to protect against cardiovascular disease, although the extent is still uncertain, and also appears to play an important role in a number of other areas including the immune system and in some viral infections.

2.5.2 Cancer

Since the 1960s a number of geographical studies have indicated a likely association of low selenium status with increased rates of cancer. Jansson and co-workers (1975) assessed death rates from all cancers across all continental US counties between 1950 and 1969, and found the highest rates of colon and rectal cancer occurred in the areas of lowest soil and plant selenium concentrations. Soon after, Schrauzer and colleagues (1977) also found inverse correlations between estimated dietary selenium intakes and a number of cancers, in particular colorectal, prostate and breast cancer. More recently, Clark et al (1991) reanalysed, more thoroughly, the data used by Jansson, and found significant inverse associations between low forage selenium levels and adjusted rates of cancer of the lung, rectum, bladder, oesophagus, breast and cervix. In China, a study encompassing 65 counties by Kneller and co-workers (1992) found geographical areas where populations had higher blood selenium levels had strongly significant decreases in mortality rates for stomach cancers.
While not conclusive, these findings have since found support from numerous human case control and intervention studies, and from many animal studies.

An early Chinese trial of 29,584 adult subjects assessing the effects of combinations of vitamins and minerals on cancer hinted further at this possible protective ability of selenium. The Nutrition Intervention Trial (Blot et al. 1993) was conducted in Linxian County, China between 1984 and 1991. In this region of China the mortality rates for oesophageal cancer are 100 fold that of the white North American population. The nutrient treatment groups consisted of (A) retinol and zinc; (B) riboflavin and niacin; (C) vitamin C and molybdenum; and (D) beta-carotene, vitamin E and selenium (50 μg). A total of 2127 deaths were recorded during the trial; 1298 cases of cancer were detected and it ranked as the major cause of death, responsible for 32% of deaths. It was found that treatment A, B and C had no significant effects on total mortality rates. Those receiving treatment D had 9% lower total mortality (RR, 0.91; 95%CI, 0.84-0.99; P = 0.03) with 13% lower cancer incidence (RR, 0.87; 95%CI, 0.75-1.00); the greatest effect was seen with stomach cancer (RR, 0.79; 95%CI, 0.64-0.99). Thus a protective effect was attributed to the combination of beta-carotene, vitamin E and selenium.

Wei and co-workers (2004) later evaluated a sub-sample of this cohort, using baseline blood selenium levels, and found significant inverse relationships between serum selenium and mortality from oesophageal cancer (RR, 0.83; 95%CI, 0.71-0.98; P = 0.03) and gastric cardia cancer (RR, 0.75; 95%CI, 0.59-0.95; P = 0.02). The mean serum selenium for this cohort was 0.93 μmol/L and the risk ratios estimated the change in risk due to a 25% increase in serum selenium across the population distribution.
The study that elicited the greatest interest in the chemopreventative potential of selenium was an American trial originally designed to assess the effects of supra-nutritional selenium on the occurrence of non-melanoma skin cancers. The National Prevention of Cancer (NPC) trial by Clark et al (1996), which ran from 1983 to 1991 was a double blind, randomised placebo controlled trial involving 1312 older subjects (mean age 63 years) with histories of non-melanoma skin cancers. Intervention was oral supplementation of 200 μg/day selenium as selenized yeast or placebo. The primary end-points were incidences of basal or squamous cell carcinomas of the skin.

After 4.5 years treatment the group found no reduction in the incidence of skin cancers. There was however an effect on secondary end-points with 37% lower total cancer incidence (RR, 0.63; 95%CI, 0.47-0.85; P = 0.001), 50% lower total cancer mortality (RR, 0.50; 95%CI, 0.31-0.80; P = 0.002), 63% fewer prostate cancers (RR, 0.37; 95%CI, 0.18-0.71; P = 0.002), 58% fewer colorectal cancers (RR, 0.42; 95%CI, 0.18-0.95; P = 0.03) and 46% fewer lung cancers (RR, 0.54; 95%CI, 0.30-0.98; P = 0.04). Case numbers were too small to reach statistical significance for cancers at other sites.

After 10 years the effects were lessened but the trend still indicated a protective effect (Duffield-Lillico et al. 2002). The treatment group exhibited a 25% lower incidence in total cancers (HR, 0.75; 95%CI, 0.58-0.97; P = 0.03), prostate cancer incidence was significantly reduced by 52% (HR, 0.48; 95%CI, 0.28-0.80; P = 0.005) while reductions in lung and colorectal cancers were non-significant. The protective effect was also only evident in males (HR, 0.67; 95%CI, 0.50-0.89; P = 0.005), however there was a large gender bias in the sample (931 males; 319 females). The effect was also most pronounced in former smokers.
Significantly, the protective effect against total cancer incidence was strongest in participants in the lowest tertile (<1.33 \mu mol/L) of baseline plasma selenium (HR, 0.51; 95%CI, 0.32-0.81; P = 0.005), while there was no significant reduction in those with baseline plasma selenium above the median of 1.43 \mu mol/L. This finding is particularly interesting considering much of the Australian population (and many other countries) is likely to have selenium levels near to or below 1.33 \mu mol/L, as indicated by the very few Australian studies to date.

More recently the Selenium and Vitamin E Cancer Prevention Trial (SELECT) (Lippman et al. 2009) was conducted; a phase III randomised double-blind placebo controlled trial of supplementation with selenium (200 \mu g/day) or vitamin E (400 IU/day), or a combination of the two. The US trial, involving 35,533 male volunteers (minimum age of 50 yrs) began in 2001 and chose to study the potential for protection against prostate cancer by these two agents primarily because of the positive findings from the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC Study) and the aforementioned NPC trial. It was planned to run SELECT for a maximum of 12 years but it was recently halted after 5.5 years follow up. It found no significant differences in hazard ratios for any of the trials primary or secondary cancer endpoints. Of concern were non-significant associations with increased incidences of diabetes in subjects receiving selenium. Unfortunately SELECT did not include significant numbers of men of low or marginal selenium status, where selenium supplementation appears likely to have its greatest effect. Indeed, median baseline selenium levels (1.71 \mu mol/L) indicate most of the trial participants were likely to be selenium replete and a beneficial effect from further increasing selenium levels is probably unlikely.
A second large trial has been proposed in the UK and Europe (Rayman 2000). The Prevention of Cancer by Intervention with Selenium (PRECISE) trial is an extended repeat of the NPC trial, testing the efficacy of selenium supplementation in marginal selenium status populations, such as those in Europe. The trial aims to accrue 42,000 participants, via the internet, who will receive 100 μg, 200 μg or 300 μg/day of selenium yeast supplement or placebo for a minimum of 5 years. At this time two small pilot studies have been completed in the UK (Rayman et al. 2008) and Denmark (Ravn-Haren et al. 2008).

In addition to intervention trials there have been numerous case control studies that have assessed selenium levels, either in toenails or plasma, in relation to the incidence of various forms of cancer. Notably there is further evidence of a relationship between selenium and prostate cancer. Large studies in the United States (Yoshizawa et al. 1998; Helzlsouer et al. 2000; Nomura et al. 2000; Brooks et al. 2001; Li et al. 2004) and the Netherlands (Van den Brandt et al. 2003) all determined that subjects with lower levels of selenium had a significantly increased risk of prostate cancer. Brooks and co-workers (2001) findings regarding a potential threshold effect agreed with a later animal based study by Waters and colleagues (2005), which suggested the existence of a baseline threshold, above which further selenium supplementation provided no added protection.

Associations between low selenium status and gastric cancer (Knekt et al. 1990; Mark et al. 2000), oesophageal cancer (Mark et al. 2000; Rudolph et al. 2003) and bladder cancer (Zeegers et al. 2002; Kellen et al. 2006) have also been observed. Conversely, no association was evident in similar studies between selenium and breast (Mannisto

The mechanisms of the anticarcinogenic effects of selenium have been the focus of much interest. Monomethylated forms of selenium, such as methylselenic acid, that lead directly to the generation of the key metabolite methylselenol, appear to have numerous actions even at very low concentrations in both in vitro and in vivo studies (Ip et al. 2000). Findings from this work suggest likely actions to be the induction of cell cycle arrest and apoptosis and the inhibition of tumour invasion (Redman et al. 1997; Zhong and Oberley 2001; Jiang et al. 2002).

In cell culture, Dong and co-workers (2002; 2003) used flow cytometry and cDNA microarray technology to investigate gene expression during selenium induced growth inhibition in human breast and prostate cancer cell lines. They reported cell cycle arrest with apparent inhibition of the treated cells' progression to S phase, as well as the induction of apoptosis. From the cDNA microarray analysis, methylselenic acid was found to down-regulate cell cycle factors including cyclins and cdk proteins, inhibitors of apoptosis (survivin) and invasion activators (integrins, endonexin). Up-regulation was demonstrated for cell cycle regulators (RAD9, CHK2, GADD153), apoptosis promoters (caspase 9, c-jun, cyclin D1, cdk5) and invasion suppressors (cadherins). Several other in vitro studies have provided supporting evidence of the ability of various selenium metabolites to induce cell cycle arrest and apoptosis, specifically through their effects on the activity of cdk4 (Sinha and Medina 1997), caspases (Wang et al. 2002; Zu and Ip 2003; Ghosh 2004) and various protein kinases (Jiang et al. 2002; Gopee et al. 2004). Additionally, recent studies by Jiang and colleagues (1999; 2000) using human umbilical vein endothelial cells and rat
mammary carcinomas have demonstrated that methylselenol precursors inhibited the expression of both vascular endothelial growth factor (VEGF) and matrix metalloproteinase 2 (MMP-2), two proteins with critical roles in angiogenesis.

Selenium has also been shown by several groups (Makropoulos et al. 1996; Kim and Stadtman 1997; Otsuka et al. 1999; Gopee et al. 2004) to inhibit in vitro activity of the powerful transcription factor, nuclear factor-κB (NF-κB), thus identifying another possible mechanism by which selenium exerts an anti-carcinogenic effect. The findings to date suggest the protective effects of selenium occur at levels that are possible to achieve in vivo and therefore may represent a realistic picture of the mechanisms of the epidemiologically observed benefits of higher selenium status in humans. Coincidently, these effects on cell growth and proliferation may provide a possible explanation for the symptoms of selenosis in mammals such as hair and nail loss, neurological impairment and even death.

The findings of cancer related research so far suggest that increasing dietary selenium intakes may not decrease cancer incidence in those populations where plasma selenium levels are reasonably high, such as in South America and central North America. However, in Europe, as well as Australia, New Zealand and numerous other countries, where perhaps 50% or more of the population are considered by Combs (2001) to have low or marginal selenium status, there is reason to be optimistic about the positive effects increasing selenium intakes could provide. There is also some evidence that the benefit of increasing selenium status may be greater for men than women.
Compared to cancer, the case for a protective role for selenium in cardiovascular
disease (CVD) is more tenuous. Despite the identification of potential mechanisms for protection, studies in human populations have provided conflicting evidence.

The proposed mechanism of the protective role of selenium in CVD is based largely on the ability of selenoproteins, particularly glutathione peroxidase, to prevent oxidative damage of lipids and membranes. The oxidation of LDL is believed to be a key development early in the process of atherogenesis (Weiland et al. 1993), going on to accumulate in arterial walls where it is taken up by foam cells. The phospholipid hydroperoxide glutathione peroxidase, GPx-4, is known to function primarily in the reduction of fatty acid hydroperoxides, cholesterol hydroperoxides and other lipoprotein hydroperoxides. As discussed earlier, recent work has shown over expression of GPx-4 can delay the development of atherosclerosis in mouse models by protecting against this lipid peroxidation and by decreasing the sensitivity of vascular cells to oxidised lipids (Guo et al. 2008).

In addition to a basic protective function GPx-4 may also act to control the inflammatory reaction that is involved in the formation of atherosclerotic plaques. Through its effect on eicosanoid metabolism, GPx-4 is able to modulate the activity of cyclooxygenase and lipoxygenase (Schnurr et al. 1996; Sakamoto et al. 2000). Hence in low selenium states a reduced GPx-4 activity may lead to an imbalance in the synthesis of prostaglandins (vasodilators and inhibitors of platelet aggregation), thromboxanes (vasoconstrictors and promoters of platelet aggregation) and leukotrienes (influence vascular permeability and cell migration). In such a scenario
there would be an increased tendency for platelet aggregation and cell migration as seen in the formation of atherosclerotic plaque and associated thromboses.

Despite this theoretically important role for selenium in the prevention of CVD, evidence from epidemiological studies is not compelling. Analysis of the results (Rayman 2000) from such studies points to a relatively low threshold above which increasing selenium status has little or no effect and hence only those in populations of quite low selenium intake may benefit from selenium supplementation. Such a low threshold value seems consistent with the hypothesised selenoprotein involvement, as most have maximal activity at only moderate intakes.

The early Finnish study by Salonen et al (1982) determined a 2-3 fold increase in total cardiovascular morbidity and mortality when serum selenium levels were below 0.57 μmol/L. Similar results were found by Suadicani et al (1992) who reported that serum selenium below 1.01 μmol/L resulted in a significantly increased risk of ischaemic heart disease in Danish men. In contrast, subsequent Scandinavian studies (Marniemi et al. 1998; Kilander et al. 2001) in which serum selenium was similar to the risk threshold from Suadicani and colleagues' study, show no association with CVD. The Health Professionals Follow-up Study (Yoshizawa et al. 2003), using toenail selenium analysis, also found no association with total CVD though they could not exclude a possible association with myocardial infarction. While toenail selenium has often been used in long term epidemiological studies, the selenium in nails is in the form of Sem, and it is questionable whether this is necessarily an accurate indication of the selenium available for selenoprotein synthesis.

More recently, analysis of data from the NPC trial by Stranges and colleagues (2006) found no significant association between selenium and CVD endpoints after 7.6 years
follow up among the 1004 participants who had no baseline CVD. The study’s lower tertile baseline plasma selenium was \( \leq 1.33 \ \mu\text{mol/L} \) which, considering earlier research, may have been too high to detect a possible effect.

Meta-analysis of 25 observational studies between 1966 and 2005 identified a significant inverse association between selenium levels and coronary disease outcomes (Flores-Mateo et al. 2006). The authors also analysed 6 randomised trials and found subjects that were supplemented had a non-significant reduction in cardiovascular events.

In work regarding hypertension, Nawrot et al (2007) analysed data from 710 participants from the FLEMENGHO study in Europe and found significant associations between selenium and blood pressure. In men higher blood selenium was associated with lower systolic and diastolic blood pressure. Furthermore, a 1 SD increase in blood selenium resulted in a 37\% lower risk (\( P = 0.001 \)) of the development of hypertension.

Another recent meta-analysis, of 13 prospective cohort studies (Navas-Acien et al. 2008), also found a small inverse relationship between selenium concentrations and coronary heart disease. The authors, however, warned of confounding factors in the analysis, and pointed to recent studies which have suggested the potential for increased risk from supplementation of already replete populations, as a cause for concern.

Clearly more research is required, particularly in populations of low selenium intake, and perhaps to assess gender based differences also, before more definitive statements can be made regarding selenium and cardiovascular disease.
2.5.4 Immune function and viral infections

There is clear evidence that selenium has important associations with some aspects of immune function. It appears to be involved in the pathogenesis of one if not several viral diseases. The best described is the involvement of selenium in the formation of cardiovirulent strains of the Coxsackie B3 virus, now acknowledged as a co-contributor in the pathogenesis of Keshan disease. The work of Beck (1997) demonstrated, using mice, that a previously non-virulent strain of Coxsackie B3 virus will become virulent following mutation due to oxidative stress in selenium deficient hosts. The virulent strain may then go on to infect and cause disease in selenium-replete animals. Beck and colleagues believe RNA viruses in general are susceptible to oxidative mutations; presenting evidence of such an effect in an influenza virus (Beck et al. 2001) as well as suggesting an enterovirus implicated in an optic and peripheral neuropathy epidemic in Cuba (Beck et al. 2003) had also undergone such a process. With this in mind, the emergence of many virulent influenza viruses from China may be no coincidence, with its high population density and known regions of severe selenium deficiency. Indeed one author (Rayman 2000) has even postulated that selenium deficiency may have been a contributing factor in the first emergence of HIV in Africa.

Selenium is certainly associated with HIV and the progression to AIDS, but whether it is a causal relationship is still unclear. In both paediatric and adult HIV cases, low plasma selenium has been observed to be an independent predictor of mortality, associated with increased disease progression (Campa et al. 1999). In one study of HIV positive drug users, those who were selenium deficient had 10.8 times greater mortality rates (Baum et al. 1997). Selenium supplementation has also been found to
suppress HIV progression. Hurwitz et al (2007) supplemented with 200 μg/day of high selenium yeast in a double blind randomised placebo controlled trial of 174 participants over 9 months treatment. Supplementation resulted in increased blood selenium levels ($P < 0.001$), decreased viral load ($P < 0.02$) and increased CD4 T-cell count ($P < 0.04$).

In addition to direct pathogen associations, selenium is important in cellular immunity. Selenium deficiency results in functionally defective neutrophils, the reduction in cellular glutathione peroxidase activity thought to result in an inability to control the free radical production that is part of the neutrophils' bactericidal action (Arthur et al. 2003). Selenium supplementation has also been shown to enhance cytotoxicity of natural killer (NK) and cytotoxic T-lymphocyte (CTL) cells in animal models (Petrie et al. 1989), an effect also seen in elderly humans (Woods et al. 2000). Mouse models have also shown selenoproteins to be important for the production of mature T-cells and for the T-cell dependant antibody responses (Shrimali et al. 2008). A large body of work in veterinary science has shown selenium supplementation to enhance B cell lymphocyte function in animals, with titres of immunoglobulins increased following immunological challenges (Spallholz et al. 1973; Reffett et al. 1988; Droke and Loerch 1989; Knight and Tyznik 1990; Stabel et al. 1991). As well as aiding the fight against infectious disease, such an up-regulating effect on the immune system would likely assist in the response to the development of malignant cells.
2.5.5 Selenoprotein polymorphisms

With the developing understanding of the importance of selenoproteins in many processes required for normal health, there has been a significant effort to determine the effect of genetic variation in selenoproteins. A number of single nucleotide polymorphisms (SNPs) have been identified in various selenoprotein genes and have been shown to affect protein expression and/or cancer risk.

Polymorphisms in GPx genes have been the focus of much interest, in particular the SNP affecting codon 198 of GPx-1. Working with a cohort from the ATBC Study, Ratnasinghe and colleagues (2000) found a significant association between GPx-1 genotype and lung cancer. Participants with the heterozygous genotype Pro/Leu had an 80% greater risk of lung cancer, and homozygous Leu/Leu 130% greater risk, compared to those with the homozygous wild type Pro/Pro. The authors also found significant associations between genotype and lung cancer histological subtypes. These associations were observed in a Caucasian population, and the group have previously found ethnic Chinese do not possess this polymorphism and therefore are not susceptible to the increased risk conferred by it.

The GPx-1 Pro198Leu polymorphism was also found to be associated with bladder cancer in a Japanese population (Ichimura et al. 2004); with an adjusted 263% increase in risk compared to the wild type, and those with the Pro198Leu genotype were at greater risk of advanced disease. Furthermore, there was an interaction with the manganese superoxide dismutase (MnSOD) MnSOD Ala-9Val polymorphism; risk of bladder cancer increased to 631% when patients had at least one Ala allele in conjunction with the GPx-1 Pro198Leu genotype. Loss of Pro198 heterozygosity at the GPx-1 locus is thought to be a common occurrence in other malignant tumours.
including those of the head and neck (Hu et al. 2004), colon (Hu et al. 2005), lung (Hardie et al. 2000) and breast (Hu and Diamond 2003; Ravn-Haren et al. 2006), however there have been conflicting results in regard to breast cancer associations (Cox et al. 2004; Knight et al. 2004; Ahn et al. 2005). Other studies have observed no significant association between polymorphisms in the GPx-1 gene and cancers such as early onset prostate cancer (Kote-Jarai et al. 2002) and basal cell carcinoma (Vogel et al. 2004).

Research with Type 2 diabetics has also found the GPx-1 Pro198Leu genotype to be associated with increased risk of coronary heart and peripheral vascular disease; and in combination with another GPx-1 polymorphism (Ala6), the 198Leu gene product had 40% lower enzyme activity than wild type in in vitro functional analyses (Hamanishi et al. 2004). Hu and Diamond (2003) also noted that the 198Leu variant was less responsive to selenium supplementation. Ravn-Haren and co-workers (2006) have recently observed in the Danish 79,729 subject Diet, Cancer and Health study, that each copy of the variant Leu allele resulted in approximately 5% lower GPx-1 activity.

Sep15, another selenoprotein which appears to have functional variants, is expressed primarily in prostate, liver, kidney, testis and brain tissue. To date, some of the strongest epidemiological evidence for the involvement of selenium with cancer has come from prostate cancer. The Sep15 gene is located at p31 on chromosome 1, an area which is commonly deleted or mutated in cancers. Kumaraswamy and co-workers (2000) found significantly reduced amounts of Sep15 in malignant tumours, that polymorphisms influence the expression of the protein and that they respond differently to selenium supplementation. Another group observed a significant
difference in allele frequency by ethnicity, and found a significant reduction in heterozygosity in tumours of African Americans (Hu et al. 2001).

More recently, other selenoprotein polymorphisms have been observed to modify the response of selenoproteins to selenium supplementation, an important finding given the numerous studies suggesting a benefit from supplementation, and also disease risk, particularly in conjunction with other factors. This includes recent reports of a GPx-4 SNP (Meplan et al. 2008) and two common SelP SNPs (Meplan et al. 2007) which appear to affect not only selenoprotein activity but their responses to selenium supplementation. In smokers, the homozygous mutant genotype of the Sep15 1125G/A polymorphism is reported to benefit from higher selenium status, while in the other genotypes higher selenium status was associated with increased lung cancer risk (Jablonska et al. 2008). Another SelP SNP (Ala234), in association with a superoxide dismutase (SOD) SNP (Ala16Val), increases the overall risk of prostate cancer and that of aggressive prostate cancer in subjects with marginal selenium status, particularly in current or former smokers (Cooper et al. 2008).

Clearly the identification of the ~25 selenoprotein genes has been an important step and is one that is likely to shape the future research regarding selenium and quite possibly the view on the optimal selenium intake for different groups of people. With emerging data indicating that higher selenium status may be associated with adverse health effects (Stranges et al. 2007; Lippman et al. 2009), a greater understanding of the effects of genetic variation on selenoprotein function and its relation to health and disease could lead to the point where optimum selenium intakes are determined on an individual basis using genetic and other factors.
The assessment of selenium status is commonly a multifaceted one. The estimation of dietary intake is useful but is generally used in conjunction with one or more biochemical measures. A functional measure is preferred to give greater context to the analyte measured, and this is possible for selenium, through the measurement of various selenoproteins.

### 2.6.1 Dietary assessment

There are a number of different methods that may be employed in order to estimate dietary nutrient intake including diet records, duplicate diets and food frequency questionnaires. All such methods have various shortcomings, including random and systematic error, which may arise from limited periods of data collection, daily variation, failure to report usual habits or a change in habits, inaccurate estimation of portion sizes or the use of food composition data which does not accurately reflect the actual composition of food eaten (Bingham 2002).

Diet records may utilise weighed or estimated food amounts and weighed diet records are considered, although imperfect, as the dietary assessment 'gold standard'. Recording periods are typically 3-7 days in duration, though longer periods may be used to increase representativeness but are associated with a decrease in reported intake due to respondent fatigue (Thompson and Subar 2008). Such records are usually comprised of a detailed description of the food type and amount. In the case of weighed records each portion is weighed on scales prior to the meal. For estimated portion sizes, pictures of varying portion sizes or measuring cups and spoons are commonly provided. The final calculation of the estimated nutrient intake usually
utilises standard food composition data. Weighed diet record methods impose a relatively high load upon the participants in terms of time taken to fulfil requirements, which may result in altered intake or reduced compliance. Estimated records reduce the participant burden but overall the process is still quite time-consuming and expensive. Such methods are widely used and generally provide a reasonable indication of dietary nutrient intakes at the time of assessment, however seasonal variations in eating habits will not be detected. A strength of the diet record is that meals are recorded as they are consumed, however it is thought in some cases this may alter the amounts normally eaten (Bathalon et al. 2000; Goris et al. 2000) and hence lead to the underreporting that has been observed in several studies (Bathalon et al. 2000; Goris et al. 2000; Hill and Davies 2001; Trabulsi and Schoeller 2001).

The 24 hour diet recall asks the subjects to remember all food and beverages consumed over the previous 24 hours. The diet information is generally collected by an interviewer, and the ability of the interviewer to extract all relevant information from the subject is important to ensure the accuracy of the data. Although this method requires direct contact and a well trained interviewer, its strength is that the data is collected soon after the period of interest and does not rely on the ability of the subject to interpret the question or record the response correctly (Thompson and Subar 2008). Despite this strength, the 24 hour recall cannot be used to estimate usual diet because of day to day variations. The 24 hour recall has also been observed to under report when compared with biomarker measures (Bathalon et al. 2000; Tran et al. 2000; Trabulsi and Schoeller 2001; Hebert et al. 2002; Subar et al. 2003).
A food frequency questionnaire in its most basic form is a list of foods in combination with a number of options that relate to the frequency of their consumption. The questionnaire provides consumption options between 'several times a day' to 'once a month'. Semi-quantitative food frequency questionnaires collect information on serving sizes, commonly by including photographs of portions of varying sizes. For foods in which portion size information is not collected, standard portion size data is utilised to estimate daily food intakes (Thompson and Subar 2008). The calculation of daily nutrient intakes also requires the use of standard food composition data. This method provides an assessment of eating habits over a long period, generally taking into account a large number of foods and may allow for some seasonal variation depending on the instruction given at the time of assessment. As a one off, self-administered questionnaire, participant burden is lower than other methods. Low cost and ease of the distribution and processing enables large numbers of participants to be easily assessed. The major drawbacks of the food frequency questionnaire are the reliance on estimated food portion sizes and food composition data of varying accuracy. Subjects may also find it difficult to estimate how frequently they eat particular foods, and mixed food items such as pizzas and tacos may not be represented well. The close-ended nature of most FFQ also means unusual or specialist foods (eg gluten free foods) can go unreported.

Duffield and Thomson (1999) conducted a comparison of methods of dietary assessment specifically for the estimation of selenium intake. Comparisons were made between a food frequency questionnaire (n = 110) and 3-day diet records and duplicate diets (n = 43). In addition to dietary intake, the selenium status of each participant was assessed using whole blood, plasma and urinary selenium analysis.
From dietary analysis, mean selenium intakes were similar for both duplicate diets (29 ± 13 μg/day) and diet records (28 ± 15 μg/day), but the mean estimated intake from the food frequency questionnaire was 75-80% greater (51 ± 26 μg/day). Interestingly, all three methods found significantly different intakes between sexes, a difference that was not reflected in blood selenium levels and was suggested to be due to body mass variation between sexes.

Significant correlations were found for selenium intake by duplicate diets (r 0.3, P < 0.05) and diet records (r 0.4, P < 0.05) with plasma selenium concentration, and selenium intake from the food frequency questionnaire with whole blood selenium (r 0.3, P < 0.05). There was a strong correlation between duplicate diets and diet records (r 0.7, P = 0.0001), however Bland and Altman difference plot analysis indicated a lack of agreement. The authors suggested the large difference in estimated intakes may be due to the food frequency questionnaire providing a measure of longer term intakes compared to the other methods.

The reliability of the FFQ as an assessment tool has however been questioned for some time (Byers 2001; Kristal et al. 2005). In general comparisons of FFQs with reference methods, poor overall agreements between the methods are reported (Riboli et al. 1997; Subar et al. 2001; Schatzkin et al. 2003; McNaughton et al. 2005), but high cost and subject burden of the diet records which are considered more reliable, ensure that the FFQ is still commonly employed in epidemiological studies. Thus, while possibly not suitable for a stand alone assessment of dietary selenium exposure, a well designed and administered food frequency questionnaire could still provide a convenient and cost effective method for ranking subject intakes but some may lack accuracy in providing absolute values.
Due to the error inherent in all dietary assessment methods there has been considerable effort toward the development of biomarkers for validating estimates of dietary intake (Schatzkin et al. 2003; Subar et al. 2003; Neuhouser et al. 2008). Such markers include doubly labelled water for estimating energy intake and expenditure, 24 hour urine nitrogen for nitrogen and hence protein intake, and 24 hour urine potassium (Bingham 2002).

Another major issue for all dietary analysis of selenium is the limited data on selenium in foods and the variations in food selenium content between different geographical areas.

2.6.2 Biochemical assessments

The primary measure of selenium status is the determination of selenium within tissues of the body. There are a number of commonly used specimens including hair, toenails, blood and urine; however, estimation of selenium in plasma or whole blood is the preferred measure for most studies. Plasma or serum selenium is widely used and reliably reflects short-term intake. Whole blood, due to the turnover time of the red cell population, is thought to provide a longer-term measure (Thomson 2004a) but unfortunately also brings with it analytical difficulties.

Measures of plasma or serum selenium have been used as the accepted biomarker of selenium status in many studies, from some of the earliest to the most recent (Salonen et al. 1982; Knekt et al. 1990; Thompson et al. 2002; Wallace et al. 2003; Bleys et al. 2008; Rasmussen et al. 2009), including the key studies associated with selenium and cancer risk, such as the NPC trial (Clark et al. 1996) and the recent SELECT trial (Lippman et al. 2009).

The measurement of toenail selenium has been used in numerous long-term studies (Yoshizawa et al. 1998; Mannisto et al. 2000; Zeegers et al. 2002; Van den Brandt et
al. 2003; Yoshizawa et al. 2003), and is considered a reliable indicator of selenium status over the 4-12 month period that is required for nail growth (Ovaskainen et al. 1993). Toenail samples have proven particularly useful in large studies due to the ease of collection and storage; however analysis of toenail specimens is more involved than methods for fluid samples as the solid nail sample requires digestion before analysis.

Hair, like toenails, should provide a convenient and reasonably reliable index of selenium status but in reality its usefulness is limited due to various factors that complicate the analysis and interpretation of hair selenium content. The selenium content of hair appears easily altered by selenium-containing shampoos and the selenium stripping action of some dyes (Borella et al. 1998), both of which have a wide usage, especially in developed countries. Additionally, concerns over the inadequacy of pre-digestion washing procedures, make inter-laboratory comparisons very problematic.

The measurement of urine selenium has been considered to be of little use in the assessment of nutritional status (Neve 2000). It lacks the necessary sensitivity for detecting nutritional deficiency (Sheehan and Halls 1999); and due to the complexities of the relationship between urine selenium excretion and selenium intake, interpretation is difficult. Urinary selenium excretion is however used as a method for screening for excessive occupational exposure.

There are a number of analytical methods in use for determining selenium in biological tissues and fluids. Techniques include molecular fluorescence spectrometry (MFS), hydride generation (HGAAS) and graphite furnace atomic absorption spectrometry (GFAAS) and, in more recent years, inductively coupled plasma-mass spectrometry (ICP-MS) (Sieniawska et al. 1999). All methods, provided calibration is
addressed correctly, perform well, with MFS, HGAAS and ICP-MS considered being perhaps slightly superior in terms of sensitivity. Currently though, due to the ease of sample preparation and throughput, GFAAS remains the method of choice in most laboratories, particularly for plasma and serum samples (Sheehan and Halls 1999).

With no commonly accepted reference range for blood selenium levels, a functional marker is often measured in conjunction with selenium itself. The discovery of numerous selenoproteins has in theory provided multiple options for such a functional index of selenium status. The classic functional marker of selenium status is glutathione peroxidase in plasma (GPx-3), red cells or platelets (GPx-1). It remains the preferred method due to ease of analysis, responsiveness to selenium intake and its positive correlation with blood selenium up to an activity saturation point (Neve 2000). Glutathione peroxidase may be measured using spectrophotometric (enzyme activity) or ELISA based assays (protein levels). The most widely used methods measure enzyme activity using variations of the classic method of Paglia and Valentine (1967), which is based on the ability of GPx to catalyse the glutathione dependent reduction of an organic hydrogen peroxide.

The correlation between glutathione peroxidase activity and selenium levels below a threshold value makes it a particularly useful marker in populations with marginal selenium intakes. The lower ranking of glutathione peroxidase in the selenoprotein hierarchy means it requires higher blood selenium levels to reach saturation of activity in comparison to other selenoproteins.

Other selenoproteins that have been considered as alternative or additional measures of selenium status are Selenoprotein P (SelP) and some of the selenium containing thyroid hormones. SelP is likely a storage protein for selenium and can be measured
in plasma using a number of methods (HPLC-ICPMS, radioimmunoassay, luminometric immunoassay). Previous studies have shown it to be significantly associated with other indices such as plasma selenium, GPx-1 and GPx-3 (Hill et al. 1996; Neve 2000). Our improving understanding of SelP has seen it begin to be used as a biomarker for selenium status (Koyama et al. 2009; Rasmussen et al. 2009); it is believed by some to be a more sensitive index than GPx (Xia et al. 2005).

The potential of monitoring thyroid hormone levels as a rather indirect assessment of selenium status has been suggested (Neve 2000). Duffield and co-workers (1999) investigated thyroid hormones as an indicator of selenium status as part of a supplementation trial using members of the selenium-depleted New Zealand population. Supplementation appeared to cause a decrease in T4 levels, probably due to increases in deiodinase activity. The group concluded that the iodothyronine deiodinases would be maximised at a selenium level of approximately 0.85 μmol/L. These proteins therefore appear to be unsuited for assessing selenium status in many populations.

At this point, cellular or extra cellular GPx activity remains the most common functional measure, due to the well established relationship with selenium and the ease of analysis; the measurement of SelP is however becoming more common due to improved methods and better understanding of the protein response to selenium intake.

**2.7 Dietary selenium intakes in Australia**

As the selenium content of food is dependent on the soil content where crops and livestock are raised, geological differences around the world result in corresponding variations in human dietary intakes. The increase in importation of foods in many
countries may positively influence dietary intakes in countries with low or marginal selenium status, but it is unlikely to be a method with which to rectify such a problem completely. Countries including New Zealand, Zaire and parts of China consume diets with much lower selenium content in comparison to seleniferous countries on the continents of South and North America. The global variations in dietary selenium intake and selenium status as compiled and reviewed by Combs (2001) indicates numerous European countries only consume approximately 50 μg/day of selenium in their normal diets. Combs goes on to estimate that in over 20 countries worldwide the prevalence of low selenium status is >50%. Conversely, many people in certain regions of the United States, Canada and especially Venezuela consume over 200 μg/day in normal diets.

In Australia there have been few surveys that have attempted to estimate daily selenium intake. Fardy, McOrist and Farrar (1989) analysed 50 representative foods using neutron activation analysis as part of the NHMRC Market Basket survey. They estimated a daily selenium intake of 87 and 57 μg/day for men and women respectively. Reilly (1992) used a similar approach by analysing local foods in Brisbane, Queensland, estimating the daily intake to be 89 and 59 μg/day for males and females respectively.

Market basket surveys have major limitations, however, including that they may not be an accurate representation of population consumption, nor take into account geographical variations in food content levels.

The 19th Australian Total Diet Survey (ANZFA 2001), formerly the Australian Market Basket Survey, reported selenium as a contaminant in/on food. Daily intake was expressed as a range in units of μg/kg body weight. Selenium content below 0.01
mg/kg of food was the lower limit for reporting, and intakes were based on food consumption data from the 1995 National Nutrition Survey (NNS). The estimated dietary exposure was 1.2-1.7 μg/kg body weight/day and 0.97-1.4 μg/kg body weight/day for adult (25-34 years) males and females respectively. The subsequent 20th Australian Total Diet Survey (FSANZ 2003) estimated the selenium dietary exposure as 1.17-1.41 μg/kg body weight/day and 0.96-1.18 μg/kg body weight/day for adult (25-34 years) males and females respectively.

The mean estimated selenium intakes for both surveys surpass the Australian RDI — expressed in μg/kg body weight/day as 0.85 μg/kg body weight/day and 0.89 μg/kg body weight/day for adult males and females respectively. Thus, although Australian data on selenium intake is sparse, and has methodological limitations it suggests a moderate standing on a world scale, with many Australians from the surveyed areas likely to meet the national EAR values, but likely to fall short of intakes that may be optimal for disease prevention.

There has been no previous detailed assessment of dietary intakes of selenium in Tasmania. For a number of years Tasmania has been considered at risk of low selenium status, due to deficiency in grazing animals. The Tasmanian climate and topography is such that leaching of trace elements is highly probable; the state also has a well documented history of recurring iodine deficiency (Guttikonda et al. 2002).
In accordance with the widely fluctuating selenium intakes reported from around the world, blood selenium levels between regions are also starkly different. Combs (2001) has, to date, compiled the most comprehensive list of blood selenium analyses performed around the world. Not surprisingly, countries with well-documented histories of animal or human deficiency diseases have recorded selenium levels below, and in some cases far below, the range of 1.14 - 1.27 μmol/L which has been suggested as the physiological requirement (Rayman 1997; Duffield et al. 1999). In New Zealand, following recognition of low selenium intakes, mean plasma selenium levels have risen to between 0.84 and 1.12 μmol/L (Thomson 2004b). In the Keshan disease areas of China, plasma selenium values as low as 0.20 μmol/L are observed (n = 44). Many other countries are seen to have marginal to adequate levels (0.89 μmol/L to 1.27 μmol/L) while studies in seleniferous areas such as the United States (n = 142) and Zaire (n = 75) have recorded mean levels as high as 2.51 μmol/L and 2.56 μmol/L respectively (Combs 2001).

In Australia, again there is very little data from representative adult populations. The most comprehensive data appears to originate in South Australia. Here, Lyons and co-workers (2004) have compared plasma and whole blood selenium levels from a sample of the population in Adelaide in 2002 to previous levels from 5 studies carried out between 1977 and 1988 (total n = 834). The 2002 whole blood mean ± sd of 1.58 ± 20 μmol/L represented an approximate 20% decline from the 1977 value of 1.96 ± 27 μmol/L. While there was a change in methodology between analyses performed pre-2002 and those in 2002 (fluorometric v. hydride ICPOES), reanalysis of a batch of
samples found they gave similar results. The authors suggest the decline may be due
to a decrease in wheat selenium content as a result of farming practices altering the
availability of selenium in the soil. The possible gender bias in the studies of 1977 and
1979 resulting in a higher ratio of men to women was unlikely to be a cause as men
had only marginally higher selenium levels than women. The mean plasma selenium
values of the four most recent studies (1987; 1.24 ± 0.19 μmol/L, 1987; 1.22 ± 0.18
μmol/L, 1988; 1.15 ± 0.15 μmol/L and 2002; 1.30 ± 0.14 μmol/L) compare
reasonably with Daniels and colleagues (2000) mean values of 1.11 ± 0.25 μmol/L for
19 South Australian adults. Included in this small study were 25 adult Tasmanian
blood donors whose mean plasma selenium was a significantly lower 0.98 ± 0.16
μmol/L (P < 0.05).

The study by Dhindsa and co-workers (1998) sampled a population of Sikh (India)
migrants in Sydney. Despite vegetarianism being common amongst this ethnic group
the results indicated that both sexes appear to have a marginal selenium status. The
overall population (n = 196) mean was 1.16 ± 0.19 μmol/L while the male (n = 115)
value of 1.19 ± 0.21 μmol/L was significantly higher (P < 0.01) than the 1.12 ± 0.16
μmol/L values of females (n = 81). Lux and Naidoo (1995) reported a very limited
study using a sample of twelve New South Wales adults to determine a mean plasma
selenium value of 1.28 ± 0.13 μmol/L.

The most recent Australian study reported the selenium status of two cohorts in
Queensland (Lymbury et al. 2008). No significant difference in mean plasma
selenium was found between cohorts of 140 healthy (1.27 ± 0.02 μmol/L) and 112
CVD patients (1.26 ± 0.02 μmol/L). The authors suggested a bell-curve effect
between age and selenium status with lowest selenium status found in the youngest
and oldest age groups and selenium levels peaking in middle age groups. They also reported that just over half (53%) of the healthy subjects had selenium levels higher than 1.27 μmol/L, a level associated with optimal GPx activity.

In the largest Tasmanian study (McGlashan et al. 1996), the serum of 390 newly-pregnant Tasmanian women was analysed in an attempt to detect a possible relationship between maternal selenium levels and sudden infant death syndrome (SIDS). Included as a normal component were 171 female blood donors who were found to have significantly lower serum selenium (1.02 ± 0.23 μmol/l) compared to those from other Australian States (1.10 ± 0.21 μmol/l; n = 174); however, the authors did not collect other demographic and dietary data.

Recently Jacobson and co-workers (2007) reported a mean plasma selenium level of 1.39 ± 0.24 μmol/L from a sample of 335 adults living in the Greater Hobart area, Tasmania; which is significantly higher than other reported plasma levels in Australia. However, the group used the more recently developed magnetic sector ICP-MS method rather than the commonly used GF-AAS method, and had concerns over its reliability; uncertainty over methodology differences makes it difficult to compare these results directly with other Australian data. The response rate of this study was 26% of the original 1500 contacted, with a large proportion of positive responses being older (46 - 65 yrs) and more highly educated. This groups findings indicated that both lower educational status and younger age were associated with lower plasma selenium levels, so it is likely a more representative population sample could reveal a lower mean selenium level.

While this study provided demographic and other health related data in reasonable detail, the method for dietary analysis was less thorough. A basic questionnaire was used to determine the frequency with which subjects consumed some major food
types, and an estimated daily selenium intake was not reported. Given the sample composition, limited dietary data and particularly the analytical method differences, it is difficult to interpret these results on a comparative population basis.

Australian data is limited but it appears mean plasma selenium levels for the population may range between 1.1 and 1.3 μmol/L; the upper level of this range being similar to the physiological requirement. There would therefore be a significant proportion of the population considered selenium deficient by the definition of not meeting selenoprotein physiological requirements, which concurs with the views of Combs (2001).

There is insufficient data to determine whether Tasmanians do have lower selenium status than their mainland counterparts. It does seem likely however, that a significant proportion of the Tasmanian population, like much of the rest of Australia, fail to consume levels of selenium associated with both maximizing selenoprotein activity and chemoprevention. Further study is therefore warranted to provide more comprehensive information on the selenium status in this population.
Table 2.3: Recent Australian data for selenium intakes and blood levels.

**Previous Australian Estimated Daily Intakes**

<table>
<thead>
<tr>
<th>Location</th>
<th>Male</th>
<th>Female</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brisbane</td>
<td>96 µg</td>
<td>63 µg</td>
<td>(Fardy et al. 1989)</td>
</tr>
<tr>
<td></td>
<td>89 µg (range 35-204)</td>
<td>59 µg (range 23–141)</td>
<td>(Reilly, 1992)</td>
</tr>
<tr>
<td>19th ATDS</td>
<td>1.2-1.7 µg/kg body-wt</td>
<td>0.97-1.4 µg/kg body-wt</td>
<td>(ANZFA, 2001)</td>
</tr>
<tr>
<td>20th ATDS</td>
<td>1.17-1.41 µg/kg body-wt</td>
<td>0.96-1.18 µg/kg body -wt</td>
<td>(FSANZ, 2003)</td>
</tr>
</tbody>
</table>

**Plasma Selenium Levels**

<table>
<thead>
<tr>
<th>Location</th>
<th>Level (µmol/L)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sydney - Sikh migrants (n = 196)</td>
<td>1.16 ± 0.19 µmol/L</td>
<td>(Dhindsa et al. 1998)</td>
</tr>
<tr>
<td>South Australia (n = 19)</td>
<td>1.11 ± 0.25 µmol/L</td>
<td>(Daniels et al. 2000)</td>
</tr>
<tr>
<td>South Australia (n = 288)</td>
<td>1.30 ± 0.14 µmol/L</td>
<td>(Lyons et al. 2004)</td>
</tr>
<tr>
<td>Queensland</td>
<td></td>
<td>(Lymbury et al. 2008)</td>
</tr>
<tr>
<td>Healthy (n = 140)</td>
<td>1.27 ± 0.02 µmol/L</td>
<td></td>
</tr>
<tr>
<td>CVD patients (n = 112)</td>
<td>1.26 ± 0.02 µmol/L</td>
<td></td>
</tr>
<tr>
<td>Tasmania - female blood donors (n = 171)</td>
<td>1.02 ± 0.23 µmol/L</td>
<td>(McGlashan et al. 1996)</td>
</tr>
<tr>
<td>Tasmania - adult blood donors (n = 25)</td>
<td>0.98 ± 0.16 µmol/L</td>
<td>(Daniels et al. 2000)</td>
</tr>
<tr>
<td>Tasmania (n = 335)</td>
<td>1.39 ± 0.24 µmol/L</td>
<td>(Jacobson et al. 2007)</td>
</tr>
</tbody>
</table>
2.9 Copper

2.9.1 A brief history

With an atomic weight of 63.55, copper belongs to group 11 of the periodic table; other members of this group include the precious metals silver and gold. Copper has been in use for approximately 5000 years, first as a material for artistic objects and then as a component of the alloy bronze; the use of which spelt the end of the Stone Age and the beginning of the Bronze Age in human history (Stern et al. 2007). Copper remains an important commodity today, especially for its conductive properties.

2.9.2 Essentiality of copper

That copper is an essential trace element in mammals was not revealed until 1927 where it was discovered that copper was required for erythropoiesis in rats (Hart et al. 1927). Copper is a transitional metal with three oxidation states; it is the ability to cycle between Cu$^{1+}$ and Cu$^{2+}$ that gives rise to its importance in biological roles. With this ability copper can act as an electron donor or acceptor in redox reactions which are at the heart of many enzymatic reactions (Uauy et al. 1998).

Copper is an essential component, either as a cofactor or as an allosteric component, of cuproenzymes. At least 11 cuproenzymes are known, with functions ranging from antioxidant protection and copper transport to iron metabolism, electron transport and pigment synthesis (Stern et al. 2007). The three major cuproenzymes are active in redox reactions: caeruloplasmin (also known as ferro-oxidase I), Cu-Zn-superoxide dismutase (Cu-Zn SOD) and cytochrome c oxidase.

The 132 kDa caeruloplasmin is the major copper containing protein, with six copper ions in each molecule, accounting for >95% of copper in plasma. The six copper ions
are located at two separate molecular sites; three at a site involved in electron transfer; the remaining three at a location involved in oxygen activation (Hellman and Gitlin 2002). These active sites give caeruloplasmin its oxidase function and a radical scavenging ability.

Caeruloplasmin is an extracellular protein which is involved in the acute phase reaction in inflammation and may protect against oxidative damage by scavenging free radicals (Stern et al. 2007). Its major roles appear to be copper transport from the liver to the cells, and as a ferroxidase, active in iron metabolism by oxidising Fe (II) from ferritin to Fe (III) which then binds to transferrin to be distributed around the body (Hellman and Gitlin 2002). Deficiency in caeruloplasmin activity therefore results in iron metabolism abnormalities, seen commonly as a normochromic, normocytic anaemia with lowered serum iron levels (Hellman and Gitlin 2002). In the case of hereditary forms of acaeruloplasminaemia, the disturbed iron metabolism leads to parenchymal iron deposition in most tissues with clinical symptoms of diabetes, myeloneuropathy and retinal degradation (McNeill et al. 2008). Cu-Zn SOD is a cytoplasmic 32 kDa homodimer; each subunit of the enzyme normally containing one copper and one zinc ion at active sites where the substrate, the superoxide ion, binds to copper, and catalysis of the dismutation of superoxide (O₂⁻) to hydrogen peroxide (H₂O₂) occurs (Zelko et al. 2002). Other major antioxidant enzymes such as the selenoprotein GPx or catalase may then complete the removal of the oxidant threat by catalysing the reduction of the resultant H₂O₂ to water.

Cu-Zn SOD knockout studies in mice have described phenotypically normal animals, but mutations in the gene are associated with disease in humans. In cases of amyotrophic lateral sclerosis (ALS) at least 100 mutations have been identified in the
SOD1 (Cu-Zn SOD) gene (Sandelin et al. 2007). In many cases mutations in this gene result in reductions in SOD activity of 20% to 50% (Forsberg et al. 2001).

Cytochrome c oxidase (COX) is a critical protein located on the inner mitochondrial membrane which, as the terminal enzyme of the respiratory chain, acts to catalyse the reduction of elemental oxygen to water and generate a transmembrane proton gradient to drive ATP generation. An integral membrane protein, COX consists of 13 protein subunits, and contains 3 copper atoms (Michel et al. 1998). Defects or deficiencies in COX may result in muscle weakness, hypothermia and neurological abnormalities (Stern et al. 2007). In Menkes disease, the classic hereditary copper metabolism disease, a major contributor to the severe neurological damage that occurs due to copper deficiency is thought be decreased COX activity leading to impaired myelination of neurons (Kaler 1998).
Table 2.4: Function of known cuproenzymes (modified from Stern et al (2007)).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Function</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (Cu-Zn SOD)</td>
<td>Destruction of superoxide radicals</td>
<td>Found in cytoplasm; during dismutation of superoxide radical (O2), Cu at active site of enzyme is reduced to yield H2O2.</td>
</tr>
<tr>
<td>Caeruloplasmin (CP)</td>
<td>Copper transport, oxidation</td>
<td>Contains 65–90% of vertebrate serum copper; human plasma level □ 200–500 mg/L.</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>Electron transport, terminal oxidase</td>
<td>Terminal enzyme in the respiratory chain, located in the inner membrane of mitochondria and bacteria. Catalyses the reduction of O2 to water; pumps one proton across membrane for each proton consumed in the reaction to generate electro-chemical gradients that can be used for other cellular purposes (e.g., ATP synthesis).</td>
</tr>
<tr>
<td>Dopamine β-hydroxylase</td>
<td>Norepinephrine synthesis</td>
<td>Catalyses conversion of dopamine to norepinephrine.</td>
</tr>
<tr>
<td>Lysyl oxidase (LOX)</td>
<td>Cross-linking of collagen and elastin</td>
<td>Secreted enzyme catalyses the deamination of peptidyl lysine residues forming inter- or intra-chain covalent cross-links in elastin and collagens</td>
</tr>
<tr>
<td>Monoamine oxidase</td>
<td>Neurotransmitter synthesis</td>
<td>Found predominantly on the outer mitochondrial membrane and throughout CNS. Two isozymes both of which oxidize the neurotransmitters dopamine, epinephrine, tyramine, and tryptamine</td>
</tr>
<tr>
<td>Peptidylglucose a amidating monooxygenase (PAM)</td>
<td>Neuropeptide processing</td>
<td>Multifunctional protein involved in the maturation of bioactive hormones (e.g., neurotransmitters and growth hormones) containing two enzymes that act sequentially to catalyse the alpha-amidation of neuroendocrine peptides.</td>
</tr>
<tr>
<td>Polyphenol oxidase/tyrosinase</td>
<td>Pigment (melanin) synthesis; amino acid</td>
<td>Oxidizes tyrosine to the pigment melanin in mammals. Deficiency leads to hypopigmentation.</td>
</tr>
<tr>
<td>Prostaglandin reductase</td>
<td>Prostaglandin biosynthesis</td>
<td>Responsible for reduction of 15-oxoprostaglandins to 13,14-dihydro derivatives.</td>
</tr>
<tr>
<td>Amine oxidase</td>
<td>Deamination of primary amines</td>
<td>Catalyses the oxidation of amines (tyramine, histidine, and polyamines) producing oxidized organic products such as aldehydes, and generating NH3 and H2O2 as by-products</td>
</tr>
<tr>
<td>Ceramide glucosyltransferase</td>
<td>Myelin synthesis</td>
<td>Synthesis and maintenance of phospholipid membranes.</td>
</tr>
</tbody>
</table>
2.9.3 Copper intakes and requirements

Copper is found, in varying amounts, in most foods; those in human diets with particularly high copper content include seafood (especially molluscs and crustaceans), organ meats (eg liver) and nuts (FSANZ 2006). Foods that are cereal or grain based are also good contributors to overall intake due to their importance in many diets. Humans may also consume some copper in drinking water, particularly when it is carried in copper pipes. Depending on the amount and the bioavailability of the ingested copper, the human gastrointestinal tract can absorb between 12% - 65%; mostly in the duodenum and stomach (van den Berghe and Klomp 2009).

Previous estimates, derived from depletion/repletion experiments, have suggested dietary copper requirements in adults range between 1.2 - 2.0 mg/d (Milne 1998). The World Health Organization (WHO) has suggested men and women (aged 25 - 50 yrs) require between 1.5 - 3.0 mg/d (Solomons and Ruz 1998). A relative lack of comprehensive data in this area had led to several countries not publishing recommended dietary intakes, a situation now rectified in the US, but persisting in Australia and New Zealand. The US recommended dietary allowance (RDA) for adults is 0.9 mg/d (Food and Nutrition Board 2000a), but in Australia, EAR and RDI values have not yet been formulated. The Nutrient Reference Values for Australia and New Zealand (NHMRC 2006) provide an adequate intake (Al) - an intake level that is based on observed or estimated intakes of an apparently healthy group of people; used when evidence is considered insufficient for the establishment of an EAR and subsequently an RDI value. Currently the Al for men is 1.7 mg/d and 1.2 mg/d for non-pregnant women (Table 2.5). Safe upper levels of intake were derived from data relating to liver effects as those which produced a no observable adverse effect level (NOAEL) of 10 mg/d. Values for children were extrapolated from the adult values.
Table 2.5: Australian nutrient reference values for copper (NHMRC 2006).

<table>
<thead>
<tr>
<th>Infants</th>
<th>Adequate Intake</th>
<th>Upper level of intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-6 months</td>
<td>0.20</td>
<td>-</td>
</tr>
<tr>
<td>7-12 months</td>
<td>0.22</td>
<td>-</td>
</tr>
</tbody>
</table>

**Children and Adolescents**

**All**
- 1-3 yrs: 0.7, 1
- 4-8 yrs: 1.0, 3

**Boys**
- 9-13 yrs: 1.3, 5
- 14-18 yrs: 1.5, 8

**Girls**
- 9-13 yrs: 1.1, 5
- 14-18 yrs: 1.1, 8

**Adults**

**Men**
- 19-30 yrs: 1.7, 10
- 31-50 yrs: 1.7, 10
- 51-70 yrs: 1.7, 10
- >70yrs: 1.7, 10

**Women**
- 19-30 yrs: 1.2, 10
- 31-50 yrs: 1.2, 10
- 51-70 yrs: 1.2, 10
- >70yrs: 1.2, 10

**Pregnancy**
- 14-18 yrs: 1.2, 8
- 19-30 yrs: 1.3, 10
- 31-50 yrs: 1.3, 10

**Lactating**
- 14-18 yrs: 1.4, 8
- 19-30 yrs: 1.5, 10
- 31-50 yrs: 1.5, 10

Units for all intakes - mg/d
2.9.4 Disease associations

Overt copper deficiency is rare, but has been observed in very low birth weight infants and in patients receiving total parenteral nutrition (TPN) with inadequate copper content (Stern et al. 2007). Of more interest recently is marginal copper deficiency, which is thought to be much more common and has been associated with haematological abnormalities (Halfdanarson et al. 2008), altered immunity (Percival 1998; Bonham et al. 2002), hypercholesterolemia and cardiovascular disease (Klevay 2000; Uriu-Adams and Keen 2005). Such deficiency may result from low dietary intakes and also from reduced absorption (e.g. in coeliac disease), the rare X-linked Menkes syndrome (Ala and Schilsky 2004) and from high zinc intakes (Stern et al. 2007). Although uncommon, copper toxicity generally occurs as a result of accidental ingestion of copper salts, the consumption of drinking water with high copper content, or industrial exposure. Wilson disease, a hereditary condition of altered copper metabolism, is also a rare cause of excessive copper accumulation in major organs (Ala and Schilsky 2004).

2.9.5 Biochemical markers of copper status

Biomarkers for copper status have often been considered to lack the sensitivity required to provide reliable indications of copper status, a situation that has contributed to the uncertainty over recommended intakes in some countries. Among those currently used for this purpose or suggested as possible markers are serum/plasma/red cell copper, caeruloplasmin, Cu-Zn SOD and COX (Danzeisen et al. 2007; Harvey et al. 2009).

A recent meta-analysis included 16 studies (including randomised controlled trials, controlled clinical trials and before and after studies) covering possible copper
biomarkers (Harvey et al. 2009). Due to a paucity of well designed studies on copper biomarkers, the authors were unable to reach a conclusion on the usefulness of numerous markers including plasma, red cell, and platelet copper and COX. They did however find that both serum copper and caeruloplasmin appeared to reflect changes in copper status, albeit to a different extent. Caeruloplasmin appeared to reflect changes in highly depleted subjects whereas serum copper reflected changes in both depleted and replete individuals. As a result, the authors suggested serum copper appeared to be the most useful biomarker of copper status at the population level.

Previously, the most commonly used methods for the determination of trace elements such as copper were atomic absorption, using either flame or electrothermal atomisation, however newer methods such as inductively coupled plasma mass spectrometry (ICP-MS) are also becoming more widespread, allowing rapid multi-element analysis (Huang and Beauchemin 2003).

2.9.6 Australian data

Published data regarding trace element status in Australia is sparse. To date there are very few reports of copper intake or measures of blood copper levels in the Australian population.

Of the very few studies, an early one assessed copper intakes for female residents (n = 47) of an Australian nursing home; measuring intakes by duplicate diet analysis (using AAS) and reported limited findings which included mean intakes of 1 mg/d (Roesch et al. 1984). The largest study was of a cohort of 556 women (aged 20-88 yrs) from the Barwon electorate in Victoria. Cleverdon and Ball (2004) assessed dietary copper intake using a semi-quantitative FFQ and reported mean copper intake to be 1.55 ± 0.55 mg/d; suggesting the intakes of the majority of subjects met the AI
for Australia. Those considered at greatest risk of low copper intakes in this cohort were younger women (<40 yrs).

The only other published data on copper status from Australia comes from a study which assessed the relationship between maternal copper and zinc status and neural tube defects in 592 pregnant women (McMichael et al. 1994). This study reported a mean serum copper concentration of 32.2 μmol/L in pregnant women with healthy foetuses; well above reference ranges but likely elevated by female hormones.

Data from a representative population sample, including both genders and across a range of age groups is lacking. This is of concern, particularly with some suggestion that copper intakes have fallen in the last century (Klevay and Medeiros 1996) and that food content may be lower than estimated (Klevay 1998), combined with the proposal that copper may be related to cardiovascular disease.
2.10 Zinc

2.10.1 A brief history

Zinc has an atomic weight of 65.39, and belongs to group 12 of the periodic table; other members of this group include cadmium and mercury (Brown et al. 2001). Zinc shares similar chemistry with the neighbouring Group 11 transition metal, copper. Zinc ores have been used in the manufacture of brass for many centuries. Metallic zinc was extracted in India as early as the 1200’s and in Europe several centuries later (Deshpande 1996).

2.10.2 Essentiality of zinc

Zinc appears to be essential for all life. It was reported to be essential for growth in mammals in 1933 (Todd et al. 1933). Because of the wide distribution of zinc it was thought that deficiency in man would be an unlikely occurrence, however cases of dwarfism due to zinc deficiency were described in adolescent Egyptian males in the 1960’s (Sandstead et al. 1967).

Due to its chemical properties, and in contrast to copper or iron, zinc is redox-inert in the body making it comparatively non-toxic; these same characteristics make it particularly useful for catalytic, structural and regulatory roles (Andreini et al. 2006). Such adaptability sees it involved in all six enzyme classes (oxidoreductases, transferases, hydrolases, lysases, isomerases, and ligases) (Brown et al. 2004). Zinc may be active at the catalytic site of enzymes or be involved in maintaining tertiary structure in proteins. Intracellular zinc also participates in cellular signalling and second messenger, protein kinase and protein phosphatase activity (Hambidge and Krebs 2007). Zinc metalloproteins include zinc finger motifs, which are contained in 3% of all known human genes (Maret 2001), and hence influence gene expression.
greatly. One group of researchers has estimated the existence of a minimum of 2840 metalloproteins and that potentially 10% of the human proteome is zinc-binding (Andreini et al. 2006). Notable zinc metalloproteins include RNA polymerase, carbonic anhydrase, alcohol dehydrogenase and alkaline phosphatase.

2.10.2 Zinc intakes and requirements

Most foods contain zinc; those that contain high concentrations include muscle meats and fish. Other food types that constitute significant proportions of normal diets, such as cereal based foods, can also contribute significantly to zinc intakes. Zinc in an animal protein rich diet will generally have a greater bioavailability than when consumed as part of a vegetarian diet (Lonnerdal 2000). The absorption rate of dietary zinc has been estimated as 24% and 31% for men and women respectively for mixed/refined vegetarian diets (Brown et al. 2004). This estimated absorption drops to 16% and 21% respectively for diets based on unrefined cereal. Factors affecting the absorption of zinc, other than the animal protein content of the diet, include actual zinc content (high zinc content will result in lower absorption rate) as well as dietary phytate and calcium (Brown et al. 2004).

The physiological requirement, defined as the amount of zinc needed to be absorbed to balance zinc loss, has been estimated as 2.69 mg/d and 1.86 mg/d for men (65 kg) and women (55 kg) respectively (Brown et al. 2004).

The Australian EAR figures are derived using the zinc absorption and physiological requirements estimated by the International Zinc Nutrition Consultative Group (IZiNCG) (Brown et al. 2004). The Australian EAR for men is 12.0 mg/d and 6.5 mg/d for women; the RDIs calculated from these figures are 14.0 mg/d and 8.0 mg/d for adult males and females respectively (Table 2.6). The safe upper level of intake,
based on data relating to a reduction in Cu-Zn SOD activity, is 40 mg/d for both men and women (NHMRC 2006). A joint WHO/UNICEF/IAEA/IZiNCG meeting determined risk of zinc deficiency is elevated if >25% of a population consumes <EAR of zinc (de Benoist *et al.* 2007).
Table 2.6: Australian nutrient reference values for zinc (NHMRC 2006).

<table>
<thead>
<tr>
<th>Infants</th>
<th>EAR</th>
<th>RDI</th>
<th>Upper level of intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-6 months</td>
<td>2.0*</td>
<td>-</td>
<td>4.0</td>
</tr>
<tr>
<td>7-12 months</td>
<td>2.5</td>
<td>3.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Children and Adolescents

<table>
<thead>
<tr>
<th>All</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3 yrs</td>
<td>2.5</td>
<td>3.0</td>
<td>7.0</td>
</tr>
<tr>
<td>4-8 yrs</td>
<td>3.0</td>
<td>4.0</td>
<td>12.0</td>
</tr>
</tbody>
</table>

Boys

| 9-13 yrs           | 5.0 | 6.0 | 25.0                  |
| 14-18 yrs          | 11.0| 13.0| 35.0                  |

Girls

| 9-13 yrs           | 5.0 | 6.0 | 25.0                  |
| 14-18 yrs          | 6.0 | 7.0 | 35.0                  |

Adults

<table>
<thead>
<tr>
<th>Men</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>19-30 yrs</td>
<td>12.0</td>
<td>14.0</td>
<td>40.0</td>
</tr>
<tr>
<td>31-50 yrs</td>
<td>12.0</td>
<td>14.0</td>
<td>40.0</td>
</tr>
<tr>
<td>51-70 yrs</td>
<td>12.0</td>
<td>14.0</td>
<td>40.0</td>
</tr>
<tr>
<td>&gt;70yrs</td>
<td>12.0</td>
<td>14.0</td>
<td>40.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Women</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>19-30 yrs</td>
<td>6.5</td>
<td>8.0</td>
<td>40.0</td>
</tr>
<tr>
<td>31-50 yrs</td>
<td>6.5</td>
<td>8.0</td>
<td>40.0</td>
</tr>
<tr>
<td>51-70 yrs</td>
<td>6.5</td>
<td>8.0</td>
<td>40.0</td>
</tr>
<tr>
<td>&gt;70yrs</td>
<td>6.5</td>
<td>8.0</td>
<td>40.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pregnancy</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>14-18 yrs</td>
<td>8.5</td>
<td>10.0</td>
<td>35.0</td>
</tr>
<tr>
<td>19-30 yrs</td>
<td>9.0</td>
<td>11.0</td>
<td>40.0</td>
</tr>
<tr>
<td>31-50 yrs</td>
<td>9.0</td>
<td>11.0</td>
<td>40.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lactating</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>14-18 yrs</td>
<td>9.0</td>
<td>11.0</td>
<td>35.0</td>
</tr>
<tr>
<td>19-30 yrs</td>
<td>10.0</td>
<td>12.0</td>
<td>40.0</td>
</tr>
<tr>
<td>31-50 yrs</td>
<td>10.0</td>
<td>12.0</td>
<td>40.0</td>
</tr>
</tbody>
</table>

*AI (adequate intake); all intakes in mg/d
2.10.3 Disease associations

Due to a very broad involvement of zinc in human metabolism, even mild zinc deficiency has been recognised as a significant public health concern throughout much of the world, especially in developing countries; with 20% of the world's population estimated to be affected (Sanghvi et al. 2007). Arising from inadequate diets and poor bioavailability, it is associated with increased morbidity with stunted growth and development (MacDonald 2000; Beyersmann and Haase 2001), delayed wound healing (Lansdown et al. 2007) and altered immune function (Ibs and Rink 2003).

More severe or overt zinc deficiency is uncommon. Classically seen in the rare genetic disorder of zinc absorption, acrodermatitis enteropathica (Maverakis et al. 2007), but also caused by inadequate zinc in TPN formulations (Jeejeebhoy 2009) and by chelation therapy, it causes major dermal, gastrointestinal and neurological disorders (Brown et al. 2004).

Zinc toxicity from a normal diet is very unlikely but can occur due to excessive consumption of zinc supplement preparations, from contaminated food and drink sources, and from industrial exposures (Cai et al. 2005). Symptoms of zinc toxicity include severe gastrointestinal effects such as nausea, vomiting, gastric pain and gastroenteritis (Brown et al. 2004).

2.10.4 Biochemical markers of zinc status

The apparent importance of zinc in human biology has seen many putative measures of zinc status employed in various studies; the tight homeostatic control over zinc levels in the body perhaps complicating the use of some markers for nutritional assessments.
A recent meta-analysis of 48 studies (randomised control studies and before and after studies) of 32 potential zinc biomarkers found data was insufficient to make clear conclusions of the viability of many markers for assessing zinc status (Lowe et al. 2009). The most conclusive evidence related to the use of serum/plasma zinc. This was found to reflect zinc intakes across gender and age groups as well as those with low to moderate zinc status. These findings concur with those of the joint WHO/UNICEF/IAEA/IIZiNCG meeting (de Benoist et al. 2007) which concluded that serum zinc is the biochemical marker of choice for zinc status at a population level and recommended serum zinc concentration age/gender/time of day specific cut-offs for determining populations at risk of low zinc status. Where >20% of a population subgroup has serum zinc below the cut-off, the population subgroup is considered to be at risk of zinc deficiency (Gibson et al. 2008).

Table 2.7: WHO cut-off values for assessment of zinc deficiency using serum zinc concentrations (Gibson et al. 2008).

<table>
<thead>
<tr>
<th></th>
<th>Lower cut-offs for serum Zn (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Children &lt; 10 yrs</td>
</tr>
<tr>
<td>Morning fasting</td>
<td>n/a</td>
</tr>
<tr>
<td>Morning non-fasting</td>
<td>9.9</td>
</tr>
<tr>
<td>Afternoon</td>
<td>8.7</td>
</tr>
<tr>
<td>Pregnancy</td>
<td></td>
</tr>
<tr>
<td>1st Trimester</td>
<td></td>
</tr>
<tr>
<td>2nd/3rd Trimester</td>
<td></td>
</tr>
</tbody>
</table>
Measurement of zinc in biological samples such as serum has most commonly been performed using flame or electrothermal AAS methods, however more recently ICP-MS methods have become more common.

2.10.5 Australian data

Assessment of zinc status by biochemical measures has not been conducted on a wide scale in Australia; the major published research has relied on zinc intake estimates as an indicator of zinc status of the population.

Dietary intakes

The 1995 National Nutrition Survey (NNS) (McLennan and Podger 1995) used a 24 hour diet recall in conjunction with a FFQ to estimate the dietary intake of 13,858 subjects (from 2 yrs of age), with all Australian states represented. Estimated intakes in Tasmanian men and women (>19 yrs) were both lower than the national average. Tasmanian men consumed 13.5 mg/d compared to 14.4 mg/d, while Tasmanian women consumed 9.1 mg/d compared to 9.7 mg/d; mean intakes for both genders nationally and in Tasmania exceeded the current EAR.

A study by English and colleagues (1997) reported mean zinc intakes of adult non-smokers (n = 4395). Using 24 hr diet recall, estimated mean intakes for men were 15.1 mg/d and 10.5 mg/d for women, suggesting most of this cohort consumed sufficient zinc.

The 20th Australian Total Diet Survey (FSANZ 2003) reported mean zinc intakes for males aged 25-34 yrs of 14 mg/d; above the current Australian EAR of 12 mg/d; while women of the same age range consumed on average 8.4 mg/d; also above the EAR for women of 6.5 mg/d.
Blood zinc levels

The study by Ball and Ackland (2000) appears to be the only published Australian study to measure blood zinc levels in healthy adults. The authors reported mean serum zinc from a small sample of 13.0 ± 2.1 μmol/L in men (n = 21) and 13.9 ± 2.3 μmol/L for women (n = 21). Zinc status was lower in the male subjects but both mean and median serum zinc were above the WHO cut-off for morning fasting samples, and the lower reference range (11 μmol/L). The sample was unfortunately small and sufficient detail was not provided to determine the proportion of subjects below the cut-off or of age group trends.
2.11 Conclusion

Micronutrient deficiency is acknowledged as a significant concern for a large proportion of the world's population (Sanghvi et al. 2007). Because of this involvement in many metabolic functions in humans, deficiencies in trace elements such as selenium, copper and zinc are potential contributors to morbidity and mortality in susceptible populations.

Consequently, it is important to investigate populations where little data exists, particularly where there may be suspicion of increased risk of deficiency. Given the history of problems with iodine deficiency in humans and with selenium, copper and cobalt deficiency in livestock, the human population in Tasmania, or at least subsections of it, may be at risk of deficiency or suboptimal status of trace elements such as selenium. As the Tasmanian population has high rates of cancer and cardiovascular disease (A.B.S. 2006a), this provides further impetus to identify factors which may contribute to these conditions.
Chapter 3

General methodology

From a methodological point of view, the aim was to utilise methods of analysis for both biological specimens and dietary data that were well established, to ensure the reliability of the data produced. Most analyses were performed by the candidate; as was the case for the trace element analyses, serum lipids, serum TAS, HFE genotyping and dietary estimates. The measurement of glutathione peroxidase and superoxide dismutase enzyme activity, and the genotyping of participants for the GPx-1 Pro/Leu polymorphism, was performed in collaboration with another study of antioxidant enzymes. The candidate also coordinated the recruitment of participants for both the pilot and population studies.

For routine biochemical assays such as serum lipids and glucose, specialised antioxidant assays, as well as molecular techniques, the specific methods utilised are in common usage in many laboratories, and have been in use in the Human Life Sciences laboratory in their current form on existing analysers for several years.

The methods employed for trace element analysis were carried out in a National Association of Testing Authorities (NATA) and Royal College of Pathologists of Australasia (RCPA) accredited hospital laboratory, have been in use for many years and perform well in external quality assurance programs.

Where consideration was given to the use of an alternate method to that accepted as standard; as was case for the prospective use of colorimetric methods for serum copper and zinc determination; a full evaluation of the alternate method was performed in comparison to an accepted standard method.
Dietary analysis utilised a validated food frequency questionnaire sourced from the Cancer Council of Victoria Epidemiology Centre. As well as providing estimates on many standard nutrients, the raw food intake data from this analysis, in conjunction with standard food content tables, was used to estimate intakes for the trace elements of interest.

The methodology adopted for the collection of all data, including subject recruitment, dietary estimates, standard biochemical and molecular techniques as well as statistical analysis are addressed in this chapter. The subsequent chapter (Chapter 4) details the method evaluation of the colorimetric serum copper and zinc assays which were considered as alternate methods to the more established atomic absorption spectroscopy which was eventually employed.

3.1 Subject recruitment

3.1.1 Pilot Study

Recruited in a convenience sample, the subjects for the pilot study were 198 non-institutionalised male and female adults residing in north and northwest Tasmanian townships. A significant number of these subjects were recruited from a database containing haemochromatotic and non-haemochromatotic individuals as part of the research project - 'The Influence of Diet, Health and Lifestyle Factors on Clinical Disease Development in the Tasmanian Population with Haemochromatosis Genes'. A substantial portion of the non-haemochromatotic subjects (n = 82) were recruited from outside this database, from other University of Tasmania studies.

Ethics approval was provided by the Human Research Ethics Committee (Tasmania) Network (EC Ref: H0006375).
Blood samples were collected at the Pathology department of the Launceston General Hospital, Launceston, Tasmania, at NorthWest Pathology centres in Burnie and Devonport, Tasmania and at the School of Human Life Science, University of Tasmania. Blood samples from all participants consisted of a single 4 mL lithium heparin tube (BD Vacutainer) and two 7 mL trace element serum tubes (BD Vacutainer). Following collection, samples were kept at 4°C for a maximum of 24 hours before processing.

3.1.2 Population Study

The subjects for the population study were 498 non-institutionalised male and female adults residing in north, northwest and northeast Tasmanian townships. Recruitment was via a random sample taken from an extract of the Australian electoral roll provided by the Australian Electoral Commission (AEC). Ethics approval was provided by the Human Research Ethics Committee (Tasmania) Network (EC Ref: H0009038).

Blood samples were collected at the Pathology department of the Launceston General Hospital, Launceston, Tasmania; at NorthWest Pathology centres in Burnie, Devonport and Latrobe, Tasmania; at Scottsdale Doctors Surgery, Scottsdale; and at the Campbell Town Health and Community Services Centre, Campbell Town. Blood samples provided by subjects consisted of a single 6 mL lithium heparin Vacutainer tube, one 7 mL trace element serum tube, one 7 mL SST serum tube and one 4 mL EDTA tube (Becton Dickinson, Rutherford, USA). Following collection, samples were kept at 4°C for a maximum of 24 hours before processing.
3.2 Dietary analysis

Normal dietary nutrient intake over a 12 month period was estimated with a self-administered validated semi-quantitative food frequency questionnaire (FFQ) (Giles and Ireland 1996). The FFQ consisted of 121 types of food and beverages. For items such as milk, bread, sugar and alcohol, subjects indicated the amount normally consumed per day. Pictures were used to assist in the establishment of normal portion sizes for meals of potatoes, other vegetables and steak. For other foods and beverages, subjects were asked “Over the last 12 months, on average, how often did you eat the following foods?” Ten response options were provided, ranging from ‘never’ to ‘3 or more times per day’. Analysis for energy and nutrient intakes, excluding selenium, copper and zinc, was performed by the Cancer Council of Victoria.

Data from NUTTAB 2006 food composition tables (FSANZ 2006) and responses from the FFQ were used to estimate daily selenium, copper and zinc intakes. All participants were asked specifically about Brazil nut consumption, due to their very high selenium content. Details of vitamin and mineral supplement use were also documented. Dietary selenium, copper and zinc estimates were calculated using Microsoft Excel 2003 software.

3.3 Health and anthropometric data

In addition to dietary information, health and lifestyle questionnaires were used to collect data on age, height, weight, smoking habits, basic medical histories covering major diseases, current medications and multivitamin usage.
3.4 Biochemical methods

3.4.1 Sample processing

Blood samples were processed within 24 hours of collection to produce serum and red cell lysates. Samples collected into trace element free serum tubes, SST serum tubes and lithium heparin tubes were centrifuged at 2500 rpm at 4 °C for 15 minutes in a refrigerated centrifuge. Five hundred microlitre aliquots of the resulting serum were stored at -80 °C until subsequent analysis for selenium, glutathione peroxidase (GPx), total antioxidant status (TAS) and lipid studies. Aliquots of 800 µL were also stored for serum copper and zinc determinations. Lithium heparinised blood was used to produce a red cell lysate using a modification of the method of Abiaka and co-workers (2000). After centrifugation, plasma and buffy coat were discarded and red cells washed twice in 1 volume of cold 0.9% NaCl solution. Five hundred microlitres of washed red cells were lysed in 1 volume of cold reverse osmosis water to produce a 50% lysate. The lysate was vortex mixed, centrifuged at 13000 rpm for 3 minutes to remove cell debris and stored in aliquots of 500 µL at -80 °C, until analysis for Red cell GPx, superoxide dismutase (SOD) and haemoglobin.

3.4.2 Haemoglobin

Haemoglobin concentration in assay samples for red cell GPx and SOD was determined using the Cobas Mira autoanalyser (Roche Diagnostics Ltd., Rotkreuz, Switzerland). One hundred microlitres of 50% lysate were diluted in 900 µL of 20mM, pH 7.4 tris(hydroxymethyl)aminomethane (Tris) buffer prior to analysis. Haemoglobin concentration was measured using a wavelength of 550 nm after incubation at 37 °C. Within-run and between-run precision for haemoglobin was 0.89% (n = 20) and 3.5% (n = 35) respectively.
3.4.3 Glutathione peroxidase

Analysis for GPx was performed at 37 °C using a Data Pro random access chemistry analyser (Thermo-Electron Corporation, Melbourne, Australia). Due to instability of the enzyme, GPx activity in red cells and serum was determined within 7 days of sampling. Serum and lysate for each subject were analysed within the same assay run. The method measured absorbance reduction at 340 nm over 3 minutes, due to oxidation of NADPH, using a coupled enzyme reaction with cumene hydroperoxide as a substrate (Ransel; Randox Laboratories Ltd., Crumlin, United Kingdom). One activity unit was defined as 1 µmol NADPH oxidised per minute per litre of serum or lysate. Ransel control (Randox Laboratories Ltd., Crumlin, United Kingdom) was used for internal quality control.

Red cell glutathione peroxidase

A 50 µL volume of red cell lysate was diluted with 1 mL of Ransel diluting agent and incubated at room temperature for 5 min to ensure the reduction of glutathione prior to reaction. This was followed by the addition of 1 mL of double strength Drabkins reagent, which inhibits other non-specific peroxidases. The Drabkins reagent was prepared by adding 250 µL of 500g/L Brij 35 to 100 mg potassium ferricyanide, 25 mg potassium cyanide and 70 mg potassium dihydrogen phosphate in 500 mL of reverse osmosis water (adjusted to pH 7.0). The final dilution of the whole blood sample was 1:82. Stored aliquots of pooled lysate analysed to determine between-run precision provided a CV of 4.3% (n = 21). Within run precision was 5.1% (n = 17). Activity was expressed as U/g Hb.
Serum glutathione peroxidase

Three hundred microlitres of serum was diluted with 1 volume of Ransel diluting agent (Ransel reagent: Randox). If absorbance change per minute exceeded 0.1, the sample was again diluted 1:2 using Ransel diluting agent. Samples were measured in duplicate. Activity was expressed as U/L. Within run precision was 3.0% (n = 14).

3.4.4 Red cell superoxide dismutase

Analysis for red cell SOD was performed at 37°C using the Cobas Mira autoanalyser (Roche Diagnostics Ltd., Rotkreuz, Switzerland). The Ransod method (Randox Laboratories Ltd., Crumlin, United Kingdom) uses the xanthine-xanthine oxidase system to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye, which is measured at 500nm. SOD in the added sample inhibits the formation of the dye; therefore activity is measured as the degree of inhibition. Calibration was performed for each assay run using the supplied kit standards (S2-S6). Assay of 0.01 M potassium phosphate buffer (pH 7.0) provided a value for an uninhibited reaction and a log calibration curve was prepared. The unknown samples were diluted to bring % inhibition, the reduction in absorbance that the unknown produces when compared to the uninhibited reaction value of the phosphate buffer, to between 30% and 60%.

Five microlitres of red cell lysate was diluted with 1.25 mL of 0.01 M potassium phosphate buffer (pH 7.0) to provide a total sample dilution of 1:500. Aliquots of pooled red cell lysate stored at -80°C were assayed for internal quality control. Control material was diluted 1:200 prior to analysis giving a total dilution of 1:400. Within-run and between-run precision was 1.9% (n = 19) and 5.7% (n = 35) respectively. Sample SOD activity was expressed as U/g Hb.
3.4.5 Total antioxidant status

Analysis for TAS was performed at 37°C using the Data Pro clinical analyser (Thermo-Electron Corporation, Melbourne, Australia), using the TAS kit (NX2332; Randox Laboratories Ltd., Crumlin, United Kingdom). Incubation of 2,2'-Azino-di-[ethylbenzthiazoline sulphonate] (ABTS) with metmyoglobin and H₂O₂ results in the formation of the radical cation ABTS⁺. The method measures, at 600 nm, the inhibition of ABTS⁺ formation after 3 minutes. The level of inhibition is proportional to antioxidant concentration in the sample, expressed as mmol/L.

Stored aliquots of approximately 500 μL serum required no pre-analytical preparation other than thawing and thorough mixing. Control serum (Randox Laboratories Ltd., Crumlin, United Kingdom) was used for internal quality control. Within-run and between-run precision was 2.8% (n = 20) and 2.8% (n = 17) respectively.

3.4.6 Serum selenium

Serum selenium concentration was determined by Zeeman-corrected graphite furnace atomic absorption spectrometry (GFAAS) in the Special Chemistry Laboratory at the Royal Hobart Hospital, Hobart, Tasmania using a Spectra 640Z spectrophotometer (Varian Inc., Palo Alto, USA) and a method based on that of Saeed and colleagues (1979). This method utilises a wavelength of 196 nm, a slit width of 1.0 nm and current of 10.0 mA. Calibration for the analysis used the standard addition method.

The addition of volumes of 0, 25, 50 and 75 μL of 25.4 μmol/L selenium stock solution to 1 mL serum, made up to 1100 μL with deionised water, produced a four point curve of 0, 0.58, 1.15 and 1.73 μmol/L respectively. Analysis of Seronorm Trace Elements control serum (Sero, Billingstad, Norway) with a certified selenium
concentration of 0.92 μmol/L gave a mean of 0.88 μmol/L (CV; 7.0%; n = 19). Within-run precision was 3.7% (n = 15).

One hundred microlitre volumes of standard, control or sample were diluted with nickel chloride modifier in acid washed plastic tubes. Modifier was prepared by addition of 0.5g NiCl₂·6H₂O and 500 μL Triton X-100 to 100 mL of distilled water, acidified with 100 μL Suprapur 65% nitric acid. All samples were analysed in duplicate to ensure reproducibility of results. Selenium concentration was expressed as μmol/L.

3.4.7 Serum copper

Serum copper concentration was determined by flame atomic absorption spectrometry (FAAS) in the Special Chemistry Laboratory at the Royal Hobart Hospital, Hobart, Tasmania using a Spectra 880 spectrophotometer (Varian Inc., Palo Alto, USA) at a wavelength of 324.8 nm, a slit width of 0.5 nm and current of 5.0 mA. Calibration for the analysis used the standard addition method. The addition of volumes of 0, 25, 50 and 75 μL of 628 μmol/L copper stock solution to 1 mL serum, made up to 1100 μL with deionised water, produced a four point curve of 0, 14.3, 28.5 and 42.8 μmol/L respectively.

Three hundred microlitre volumes of standard, control or sample were diluted with 600 μL of deionised water in acid washed plastic tubes. Analysis of Seronorm controls (Sero, Billingstad, Norway) with certified copper concentrations of 13.2 and 27.4 μmol/L gave means of 11.4 (CV; 3.8%; n = 38) and 23.8 μmol/L (CV; 3.2%; n = 38) respectively. Within-run precision was 1.8% (n = 13). Copper concentration was expressed as μmol/L.
3.4.8 Serum zinc

Serum zinc concentration was determined by flame atomic absorption spectrometry (FAAS) in the Special Chemistry Laboratory at the Royal Hobart Hospital, Hobart, Tasmania using a Spectra 880 spectrophotometer (Varian Inc., Palo Alto, USA) at a wavelength of 213.9 nm, a slit width of 1.0 nm and current of 5.0 mA. Calibration for the analysis used the standard addition method. The addition of volumes of 0, 25, 50 and 75 μL of 612 μmol/L zinc stock solution to 1 mL serum, made up to 1100 μL with deionised water, produced a four point curve of 0, 13.9, 27.8 and 41.7 μmol/L respectively.

Three hundred microlitre volumes of standard, control or sample were diluted with 600 μL of deionised water in acid washed plastic tubes. Analysis of Seronorm controls (Sero, Billingstad, Norway) with certified zinc concentrations of 14.0 and 16.1 μmol/L gave means of 13.0 (CV; 4.8%; n = 38) and 17.8 μmol/L (CV; 4.0%; n = 38). Within-run precision was 3.5% (n = 13). Zinc concentration was expressed as μmol/L.

3.4.9 Serum ferritin

Serum ferritin was determined using a two-site chemiluminescent immunometric assay (LKFE1) on the Immulite analyser (Siemens Healthcare Diagnostics; Deerfield, USA).

In the automated protocol, serum sample (10 μL) and alkaline phosphatase conjugated goat polyclonal anti-ferritin reagent were introduced into an Immulite test unit coated with monoclonal murine anti-ferritin antibodies. Following incubation at 37 °C and subsequent wash steps, the bound ferritin and conjugated alkaline phosphatase was
detected by its action on a chemiluminescent substrate. Ferritin concentration was reported in μg/L.

3.4.10 Lipid profile

The methods utilised for determining lipid profiles for subjects were all performed on serum samples using the Data Pro clinical analyser (Thermo-Electron Corporation, Melbourne, Australia).

**Serum Total Cholesterol**

Serum total cholesterol was measured at 37 °C using an enzymatic method (TR13303; Thermo-Electron Corporation, Melbourne, Australia) based on the conversion, by cholesterol oxidase, of cholesterol to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide produced combines with hydroxybenzoic acid and 4-aminoantipyrine to form the chromophore quinonemine measured spectrophotometrically at 505 nm. Total cholesterol was reported in mmol/L. Trace calibrator (TR43002; Thermo-Electro Corporation, Melbourne, Australia) was used for calibration, while internal quality control used two level Lyphochek controls (Bio-Rad, Hercules, USA) with between run precision (n = 17) of 2.9% and 2.8% for level 1 and level 2 respectively. Within-run precision was 1.8% (n = 11).

**Serum High Density Lipoprotein (HDL) Cholesterol**

Serum HDL cholesterol was measured at 37°C using an enzymatic method (TR39601; Thermo-Electron Corporation, Melbourne, Australia) based on the conversion, by cholesterol oxidase, of cholesterol to cholesterol-3-on and hydrogen peroxide. The hydrogen peroxide produced combines with N-(2-hydroxy-3-sulfopropyl)-3,5-
dimethoxyaniline (HDAOS) and 4-aminoantipyrine to form a quinone chromophore measured spectrophotometrically at 600 nm. Non-HDL lipoproteins are removed prior to the main reaction by selective reaction with cholesterol esterase and cholesterol oxidase. Serum HDL was reported in mmol/L.

Calibration used Trace HDL calibrator (1913-003; Thermo-Electro Corporation, Melbourne, Australia); internal quality control used two level Trace Lipid Control (1919-030; Thermo-Electron Corporation, Melbourne, Australia) with between run precision (n = 17) of 2.4% and 3.2% for level 1 and level 2 respectively. Within-run precision was 2.3% (n = 11).

**Serum Low Density Lipoprotein (LDL) Cholesterol**

For subjects where fasting blood samples were used, serum LDL cholesterol was calculated using the equation developed by Friedewald and colleagues (1972). This equation estimates the LDL cholesterol concentration in mmol/L using serum total cholesterol, HDL cholesterol and triglyceride values for each subject as follows:

\[
LDL = [\text{Total Cholesterol}] - ([\text{HDL cholesterol}] + 0.45 \times [\text{Triglycerides}]).
\]

Where non-fasting samples were used, serum LDL cholesterol was measured directly at 37 °C using an enzymatic method (TR53202; Thermo-Electron Corporation, Melbourne, Australia) which, as with HDL cholesterol, is based on the conversion, by cholesterol oxidase, of cholesterol to cholesterol-3-on and hydrogen peroxide. The hydrogen peroxide produced combines with N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline (TOOS) and 4-aminoantipyrine to form a quinone chromophore measured spectrophotometrically at 600 nm. Again, non-HDL lipoproteins are removed prior to the main reaction by selective reaction with cholesterol esterase and cholesterol oxidase. Serum LDL was reported in mmol/L.
Calibration used Trace HDL/LDL calibrator (1913-003; Thermo-Electro Corporation, Melbourne, Australia); internal quality control used two level Trace Lipid Control (1919-030; Thermo-Electron Corporation, Melbourne, Australia) with between run precision \((n = 17)\) of 4.2\% and 4.3\% for level 1 and level 2 respectively. Within-run precision was 3.9\% \((n = 11)\).

Serum triglycerides

Serum triglycerides were measured at 37 °C using an enzymatic method (TR22215; Thermo-Electron Corporation, Melbourne, Australia) based on the conversion of triglycerides to glycerol and free fatty acids by lipase. The glycerol undergoes conversion, with catalysis by glycerol kinase and then glycerol phosphate oxidase, to form dihydroxyacetone and hydrogen peroxidase. The hydrogen peroxidase combines with 3,5-dichloro-2-hydroxybenzene sulphate (DHBS) and 4-aminoantipyrine to form a quinonemine which is measured spectrophotometrically at 505 nm. Serum triglycerides were reported in mmol/L.

Trace calibrator (TR43002; Thermo-Electro Corporation, Melbourne, Australia) was used for calibration, while internal quality control used two level Lyphochek controls (Bio-Rad, Hercules, USA) with between run precision \((n = 17)\) of 2.1\% and 2.5\% for level 1 and level 2 respectively. Within-run precision was 1.6\% \((n = 11)\).

3.4.11 Plasma Glucose

Plasma glucose was measured at 37 °C using the hexokinase enzymatic method (TR15003; Thermo-Electron Corporation, Melbourne, Australia). Hexokinase catalyses the phosphorylation of glucose by ATP to form ADP and glucose-6-phosphate. Glucose-6-phosphate is oxidised by glucose-6-phosphate dehydrogenase
with a concomitant reduction of NAD$^+$ to NADH; the change in absorbance due to NADH was measured at 340 nm and is proportional to glucose concentration. Plasma glucose was reported in mmol/L.

Trace calibrator (TR43002; Thermo-Electro Corporation, Melbourne, Australia) was used for calibration, while internal quality control used two level Lyphochek controls (Bio-Rad, Hercules, USA) with between run precision ($n = 20$) of 4.4% and 4.3% for level 1 and level 2 respectively. Within-run precision was 1.9% ($n = 11$).

3.5 Molecular methods

3.5.1 DNA extraction

Genomic DNA was extracted from whole blood using the Blood and Tissue Genomic DNA Extraction Miniprep method (Viogene; Sunnyvale, USA).

DNA extraction was performed using two hundred microlitres of fresh or frozen (-80°C) lithium heparin or EDTA blood, which was added to an equal volume of extraction buffer and 20 μL of proteinase K (10mg/mL). After a thorough mixing by vortex, the sample was incubated at 60 °C for 20 minutes followed by 20 minutes at 70 °C. During this time the sample was vortexed frequently to ensure complete mixing. Following the addition of three hundred and ten microlitres of 100% ethanol, and further vortexing, the entire sample was pipetted onto the Miniprep column and centrifuged at 8000 rpm for 2 minutes. Washing of the column was performed twice by addition of 500 μL of wash buffer and centrifugation at 8000 rpm for 2 minutes. The column was dried by centrifugation at 13000 rpm for 2 minutes, after which time the DNA was eluted from the column with 200 μL of 70 °C molecular grade water or Tris EDTA Buffer (pH 8.0). DNA samples were stored at -20 °C.
3.5.2 *HFE* genotype

Participant genotyping for the hereditary haemochromatosis gene used a multiplex amplification refractory mutation system (ARMS) method to simultaneously detect the C282Y and H63D mutations in the *HFE* gene located on chromosome 6.

The DNA template used was genomic DNA extracted using the Blood and Tissue Genomic DNA Extraction Miniprep method (Viogene; Sunnyvale, USA), and stored at -20 °C in molecular grade water.

Sense primers for both C282Y and H63D were used together with specific antisense primers in a two-tube paired polymerase chain reaction (PCR):

**C282Y Oligonucleotide Primers**

Sense: 5’-TGGCAAGGGTAAACAGATCC-3’

Antisense Normal: 5’-GCTGATCCAGGCCTGGGTGCTCCACCTGCC-3’

Antisense Mutant: 5’-GCTGATCCAGGCCTGGGTGCTCCACCTGCT-3’

**H63D Oligonucleotide Primers**

Sense: 5’-ACATGGTTAAGGCCCTGTG-3’

H63D normal: 5’-AGTTCGGGGTGCTCCACACGGCGACTCTCAAG-3’

H63D mutant: 5’-AGTTCGGGGTGCTCCACACGGCGACTCTCAAC-3’

As an internal PCR control, primers for the human growth hormone (HGH) were also included:

**HGH Oligonucleotide Primers**

Sense: 5’-TGCTTCCCAACCACCTCCCTTA-3’

Antisense: 5’-CAGTCGGGAATTCTGTGTTGTTTCC-3’
Table 3.1: Primer allocation in two tube multiplex ARMS assay for HFE mutations.

<table>
<thead>
<tr>
<th>PCR Reaction Tube A</th>
<th>PCR Reaction Tube B</th>
</tr>
</thead>
<tbody>
<tr>
<td>C282Y sense primer</td>
<td>C282Y sense primer</td>
</tr>
<tr>
<td>C282Y Antisense ‘normal’ primer</td>
<td>C282Y Antisense ‘mutant’ primer</td>
</tr>
<tr>
<td>H63D sense primer</td>
<td>H63D sense primer</td>
</tr>
<tr>
<td>H63D Antisense ‘mutant’ primer</td>
<td>H63D Antisense ‘normal’ primer</td>
</tr>
<tr>
<td>HGH sense primer</td>
<td>HGH sense primer</td>
</tr>
<tr>
<td>HGH antisense primer</td>
<td>HGH antisense primer</td>
</tr>
</tbody>
</table>

Primers were added to Taq PCR master mix (Qiagen Pty Ltd., Melbourne, Australia) to give final concentrations for each pair of 1.05 μM for H63D, 0.52 μM for C282Y and 0.35 μM for HGH. Product fragment size for C282Y primers was 300 bp; for H63D fragments were 180 bp, while the HGH internal control produced a fragment of 434 bp.

The total reaction volume of 20 μL consisted of 2 μL of subject or positive control DNA template (or ddH₂O as negative control) and 18 μL of the primer-master mix. PCR reactions were initiated at 94 °C for 10 minutes followed by 35 cycles of 20 seconds at 94 °C (denaturation), 20 seconds at 58 °C (annealing) and 20 seconds at 72°C (elongation), using the MJ PTC-200 Thermalcycler (GMI Inc., Ramsey, USA). Reaction products were separated by electrophoresis, producing six distinct genotype patterns (Figure 3.1). Electrophoresis used a 2% Ultra Pure agarose gel (Invitrogen Pty Ltd., Melbourne, Australia) at 100V for 50 minutes, stained with ethidium bromide and visualised using a UV lamp.
3.5.3 \textit{GPx-1} genotype

Genotyping for the single nucleotide polymorphism at codon 198 of the \textit{GPx-1} gene located on chromosome 3 results in the Pro/Pro, Pro/Leu and Leu/Leu variants of the enzyme. The DNA template used was genomic DNA extracted using the Blood and Tissue Genomic DNA Extraction Miniprep method (Viogene; Sunnyvale, USA), and stored at -20 °C in Tris EDTA Buffer at pH 8.0. The primers and protocol used were developed by Amanda Crawford at the School of Human Life Sciences (University of Tasmania, Launceston, Australia).

\textit{GPx-1 Pro198Leu Oligonucleotide Primers}

Sense: 5'-CGC CAA GAA CGA AGA GAT TC-3'

Antisense: 5'-CAG GTG TTC CTC CCT CGT AG-3'

Primers were added to \textit{Taq} PCR master mix (Qiagen Pty Ltd., Melbourne, Australia) to give final concentrations for each pair of 0.5 \textmu M. The total reaction volume of 10 \textmu L consisted of 1 \textmu L of subject or positive control DNA template (or ddH2O as negative control) and 9 \textmu L of primers/master mix. The PCR reaction was initiated at 94 °C for 15 minutes followed by 30 cycles of 45 seconds at 94 °C (denaturation), 45
seconds at 56 °C (annealing) and 30 seconds at 72 °C (elongation), using the MJ PTC-200 Thermalcycler (GMI Inc., Ramsey, USA).

The PCR product was digested for 2 hours at 37 °C using 1 μL 50000 U/mL Apa-1 restriction enzyme (New England Biolabs Inc., Ipswich, USA). Digest products were separated by electrophoresis, using a 3% Ultra Pure agarose gel (Invitrogen Pty Ltd., Melbourne, Australia) at 120V for 40 minutes, stained with ethidium bromide and visualised using a UV lamp.

Electrophoresis of the restriction enzyme digestion product revealed up to four bands depending on subject genotype. The heterozygous Pro/Leu genotype resulted in four bands of 252, 208, 120 and 88 base pairs in size; homozygous Pro/Pro genotype gave three bands of 252, 120 and 88 base pairs in size, and homozygous Leu/Leu resulted in only 2 bands of 252 and 208 base pairs in size (Figure 3.2).

![Electrophoresis band patterns of restriction enzyme digestion product.](image)

**Figure 3.2**: Electrophoresis band patterns of restriction enzyme digestion product.

### 3.6 Statistical Analysis

Repeated measures ANOVA using general linear modelling (GLM) with robust standard error estimation was used to test for any differences in trace element or antioxidant status within different groups as defined by age, gender, genotype or
smoking habit (STATA version 9.2, StataCorp LP, USA). Post estimation Holm test analysis was then used to adjust p values for multiple comparisons (Aickin and Gensler 1996). The relationship between trace elements and antioxidant levels with dietary and other biochemical factors was also examined using GLM with robust standard error estimation. Z-scores of continuous independent variables [(subject variable – group mean)/standard deviation] were calculated for dietary and biochemical variables in order to show the relative effect of the actual variation of those variables within the subject population. The validity of regression assumptions was tested by post-hoc analysis to exclude significant heteroskedasticity and missing variable effects.

In the population study, the response rate varied in different age and socio-economic status groups and as a result estimates were made that were both unweighted for these analyses, and also weighted for the age/gender numbers and their socio-economic status in the study sample compared with that of the northern Tasmanian region from the 2006 census. Adjustment for socio-economic status was achieved using the census collector district of residence SEIFA (socioeconomic indexes for areas) index as a proxy for the status of the individual subjects.
Chapter 4

Evaluation of the Randox colorimetric serum copper and zinc assays against atomic absorption spectroscopy

4.1 Introduction

Serum concentrations of copper and zinc are currently the most frequently used biomarkers of the status of these essential trace elements and are useful in assessing both deficiency and toxicity.

4.1.1 Copper deficiency and toxicity

Severe copper deficiency is rare, but may be seen in low birth weight infants and in patients receiving total parenteral nutrition (TPN) with inadequate copper (Stern et al. 2007); marginal copper deficiency however, is thought to be more common and is of increasing interest due to the association of low copper status with haematological abnormalities (Halfdanarson et al. 2008), altered immunity (Percival 1998), hypercholesterolemia and cardiovascular disease (Klevay 2000). As well as resulting from low dietary intakes, deficiency may also arise from reduced absorption (e.g. coeliac disease), the rare X-linked Menkes syndrome and from high zinc intakes (Stern et al. 2007). Although uncommon, copper toxicity generally occurs as a result of accidental ingestion of copper salts, the consumption of drinking water with high copper content, or industrial exposure. Wilsons disease, a hereditary condition of altered copper metabolism, is also a rare cause of excessive copper accumulation in major organs (Stern et al. 2007).
4.1.2 Zinc deficiency and toxicity

Zinc, as a component of >300 proteins (Coleman 1992), has a wide range of roles in the body. Mild zinc deficiency is emerging as a significant public health concern throughout much of the world, especially in developing countries (Brown et al. 2001). Arising from inadequate diets and poor bioavailability, it is associated with increased morbidity with stunted growth and development (MacDonald 2000; Beyersmann and Haase 2001), delayed wound healing (Lansdown et al. 2007) and altered immune function (Ibs and Rink 2003). Severe zinc deficiency, classically seen in the rare genetic disorder of zinc absorption, acrodermatitis enteropathica, but also resulting from inadequate zinc in TPN formulations and chelation therapy, causes major dermal, gastrointestinal and neurological disorders (Brown et al. 2004). Zinc toxicity may occur due to excessive consumption of zinc supplement preparations, from contaminated food and drink sources, and from industrial exposures. Symptoms of zinc toxicity include major gastrointestinal effects such as nausea, vomiting, gastric pain and gastroenteritis (Brown et al. 2004).

The analysis of serum copper and zinc concentrations is most commonly performed using atomic absorption spectroscopy (AAS) techniques. However, established methods are not available locally. The use of colorimetric assays for serum copper and zinc were considered as alternatives which could be performed in the School of Human Life Sciences laboratory in Launceston. Colorimetric methods, such as those using the chromogen 4-(3,5-Dibromo-2-pyridylazo)-N-Ethyl-N-(3-sulphopropyl) aniline (3,5-Di-Br-PAESA) for copper, and the chromogen 2-(5-bromo-2-pyridylazo)-5-(N-propyl-N-sulfopropylamino)-phenol (5-Br-PAPS) for zinc and available in kit form from Randox (catalogue numbers CU2340 and ZN2341 respectively) are
potentially suitable for such an application. A search of the literature revealed a lack of comparative studies versus AAS for these reagent kits. Prior to embarking on clinical studies involving the analysis of >600 subject samples, it was necessary to thoroughly evaluate the proposed alternative methods in comparison to an accepted standard method such as atomic absorption spectroscopy, to determine their suitability for the purposes of the research.

4.2 Methods

Randox CU2340 and ZN2341 assay buffers, chromogens, standards and pre-treatment reagents were used (Randox Laboratories Ltd., Crumlin, United Kingdom). Forty eight blood samples were collected into Vacutainer Trace Element Free serum tubes (Becton Dickinson, Rutherford, USA); and after centrifugation at 2500 rpm for 15 min, serum aliquots from these tubes were stored at 4°C. All laboratory glassware and consumables were acid washed in 1% Nitric acid; collection and storage methods were assessed to eliminate all possible sources of contamination prior to evaluation. Comparison data was subject to Bland and Altman analysis (Bland and Altman 1986), using Microsoft Excel 2003, with statistical significance set at $P < 0.05$.

4.2.1 Atomic Absorption Spectrophotometry

The flame atomic absorption (FAAS) methods were based on those of Meret and Henkin (1971) and performed well in an external Quality Assurance Program (QAP). Results, when compared with those from the QAP program (Quality Control Technologies, Charlestown, Australia), placed the copper FAAS method within 0.56 standard normal deviates (SND) and zinc within 0.37 SND of the distribution means. Analysis was performed using a Spectra 880 atomic absorption spectrometer (Varian...
Inc., Palo Alto, USA) maintained in a NATA accredited hospital laboratory. For copper, a wavelength of 324.8 nm, a slit width of 0.5 nm and lamp current of 5.0 mA was employed. Three hundred microlitres of sample were diluted in 600 µL of deionised water prior to analysis. Calibration for the analysis used the standard addition method; 0 µL, 25 µL, 50 µL and 75 µL of a 628 µmol/L copper nitrate working standard was added to 1 mL pooled human serum to produce concentrations of 0, 14.3, 28.5 and 42.8 µmol/L respectively.

Zinc was also measured using the Varian Spectra 880; at a wavelength of 213.9 nm, using a slit width of 1.0 nm and lamp current of 5.0 mA. Again, sample volumes of 300 µL were diluted in 600 µL of deionised water prior to analysis. For calibration, a 612 µmol/L zinc nitrate standard was added in volumes of 0 µL, 25 µL, 50 µL and 75 µL to 1 mL pooled human serum to produce concentrations of 0, 13.9, 27.8 and 41.7 µmol/L.

4.2.2 Colorimetric Assay on the Data Pro

Colorimetric analysis was performed using the Thermo Electron Data Pro analyser (Melbourne, Australia) with parameters based on those outlined in the Randox package inserts (Table 4.1). Copper was determined using a direct serum measurement at 37 °C against a reagent blank and single point calibration. Using this method, copper is released from caeruloplasmin by a reducing agent (ascorbic acid) which reacts to form a chelate with the chromogen 3,5-Di-Br-PAESA with a resulting rise in absorbance measured at 590 nm.

Prior to zinc analysis, samples and standards underwent protein precipitation; 500 µL of 370 mmol/L trichloroacetic acid was mixed with 500 µL of serum and centrifuged at 13000 rpm for 12 minutes. The supernatant was assayed at ambient temperature
against a reagent blank with single point calibration. Zinc in the sample forms a
chelate with the chromogen 5-Br-PAPS and the resulting rise in absorbance is
measured at 550nm.

In an attempt to improve the calibration procedure of both assays, a simple multipoint
aqueous calibration curve was utilised. Stock standard solutions of 35 mmol/L copper
nitrate and 35 mmol/L zinc nitrate were used; dilution of 5 µL, 10 µL and 20 µL of
each stock standard to 10 mL resulted in working standards of 17.5 µmol/L, 35
µmol/L and 70 µmol/L respectively.

4.2.3 Colorimetric Assay on the Cobas Mira

The Cobas Mira (Roche Diagnostics Ltd., Rotkreuz, Switzerland) was also used to
measure zinc in a smaller set of samples, using the same method, with parameters
supplied by Randox (Table 4.1). These samples also underwent the serum
deproteinisation step described earlier.
Table 4.1: Colorimetric assay parameters as stated in Randox package notes and as used on the Data Pro and Cobas Mira analysers.

<table>
<thead>
<tr>
<th>Sample, Reagent, Chromogen</th>
<th>Package notes</th>
<th>Data Pro</th>
<th>Cobas Mira</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Copper</strong></td>
<td>Volume <strong>μL</strong>: 60</td>
<td>Incubation <strong>secs</strong>: 300</td>
<td>Temp <strong>°C</strong>: 37</td>
</tr>
<tr>
<td></td>
<td>Reagent: 1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chromogen: 250</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Zinc</strong></td>
<td>Volume <strong>μL</strong>: 500</td>
<td>Incubation <strong>secs</strong>: 300</td>
<td>Temp <strong>°C</strong>: 20-25</td>
</tr>
<tr>
<td></td>
<td>Reagent: 2500</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chromogen: -</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Copper</strong></td>
<td>Volume <strong>μL</strong>: 12</td>
<td>Incubation <strong>secs</strong>: 300</td>
<td>Temp <strong>°C</strong>: 37</td>
</tr>
<tr>
<td></td>
<td>Reagent: 200</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chromogen: 50</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Zinc</strong></td>
<td>Volume <strong>μL</strong>: 40</td>
<td>Incubation <strong>secs</strong>: 300</td>
<td>Temp <strong>°C</strong>: 20</td>
</tr>
<tr>
<td></td>
<td>Reagent: 200</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chromogen: -</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

+ Sample for copper analyses was serum; sample for zinc analyses was deproteinated serum
4.3 Results

Pipette precision testing of the Data Pro analyser using assay sample volumes of potassium dichromate and combined reagent and chromogen volumes of deionised water gave CVs of 0.80% for the copper protocol and 0.50% for the zinc protocol (n = 12).

Intra- and inter-assay precision data for both methods is presented in Table 4.2. Two level Lyphocheek (Bio-Rad, Hercules, USA) and Seronorm (Sero, Billingstad, Norway) quality control materials were utilised during analysis of copper and zinc by colorimetric methods and FAAS, respectively.

Least squares regression analysis for the comparison of the colorimetric copper method with FAAS (Figure 4.1A) yielded the following equation:

\[ y = 0.75x + 3.4 \ \mu\text{mol/L} \quad (r = 0.9227, \ n = 48, \ P < 0.01). \]

Colorimetric zinc using the Data Pro compared with the FAAS method (Figure 4.2A), produced the following regression line:

\[ y = 0.77x + 4.0 \ \mu\text{mol/L} \quad (r = 0.4376, \ n = 48, \ P < 0.01). \]

On the Cobas Mira, the comparison for the colorimetric zinc method demonstrated a poorer correlation with FAAS (Figure 4.3A), producing the regression line:

\[ y = 0.76x + 6.6 \ \mu\text{mol/L} \quad (r = 0.2541, \ n = 15, \ P = 0.06). \]
Table 4.2: Intra- and inter-assay precision data for FAAS and colorimetric analysis using QC material and pooled serum samples.

<table>
<thead>
<tr>
<th></th>
<th>Copper</th>
<th></th>
<th></th>
<th>Zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol/L</td>
<td>Target value</td>
<td>Our Assay mean %CV</td>
<td>µmol/L</td>
</tr>
<tr>
<td><strong>Intra-assay</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAAS Pooled sera</td>
<td>-</td>
<td>14.5 (0.3)</td>
<td>1.8 %CV</td>
<td>13.6 (0.6)</td>
</tr>
<tr>
<td>Colorimetric (Data Pro) Pooled sera</td>
<td>-</td>
<td>9.8 (0.3)</td>
<td>3.5 %CV</td>
<td>18.3 (0.9)</td>
</tr>
<tr>
<td><strong>Inter-assay</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAAS QC Level 1</td>
<td>11.0</td>
<td>11.1 (0.6)</td>
<td>5.4 %CV</td>
<td>12.8</td>
</tr>
<tr>
<td>FAAS QC Level 2</td>
<td>27.4</td>
<td>22.4 (1.0)</td>
<td>4.5 %CV</td>
<td>16.1</td>
</tr>
<tr>
<td>Colorimetric (Data Pro) QC Level 1</td>
<td>12.2</td>
<td>12.2 (0.7)</td>
<td>5.4 %CV</td>
<td>11.4</td>
</tr>
<tr>
<td>Colorimetric (Data Pro) QC Level 2</td>
<td>7.4</td>
<td>7.4 (0.3)</td>
<td>4.1 %CV</td>
<td>7.5</td>
</tr>
</tbody>
</table>

* Different batches of pooled sera were used for each method's inter-assay precision run. FAAS QC Level 1, 2 = Seronorm Lot # 0508348 and 0610542. Colorimetric QC Level 1, 2 = Lyphochek Lot # 14121 and 14122.

Three samples determined by FAAS to be above the normal adult upper reference value for copper of 22 µmol/L, were misclassified as normal by the colorimetric method (denoted by stars in Figure 4.1A). The colorimetric zinc method on the Cobas Mira misclassified two samples; of these two, FAAS determined one sample to be below and one sample to be within the reference range, but these were determined to
be within and above the reference range respectively, by the colorimetric method (denoted by stars in Figure 4.3A).

Results from linear regression analysis of Bland and Altman plots (Figure 4.1B, 4.2B and 4.3B) for the copper method using the Data Pro and the zinc method on both the Data Pro and the Cobas Mira are presented in Table 4.3. These results indicate significant systematic and fixed bias between the colorimetric and FAAS methods for copper. The evaluation of the zinc colorimetric method however, showed that systematic and fixed biases did not reach statistical significance. The smaller comparison using the zinc method on the Cobas Mira indicated the error observed in the evaluation on the Data Pro was not unique to the one analyser.
Figure 4.1 A: Least squares linear regression analysis of copper measured colorimetrically on the Data Pro analyser compared to the flame atomic absorption method. The analysis yielded a regression line equation of: \( y = 0.75x + 3.4 \mu \text{mol/L} \) \((r = 0.9227, n = 48, P < 0.01)\). Dashed lines represent the upper and lower reference range limits of 22 \( \mu \text{mol/L} \) and 11 \( \mu \text{mol/L} \) respectively.

B: Bland and Altman analysis of copper comparison data: correlation \( r = 0.6669 \) \((P < 0.01)\), slope = -0.2499 \((P < 0.01)\), intercept = 3.219 \((P < 0.01)\). Statistically significant systematic and fixed bias was found, with a mean difference of -1.4 ± 3.4 \( \mu \text{mol/L} \).
Figure 4.2 A: Least squares linear regression analysis of zinc measured colorimetrically on the Data Pro analyser compared to the flame atomic absorption method. The regression line for the analysis was: \( y = 0.77x + 4.0 \text{ μmol/L} \) (\( r = 0.4376 \), \( n = 48 \), \( P < 0.01 \)). Dashed lines represent the upper and lower reference range limits of 20 μmol/L and 10 μmol/L respectively.

B: Bland and Altman analysis of zinc comparison data: correlation \( r = 0.1976 \) (\( P = 0.18 \)), slope = 0.1807 (\( P = 0.18 \)), intercept = -1.922 (\( P = 0.33 \)).
Figure 4.3 A: Least squares linear regression analysis of zinc measured colorimetrically on the Cobas Mira analyser compared to the flame atomic absorption method. The regression line for the analysis was: $y = 0.76x + 6.6 \mu\text{mol/L}$ ($r = 0.2541$, $n = 15$, $P = 0.06$). Dashed lines represent the upper and lower reference range limits of 20 $\mu\text{mol/L}$ and 10 $\mu\text{mol/L}$ respectively.

B: Bland and Altman analysis of zinc comparison data: correlation $r = 0.4379$ ($P = 0.10$), slope = 0.5294 ($P = 0.10$), intercept = -4.074 ($P = 0.37$).
Table 4.3: Results from Bland and Altman analysis

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Correlation r</th>
<th>P</th>
<th>Slope</th>
<th>P</th>
<th>Intercept</th>
<th>P</th>
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<tbody>
<tr>
<td>Data Pro vs FAAS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copper</td>
<td>48</td>
<td>0.6999</td>
<td>&lt;0.01</td>
<td>-0.2499</td>
<td>&lt;0.01</td>
<td>3.219</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Zinc</td>
<td>48</td>
<td>0.1976</td>
<td>0.18</td>
<td>0.1807</td>
<td>0.18</td>
<td>-1.922</td>
<td>0.33</td>
</tr>
<tr>
<td>Cobas Mira vs FAAS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>15</td>
<td>0.4379</td>
<td>0.10</td>
<td>0.5294</td>
<td>0.10</td>
<td>-4.074</td>
<td>0.37</td>
</tr>
</tbody>
</table>
4.4 Discussion

4.4.1 Copper

The Bland and Altman analysis of the copper data (Figure 4.1B) determined that a statistically significant negative systematic bias and a positive fixed bias affected the colorimetric copper method, as suggested by the initial linear regression of the comparison data (Figure 4.1A). The spread of data points across a reasonably wide range of copper concentrations enabled the analysis to reveal these biases between the colorimetric and FAAS methods leading to the conclusion that the colorimetric method was unsuitable for our purposes.

4.4.2 Zinc

A major issue with our zinc data was that it only covered about half the concentration range of the corresponding copper data; the zinc data ranged from approximately 10 - 19 µmol/L whereas the copper data ranged from about 9 - 37 µmol/L. The smaller range for zinc meant that the systematic bias equivalent to 77% of the FAAS result and a fixed positive bias of 4.0 µmol/L, as suggested by the linear regression equation associated with the data plotted in Figure 4.2A, was lost when the Bland and Altman study was conducted. We confirmed that this kind of effect could be replicated in the corresponding copper data when only copper data points between 10 and 19 µmol/L were subjected to Bland and Altman analysis; the P value of the regression in that modified analysis then rose to insignificance at P = 0.15. We therefore made the decision that on the basis of the linear regression equation for the data in Figure 4.2A there was enough statistical evidence of systematic and fixed biases between the two zinc methods as to make the colorimetric method unsuitable for our purposes for
research measuring zinc levels in a population suspected of having lower than normal levels.

4.4.3 Calibration procedures

When multipoint calibration procedures were trialled for both methods rather than the supplied single aqueous standard, the comparison with the atomic absorption methods appeared to improve slightly but did not eliminate the sizeable differences evident between the methods. This suggested that the supplied aqueous standards probably did not compensate for the matrix effect of serum in the colorimetric analysis. Serum calibrators, which have since been recommended by Randox, but are not listed in the standard package inserts, would most likely improve assay performance; in particular for the copper method which in our data exhibited statistically significant systematic bias when compared with FAAS, though it seems likely the zinc method would also improve similarly. The two methods evaluated however, also appear to suffer from substantial random error when performed on the Data Pro analyser; the magnitude of which did not seem entirely attributable to the pipetting imprecision of the Data Pro, but rather may have resulted from individual patient specific random interference.

4.4.4 Decision on analytical method

The overall performance of the two colorimetric methods was such that even with some improvements to the standardisation protocol they do not appear to allow reliable determination of copper or zinc in a complex matrix such as human serum using the Data Pro analyser. Given the intention of the present research was to determine the trace element status of a population and identify groups within that
population at risk of low copper or zinc status, the performance characteristics of the
colorimetric assays were such that these methods were deemed to be inappropriate
choices for this research. In order to ensure the reliability of the data collected, the use
of colorimetric methods was abandoned and all further analyses of copper and zinc
were performed using the flame atomic absorption method as described in this chapter
and the general methodology chapter (Chapter 3).
Chapter 5

Pilot study of trace element status in northern Tasmania

5.1 Introduction

The selenium content of foods consumed by human populations is dependent on the selenium content of soils in which the food is grown. Selenium, like other trace elements may be removed from soils by leaching and the effects of glaciation, or have reduced bioavailability due to acidification of soils as a result of common agricultural practices (Judson and Reuter 1999). Selenium deficiency in livestock in Tasmania (Mason 2007), particularly in sheep, where it causes white muscle disease and decreased fertility, indicates that soil content and bioavailability of selenium is most likely low in Tasmania. This has led to the hypothesis that human populations in this state may be at risk of marginal deficiency for this essential trace element.

With mounting evidence of the importance of selenium for maintaining good health via its involvement in antioxidant protection, thyroid metabolism, immune function, cell signalling, reproduction (Rayman 2000; Gromer et al. 2005; Moghadaszadeh and Beggs 2006), and a potential role in preventing chronic disease such as cancer (Whanger 2004), combined with the high incidence of chronic disease in an aging Tasmanian population (A.B.S. 2006a), research to determine the potential susceptibility is warranted.

A pilot study was designed which drew subjects from an established database of individuals from across northern Tasmania, who had taken part in other studies previously carried out in conjunction with the University of Tasmania, and hence were easily accessible to the researchers for the recruitment into the current study. It
was considered that the collection of a convenience sample was appropriate to allow timely and efficient recruitment of subjects and provide reliable indications of the viability and requirements of a larger study. The nature of the database from which many subjects were recruited also opened up further opportunity to investigate a secondary but related issue.

The University of Tasmania study, 'The Influence of Diet, Health and Lifestyle Factors on Clinical Disease Development in the Tasmanian Population with Haemochromatosis Genes'; designed to assess the factors involved in development of disease in those with hereditary haemochromatosis, a disease common in Tasmania, created a database of recently diagnosed individuals. Included in this database with symptomatic (iron overloaded) subjects with abnormal *HFE* genotypes (C282Y and H63D), were asymptomatic subjects with abnormal *HFE* genotypes and other control members in which abnormal *HFE* genotypes and iron overload symptoms were absent. It was the control and asymptomatic component of the database which formed the sample for the pilot study of selenium (and other trace element) status to determine the area of interest for a larger population study. The inclusion of subjects with abnormal *HFE* genotypes (both symptomatic and asymptomatic) would allow the study, secondarily, to test the hypothesis that the *HFE* genotype and the increased intestinal iron absorption associated with such genotypes, could cause alterations in the status of other trace elements such as selenium, copper or zinc.

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5.2 Study aims

The primary aim for this preliminary study was;

- to investigate the selenium status of a healthy convenience sample, including the measurement of dietary selenium intakes and biomarkers of selenium status such as serum selenium and selenoprotein activity.

Secondary aims were;

- to conduct an opportunistic study to assess status of two other important trace elements, copper and zinc, in this convenience sample, including the measurement of dietary intakes and biomarkers such as serum copper and zinc,

- to investigate the potential effect of hereditary haemochromatosis and the related increase in iron absorption on the status of selenium, copper and zinc.

Research conducted to achieve the primary aim, and two secondary aims as outlined here will be presented consecutively in this chapter as three separate studies:

Study A: Selenium status in a convenience sample from Northern Tasmania
Study B: Copper and zinc status in a convenience sample from Northern Tasmania
Study C: Effect of increased iron absorption in hereditary haemochromatosis on trace element status
5.3 Methods

All methods for data collection and handling, including all dietary and biochemical analyses, and statistical analysis performed for the pilot study of selenium, copper and zinc status referred to in this chapter, were described in Chapter 2: General methodology.

5.3.1 Subject recruitment

Participants in studies A, B and C (n = 198) were permanent residents of urban and rural areas in the north and north western region of Tasmania, Australia.

Recruitment was initially from the database of the study, ‘The Influence of Diet, Health and Lifestyle Factors on Clinical Disease Development in the Tasmanian Population with Haemochromatosis Genes’. A total of 161 potential participants from the haemochromatosis database were contacted via mail, of which 117 provided positive responses. The information letter provided an introduction to the study and instructions for interested persons to complete a brief diet-based questionnaire, to be returned by mail, and present to pathology to provide a blood sample within 4 weeks. Those choosing not to participate generally provided significant reasons such as ill health or having moved from the area. The convenience sample was added to, by recruiting from other studies at the School of Human Life Sciences, University of Tasmania (Launceston, Tasmania). Subjects provided fasting blood samples, prior to any treatment phases in other studies, at various public and private pathology laboratories, or at the School of Human Life Sciences.

Recruitment and analysis for all three studies occurred concurrently. Subjects were considered abnormal and excluded from study A and B if they had abnormal iron
status (serum ferritin >300 μg/L and 200 μg/L in men and women respectively). For study C, all subjects were included and classified by ferritin levels and \textit{HFE} genoty
5.4 Study A: Selenium status of a convenience sample in Northern Tasmania

5.4.1 Background

Despite problems with selenium deficiency in livestock and a history of human deficiency of another important trace element, iodine, data relating to selenium status (and other trace elements) in Tasmania is sparse; no study of selenium status in the north of the state has occurred to date. The two previous Tasmanian studies on selenium status have had limited scope, used different methodologies and provided conflicting results; the study of McGlashan and co-workers (1996) reported a relatively low mean selenium level of 1.02 ± 0.23 μmol/L in 171 female subjects while the most recent study found comparatively high selenium status (mean plasma: 1.39 ± 0.24 μmol/L) in the south of the state (Jacobson et al. 2007). Both were undertaken in the south of the state centred on Hobart. Neither study included detailed dietary data or measures of functional biomarkers, such as GPx, the latter acknowledged for its value in assessing potentially marginal populations.

There is no data from the north of the state. A study using a convenience sample from the northern and north west regions of Tasmania with a population over 120,000 or approximately 25% of the Tasmanian population was thus conducted, using detailed dietary and biomarker assessment.
5.4.2 Results

Anthropometric data

Study subjects had a mean ± sd age of 54.2 ± 13.3 yrs and tended to be slightly over
weight with a mean BMI of 26.4 ± 4.3 (Table 5.1). Of the 160 subjects, 19 (12%) were current smokers. Men were significantly taller and heavier than women (both P < 0.001), but there was no significant difference in age or body mass index.

Dietary Intake

The FFQ analysis indicated men consumed significantly more energy than women (P < 0.001). Men also consumed significantly greater amounts of selenium (P = 0.004). The mean estimated dietary intake of selenium for both men (75.5 ± 25.3 μg/d) and women (63.5 ± 24.7 μg/d) was above the Australian EAR and RDI for selenium (60 and 70 μg/day respectively for men; 50 and 60 μg/day respectively for women). By these estimates over one quarter (28% of both men and women) consumed less than the EAR value, and around a half (48% and 53% for men and women respectively) consumed less than the RDI.

Adjusted for bodyweight, selenium intakes were similar for both men and women at approximately 1 μg/kg bodyweight. However, when intakes were adjusted for energy intake, women demonstrated a greater nutrient density, consuming significantly more selenium per MJ of energy (P < 0.001).

Men in the youngest age range (25-34 yrs) had the lowest mean selenium intake of 59 μg/d; significantly lower than men in the 35-44, 45-54 and 55-64 yrs age ranges (P < 0.001, P = 0.02 and P = 0.03).
Biochemical measures

The mean serum selenium concentration was 1.09 µmol/L, below the level associated with maximal GPx expression (~1.27 µmol/L), indicating that a substantial proportion of the study subjects had serum selenium levels below the value required for maximal selenoprotein expression. This was supported by the positive association between serum GPx activity and serum selenium concentrations (P = 0.022); the association between RBC GPx and serum selenium was also positive but was not statistically significant (P = 0.332). Eighty five percent of subjects had serum selenium levels below the threshold associated with maximal GPx activity. Only 2 subjects had serum selenium above 1.50 µmol/L, a level suggested to represent the threshold for chemopreventative action of selenium.

Selenium intake in absolute terms was not associated with serum selenium concentrations, but was positively associated with serum GPx activity (P = 0.048). When adjusted for body weight, selenium intake was not associated with serum selenium nor RBC GPx activity but was positively associated with serum GPx activity (P = 0.009). Adjusted for energy intake, dietary selenium was positively associated with serum selenium in women, and with serum GPx and RBC GPx activity in all subjects (P = 0.019, P = 0.046 and P = 0.008 respectively).

Serum selenium was similar for men of all age range; however women in the 75-84 yrs age range had significantly higher serum selenium (mean: 1.31 µmol/L) compared to women of all other age ranges, however there were only three women in this age range.
Other associations

Mean serum selenium, serum GPx activity and RBC GPx activity were higher in non-smoking subjects; however the differences were not statistically significant.

Serum selenium and RBC GPx activity were not significantly associated with serum TAS, serum lipids, or plasma glucose levels. Serum GPx was however positively associated with serum HDL-cholesterol (P<0.001) and negatively associated with serum triglycerides (P = 0.041).

### Table 5.1: Anthropometric, dietary and biochemical characteristics of subjects.

<table>
<thead>
<tr>
<th></th>
<th>All Subjects</th>
<th>Male</th>
<th>Female</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 160</td>
<td>n = 64</td>
<td>n = 96</td>
<td></td>
</tr>
<tr>
<td>Smokers, n</td>
<td>19</td>
<td>8</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>54.2 ± 13.4</td>
<td>(25 - 79)</td>
<td>54.8 ± 13.2</td>
<td>53.9 ± 13.4</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.4 ± 4.3</td>
<td>(18.8 - 45.7)</td>
<td>26.6 ± 3.5</td>
<td>26.4 ± 4.8</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>75.2 ± 14.1</td>
<td>(49 - 133)</td>
<td>83.6 ± 12.8</td>
<td>69.9 ± 12.2</td>
</tr>
<tr>
<td>Height, cm</td>
<td>1.69 ± 0.09</td>
<td>(1.48 - 1.93)</td>
<td>1.77 ± 0.07</td>
<td>1.63 ± 0.08</td>
</tr>
<tr>
<td>Energy, MJ</td>
<td>7.94 ± 3.21</td>
<td>(2.88 - 24.90)</td>
<td>9.85 ± 3.71</td>
<td>6.66 ± 2.02</td>
</tr>
<tr>
<td>Protein, g/d</td>
<td>89.5 ± 38.0</td>
<td>(31.9 - 305.6)</td>
<td>107.7 ± 4.7</td>
<td>75.2 ± 2.4</td>
</tr>
<tr>
<td>Carbohydrate, g/d</td>
<td>208.0 ± 87.0</td>
<td>(39.0 - 649.0)</td>
<td>243.3 ± 10.9</td>
<td>180.6 ± 5.8</td>
</tr>
<tr>
<td>Fibre, g/d</td>
<td>23.0 ± 9.3</td>
<td>(5.7 - 52.6)</td>
<td>25.8 ± 1.1</td>
<td>20.8 ± 0.8</td>
</tr>
<tr>
<td>Se intake, µg/d</td>
<td>68.2 ± 25.6</td>
<td>(24.2 - 212.2)</td>
<td>75.5 ± 25.3</td>
<td>63.4 ± 24.8</td>
</tr>
<tr>
<td>Se intake, µg/kg/d</td>
<td>0.94 ± 0.43</td>
<td>(0.33 - 3.86)</td>
<td>0.92 ± 0.36</td>
<td>0.95 ± 0.47</td>
</tr>
<tr>
<td>Se intake, µg/MJ/d</td>
<td>9.20 ± 3.22</td>
<td>(4.7 - 23.3)</td>
<td>8.12 ± 2.11</td>
<td>9.89 ± 3.60</td>
</tr>
<tr>
<td>Serum Se, µmol/L</td>
<td>1.09 ± 0.18</td>
<td>(0.67 - 1.96)</td>
<td>1.10 ± 0.17</td>
<td>1.08 ± 0.18</td>
</tr>
<tr>
<td>Serum GPx, U/L</td>
<td>849.6 ± 147.4</td>
<td>(398 - 1378)</td>
<td>857.4 ± 134.6</td>
<td>844.4 ± 155.8</td>
</tr>
<tr>
<td>RBC GPx, U/g Hb</td>
<td>46.7 ± 18.5</td>
<td>(11.2 - 98.9)</td>
<td>45.7 ± 17.2</td>
<td>47.4 ± 19.4</td>
</tr>
<tr>
<td>TAS, mmol/L</td>
<td>1.25 ± 0.14</td>
<td>(0.51 - 1.61)</td>
<td>1.31 ± 0.11</td>
<td>1.22 ± 0.14</td>
</tr>
</tbody>
</table>

Values are mean ± sd, (range)
Figure 5.1: Selenium intakes across subject age ranges. * Intakes for men 25-34 yrs significantly lower than men 35-44 yrs (P < 0.001), 45-54 yrs (P = 0.02) and 55-64 yrs (P = 0.03). Values are mean ± sd.
Figure 5.2: Serum selenium across subject age ranges. Dotted line indicates selenium level associated with GPx saturation; dashed line indicates selenium level associated with potential chemopreventative effect. *P < 0.001 Women 74-84 yrs significantly different compared to all other women. Values are mean ± sd.
5.4.3 Discussion

Dietary analysis for selenium indicated that the mean intake in this sample was above the Australian EAR and RDI values for this nutrient. The median intake was however below the RDI for both genders, and a large proportion of the study subjects actually failed to consume the EAR amount for selenium. Using the EAR cut-point method (Food and Nutrition Board 2000c) the prevalence of inadequacy of selenium in this population is estimated to be 28%. In Australia, FSANZ uses the US Food and Nutrition Board/Institute of Medicine definition of nutritional inadequacy as when >3% of a population has nutrient intakes below the EAR. By this definition, the sample from the current study has an inadequate intake of selenium.

In comparison to previous Australian intake estimates, mean intakes for men in the current study are lower than those reported by Fardy and co-workers (1989) and Reilly (1992) (96 µg/d and 89 µg/d respectively), but quite similar for women (63 µg/d and 59 µg/d). When compared to intake estimates from the 20th Australian Total Diet Survey (FSANZ 2003), men from the current study again consume considerably less; 0.92 µg/kg/d compared to 1.2-1.7 µg/kg/d. Women consumed slightly less than the bottom end of the range from the 20th ATDS, at 0.95 µg/kg/d compared to 0.96-1.18 µg/kg/d.

Differences in selenium intakes across age ranges is perhaps not unexpected, as it may be considered that consumption of selenium rich foods such as red meat may decrease with age, however the only significant difference observed was a lower intake in the youngest age range of men. This effect has been reported in other studies (Murphy et al. 2002), although with different dietary analysis methodology, and may be related to different consumption patterns in particular food groups; however data from the current study is not sufficient to make a firm conclusion.
Potentially a major issue in estimating dietary intakes of selenium is the dependency of food content upon the selenium content in soils where the food is grown. As a result there is an inherent inaccuracy in estimated intakes if food content data is not derived from the same source as much of the populations food supply. The NUTTAB 2006 database (FSANZ 2006) contains food content data from direct analyses of Australian foods, from estimations based on similar food types, and also from other food content databases from other countries, for foods where local data was not available, and hence may affect the accuracy of dietary estimations. The ability of the food frequency questionnaire to capture accurate estimations of dietary intake of specific nutrients, and the prevalence of under-reporting or over-reporting by study subjects are also factors that can influence such dietary analysis. In this case the FFQ has been validated (Hodge et al. 2000), but was not compared to a gold standard method as part of this study, for the estimation of selenium intake. Furthermore, the level of under or over reporting by subjects could not be estimated from the information we collected nor from biomarkers of intake now being used, such as doubly-labelled water or 24 hr urine nitrogen (Bingham 2002; Subar et al. 2003; Neuhouser et al. 2008). The use of biomarkers such as blood selenium and a functional marker such as GPx is therefore necessary to confidently assess selenium status in such a population, and was undertaken in this study.

Mean serum selenium in this study was substantially lower than the most recent Tasmanian data of 1.39 μmol/L (Jacobson et al. 2007) and that from South Australia (1.30 μmol/L) (Lyons et al. 2004), but similar to other published data from Tasmanian populations (1.02 μmol/L and 0.98 μmol/L) (McGlashan et al. 1996; Daniels et al. 2000) as well as older South Australian (1.11 μmol/L) and NSW data (1.16 μmol/L) (Dhindsa et al. 1998; Daniels et al. 2000). Globally, selenium levels were similar to
those in recent French (1.09 μmol/L for women; 1.14 μmol/L for men) and British studies (1.13 μmol/L) (Arnaud et al. 2006; Sunde et al. 2008), where there has been recent concern regarding falling selenium intakes. Selenium levels were similar to those reported for the North Island of New Zealand (1.08 μmol/L) but higher than those from the South Island (0.84 – 0.98 μmol/L) where low selenium intakes are well documented (Thomson 2004b). A trend seen in other studies of a decrease in serum selenium levels in the elderly (Brooks et al. 2001; Bates et al. 2002) is observed in male subjects in the current study, but not in females; however this may be a result of the low female subject numbers in this age range.

The serum selenium data, in conjunction with the finding of a positive association with serum GPx indicates that, as suggested by the dietary data, the population may have an inadequacy for selenium. This inadequacy is not overt; it is not of a level that would be expected to result in the classical deficiency disease Keshan disease, but rather a moderate inadequacy that may be associated with impaired selenoprotein activity, potentially affecting their many important roles in immune function, thyroid metabolism, reproduction and antioxidant protection (Rayman 2002).

Also of interest is the failure of most subjects to reach a serum selenium of 1.50 μmol/L, a level postulated to be the threshold where chemopreventative effects against some cancers may occur (Combs 2001). This is potentially a significant finding in light of the high rates of chronic disease observed in the aging population of Tasmania. As observed elsewhere (Suadicani et al. 1992; Gamez et al. 1997; Bleys et al. 2008), selenium status was also associated with cardiovascular risk factors such as HDL-cholesterol and triglycerides, which perhaps further reflects the relationship between selenium status and other health factors. It must be noted, however, that numerous previous studies have failed to find associations between low selenium and
cardiovascular disease (Alissa et al. 2003; Navas-Acien et al. 2008), but recent work has observed some associations between higher selenium status and increased risk of diabetes and hyperlipidaemia (Bleys et al. 2007; Stranges et al. 2007; Lippman et al. 2009; Stranges et al. 2009), although mostly at higher selenium concentrations than generally observed in Australian populations.

The preliminary findings from this pilot study indicate that a more representative population study is warranted to assess the extent of marginal selenium status in the Tasmanian population.
5.5 Study B: Copper and zinc status in a convenience sample from Northern Tasmania

5.5.1 Background

The trace elements copper and zinc are essential for numerous vital metabolic processes in humans through the influence of many hundreds of metalloproteins which contain these metals. The effects of mild copper, and in particular zinc deficiency, have received greater interest as associations with growth, immune function, wound healing, fertility and cardiovascular and bone health have become more established (Shankar and Prasad 1998; Hambidge 2000; Lansdown et al. 2007; Prasad 2008; Merrells et al. 2009). Marginal zinc deficiency has emerged as a serious public health concern and is suggested to affect approximately 20% of the world’s population (Sanghvi et al. 2007).

There is a paucity of published data from Australian populations regarding copper and zinc status.

Due to a relative lack of comprehensive data world-wide regarding requirements for copper, the NHMRC has not established an RDI value for this element, opting instead to provide an AI value. To date, the only large study of copper status in Australia reported dietary copper intakes in women only, residing in the Barwon area of Victoria (Cleverdon and Ball 2004). At this point there is no published data on copper intake or status in Tasmanian populations.

Zinc status in Australia has received more interest, with dietary estimates reported from comparisons between smokers and non-smokers, and in vegetarian and non-vegetarians (English et al. 1997; Ball and Ackland 2000). The 20th Australian Total Diet Survey (FSANZ 2003) also reported estimated zinc intakes in Australia.
However the only study to provide Tasmanian data was the 1995 National Nutrition Survey (NNS) (McLennan and Podger 1995) which estimated the dietary intake of 13,858 subjects in total, and reported estimated intakes in Tasmanian men and women that were both lower than the national average.

The small study of vegetarians versus non-vegetarians (total n = 42) by Ball and Ackland (2000) appears to be the only published Australian study to measure blood zinc levels in healthy adults.

This pilot study will therefore investigate the status of two important trace elements for which there is currently very little data not only in Tasmania but elsewhere in Australia.
5.5.2 Results

Anthropometric data

The sample of 62 men and 93 women had a mean age of 54.1 ± 13.5 yrs and BMI of 26.4 ± 4.3 kg/m\(^3\). A total of 19 (12%) of subjects were current smokers. Men were significantly taller and heavier than women (P < 0.001).

Copper

Men had significantly higher energy intakes compared to women (P < 0.001), and also consumed greater amounts of copper (mean 1.50 v. 1.26 mg/d; 95%CI of difference 0.09-0.40; P = 0.002). Adjusted for bodyweight, copper intake remained higher in men (P = 0.002) but when intakes were adjusted for energy, women were observed to have a greater nutrient density (0.19 v. 0.16 mg/MJ/d; 95%CI of difference 0.02-0.05; P < 0.001). Intakes for each gender within 6 age ranges were not significantly different; however, the lowest mean intake for both genders was observed in the youngest age range (25-34 yrs), whilst the highest mean intakes were in the 35-44 yrs and 74-85 yrs age ranges for men and women respectively.

Mean intakes for men were below the Australian Al (1.7 mg/d) whilst for women were just above the AI (1.2 mg/d); thus nearly three quarters of men (72%) and one in two women (51%) consumed less than the AI value for copper.

Despite lower intakes in absolute terms and by kg/bodyweight, serum copper was considerably higher in women (mean 19.0 v. 15.5 μmol/L; 95%CI of difference 2.5-4.6; P < 0.001). Serum copper in the oldest age range in women (74-85 yrs) was significantly lower than all other age ranges of women (P < 0.05). The highest mean copper in women was in the 25-34 yrs age range.
was not statistically significant in men; the highest mean serum copper was in the 25-34 yrs age range; the lowest mean serum copper was in the 35-44 yrs age range.

### Table 5.2: Anthropometric, dietary and biochemical characteristics of study subjects.

<table>
<thead>
<tr>
<th></th>
<th>All Subjects</th>
<th>Male</th>
<th>Female</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 155</td>
<td>n = 62</td>
<td>n = 93</td>
<td></td>
</tr>
<tr>
<td>Current smoker, n</td>
<td>19</td>
<td>8</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>54.1 ± 13.5</td>
<td>55.1 ± 13.3</td>
<td>54.2 ± 13.3</td>
<td>0.624</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.4 ± 4.3</td>
<td>26.7 ± 3.5</td>
<td>26.3 ± 4.8</td>
<td>0.560</td>
</tr>
<tr>
<td>Height, m</td>
<td>1.68 ± 0.10</td>
<td>1.77 ± 0.07</td>
<td>1.63 ± 0.08</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>75.3 ± 14.3</td>
<td>83.9 ± 12.9</td>
<td>69.8 ± 12.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Energy, MJ</td>
<td>7.83 ± 2.91</td>
<td>9.60 ± 3.17</td>
<td>6.66 ± 2.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cu intake, mg/d</td>
<td>1.36 ± 0.48</td>
<td>1.50 ± 0.51</td>
<td>1.26 ± 0.44</td>
<td>0.002</td>
</tr>
<tr>
<td>Cu intake, μg/kg/d</td>
<td>18.7 ± 6.7</td>
<td>20.7 ± 7.1</td>
<td>17.3 ± 6.7</td>
<td>0.002</td>
</tr>
<tr>
<td>Cu intake, μg/MJ/d</td>
<td>0.18 ± 0.05</td>
<td>0.16 ± 0.04</td>
<td>0.19 ± 0.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Zn intake, mg/d</td>
<td>11.3 ± 3.7</td>
<td>12.8 ± 4.2</td>
<td>10.3 ± 2.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Zn intake, μg/kg/d</td>
<td>155.5 ± 50.4</td>
<td>176.4 ± 58.2</td>
<td>141.8 ± 39.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Zn intake, μg/MJ/d</td>
<td>1.51 ± 0.38</td>
<td>1.38 ± 0.28</td>
<td>1.60 ± 0.42</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum Cu, μmol/L</td>
<td>17.6 ± 3.9</td>
<td>15.5 ± 2.6</td>
<td>19.0 ± 4.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum Zn, μmol/L</td>
<td>13.4 ± 2.7</td>
<td>13.3 ± 2.4</td>
<td>13.4 ± 2.9</td>
<td>0.831</td>
</tr>
<tr>
<td>RBC SOD, U/g Hb</td>
<td>1246 ± 329</td>
<td>1245 ± 309</td>
<td>1247 ± 342</td>
<td>0.960</td>
</tr>
<tr>
<td>Serum TAS, mmol/L</td>
<td>1.25 ± 0.14</td>
<td>1.31 ± 0.11</td>
<td>1.22 ± 0.14</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are mean ± sd, (range)
Figure 5.3: Dietary copper intakes and serum copper across subject age ranges. *Serum copper in women 75-84 yrs was significantly lower than women of all other age ranges (P<0.05). Values are mean ± sd.
Zinc

Zinc intakes followed the same pattern as for copper; absolute intakes were significantly higher in men (12.8 v. 10.3 mg/d; 95%CI of difference 1.3-3.7; P < 0.001), as were intakes adjusted for subject bodyweight (176.4 v. 141.8 µg/kg/d; 95%CI of difference 18.1-51.2; P < 0.001). Again, when intakes were adjusted for energy, women had significantly higher nutrient density compared to men (1.60 v. 1.38 µg/MJ/d; 95%CI of difference 0.11-0.33; P < 0.001). As well as higher energy intakes, men also consumed an average of 33 g more protein (44%) than women (95%CI of difference 21-45; P < 0.001).

Mean zinc intakes were lowest in the 25-34 yrs age range for both genders; significantly lower than intakes in the 35-44 yrs age range for men (P < 0.001) and women (P = 0.036). One half of men (50%) consumed less than the EAR (12 mg/d) while only 8% of women failed meet the EAR (6.5 mg/d) for zinc. The prevalence of low zinc intakes in men was high (>40%) in all age ranges, excluding 35-44 yrs where only one of eleven subjects consumed less than EAR. The 35-44 yrs and 75-84 yrs age ranges in women had no subjects with low zinc intakes, while the other four age ranges had low proportions of subjects (4% -11%) with inadequate intakes.

When combined, the total proportion of subjects consuming less than the EAR of zinc was 25%. The proportion below EAR was high (33%) in the youngest age range (25-34 yrs), but dropped to 5% in the next age range (35-44 yrs) and then increased with age (19%, 26%, 36% and 33% in the 45-54, 55-64, 65-74 and 75-84 yrs age ranges respectively).
Figure 5.4: Dietary zinc intakes and serum zinc across subject age ranges. *Intakes in men 25-34 yrs were significantly lower than men in 35-44 yrs age range (P < 0.001); †Intakes in women 25-34 yrs were significantly lower than women in 35-44 yrs age range (P < 0.036). *Serum zinc in men aged 45-54 yrs was significantly higher than men 75-84 yrs (P = 0.039). Values are mean ± sd.
Although men consumed considerably more zinc on average, there was no significant difference in serum zinc between genders; mean ± sd serum zinc was 13.3 ± 2.4 μmol/L in men, and 13.4 ± 2.9 μmol/L in women. Age range differences in serum zinc were not significant in women; however men aged 45-54 yrs had significantly higher serum zinc than men aged 75-84 yrs (14.4 v. 12.1 μmol/L; 95%CI of difference 0.3-3.7; P = 0.039). The greatest proportion of men with serum zinc below the WHO morning/fasting cut-off of 11.3 μmol/L was in the younger (33% and 17% in 25-34 and 35-44 yrs age group respectively) and oldest age groups (33% in 74-84 yrs age group). In women, no subjects aged 25-34 yrs had serum zinc below the 10.7 μmol/L cut-off, but the proportions increased steadily with each successive age group, peaking in the oldest age group (11%, 13%, 14% 22% and 33%).

Other associations

The significant effects of age and gender on indices of copper and zinc status meant that analyses between copper and zinc and measures of antioxidants and risk factors for cardiovascular disease (eg. serum cholesterol, serum HDL-cholesterol) were adjusted for age and gender.

Dietary copper was not associated with antioxidant or cardiovascular markers including GPx, TAS, lipids and glucose. Serum copper however, was positively associated with both serum cholesterol (P = 0.017) and serum LDL-cholesterol (P = 0.026).

Dietary zinc intake was negatively associated with serum HDL-cholesterol (P = 0.028) and positively associated with serum LDL-cholesterol (P = 0.010), however serum zinc was not significantly associated with antioxidant or cardiovascular markers.
5.5.3 Discussion

Copper

The lack of EAR values for copper complicates the assessment of the adequacy of a population's intake. The US Food and Nutrition Board/Institute of Medicine (2000c) reports that the EAR cut point method is a reliable means of assessing likely nutrient inadequacy in a population; unfortunately this method is not suitable for use with an AI value as used in Australia. An AI is used when data is considered insufficient to set an EAR, and is an amount generally expected to meet or exceed the requirements of a healthy population.

The AI for copper in Australia, determined by the National Health and Medical Research Council (NHMRC), was based on median population copper intakes reported in an Australian Bureau of Statistics (ABS) national nutrition survey (NHMRC 2006). Results from the current study suggest that the dietary intake of the female subjects compare reasonably with national intakes given the median copper intake (1.19 mg/d) was just below the AI of 1.20 mg/d. Although definitive statements cannot be made from assessments using the AI, in this instance, where the mean intake is slightly above the AI, it is suggested that the prevalence of inadequate intakes is likely to be low (Food and Nutrition Board 2000c).

As approximately three quarters of male subjects consumed less than AI amounts of copper, male intakes in this sample appear to be lower than those in the national survey data used by the NHMRC in setting the AI. This, however, does not necessarily indicate increased risk of inadequacy as an AI value should be above the true EAR, and therefore any attempt to estimate the prevalence of inadequacy using the AI would be expected to overestimate the true prevalence. In fact, in comparison
to the U.S EAR value of 0.7 mg/d, no male subjects and just six females from this current study consumed inadequate amounts of copper. A similar comparison of Australian nutrition survey data made by FSANZ (2005) estimated that no gender or age groups consumed less than the US EAR. As it stands, and until the adoption of an EAR value in Australia, assessment of adequacy of copper intakes will be made with a degree of uncertainty due to the lack of clear and accepted intake requirements.

Studies of copper intake in Australia are limited. One of the few published studies assessed a sample of women residing in Victoria (Cleverdon 2003). The women consumed on average 1.56 mg/d, an amount only approached by the oldest age group (75-84 yrs) of the current study (1.48 mg/d), but much higher than the lowest age group intake of 1.05 mg/d (25-34 yrs). As observed in the current study, Cleverdon also found intakes increased with age, with young women most at risk of low copper intakes, and suggested dietary patterns in the younger women, which included greater consumption of refined foods and “takeaway” dishes as a likely reason.

Copper intakes have also been estimated as part of the multi-country Total Diet Study (Parr et al. 2006). In this study, direct analysis of duplicate diets from six Australian cities was used and a median intake of 1.82 mg/d was reported; again considerably higher than the intakes estimated in this current study. Unfortunately no demographic data from the surveyed sample was provided.

The 20th Australian Total Diet Survey (FSANZ 2003) reported mean dietary exposures to “pesticide residues, contaminants and other substances” which included copper. However only data for men and women 25-34 yrs was provided, with mean intakes per kg body weight estimated to be 16 µg/kg/d and 14 µg/kg/d for men and women respectively, which compares very well with estimated intakes for this age group of 16.1 µg/kg/d and 14.4 µg/kg/d respectively.
The comparison between the current intake estimates and those from previous studies is obviously complicated by the use of different methodology in each case. As well as some weaknesses in FFQs that have been discussed previously (Byers 2001; Kristal et al. 2005), the use of food content data that is, to a significant degree, not derived from the food sources for the target population is likely to affect the accuracy of the dietary intake estimates, and must be considered in all comparisons. The degree of under and over reporting by study subjects is another factor affecting such analyses but was not able to be estimated in this study.

The higher serum copper in women of most ages compared to men (Figure 5.3) was not unexpected. Female hormones, including those in oral contraceptives, have been associated with higher serum copper (Johnson et al. 1992; Louro et al. 2001). The influence of hormones is possibly related to the decrease of serum copper in women with age, where despite an apparent increase in dietary intakes, it would coincide with likely lower circulating hormone levels which would be expected particularly in the post menopausal women in the older age ranges. Serum copper in men, in contrast to women, was relatively stable across all age ranges. Differences in serum copper between men and women of each age range decrease with increasing age; the only age range where women do not have higher mean serum copper is in the 75-84 yrs age range; possibly due to the lack of hormone influence in this age range.

At this point there is not an accepted cut-off value for the estimation of copper inadequacy using any biochemical measure, including serum copper, despite suggestions that it remains the most reliable indicator of copper status, particularly in copper depleted individuals (Harvey et al. 2009). In the absence of such cut-offs, comparison with the clinical reference range of 11 – 22 µmol/L is the only method that may be employed at this time. In this study, only 3 men and no women had serum
copper below the reference range lower limit of 11 μmol/L. By this comparison, inadequacy of copper in this sample appears unlikely.

Zinc

The assessment of zinc status is a simpler task due to the adoption of EAR values for dietary intakes by the NHMRC (2006), and WHO lower cut-off values for serum zinc (Gibson et al. 2008).

The WHO considers the risk of zinc deficiency to be elevated when the prevalence of sub-EAR intakes in a population is >25% (de Benoist et al. 2007). In this study one quarter of subjects consumed low zinc intakes, however a far greater proportion of men had intakes <EAR compared to women (50% v. 8%). Zinc intakes in men were reasonably constant across the age ranges and this was reflected in the proportions of each age consuming <EAR; prevalence of low intake dropped below 40% only in the 35-44 yrs age range where overall energy intake was considerably higher than other men. This pattern was not evident in women; however, the 35-44 and 75-84 yrs age ranges which had no subjects with <EAR intakes, did have higher zinc density in their diets compared to other age ranges.

The WHO considers the risk of zinc deficiency to be elevated when the prevalence of inadequate intakes in a population is >25% (de Benoist et al. 2007). Given the overall prevalence in this sample was 25%, and using the WHO guideline, the risk of deficiency is borderline. If the genders were considered as separate sub-populations, it is clear that men, but not women, would be considered to be at risk of deficiency.

Zinc intake estimates for men in the current study were similar to the only other Tasmanian data published to date. The National Nutrition Survey (NNS) of 13,858 subjects >19 yrs, reported estimated intakes of 13.5 mg/d for men; lower than the
national average of 14.4 mg/d (McLennan and Podger 1995). Men in the current study had lower intakes (by 15% and 8% respectively) than those reported in large studies by English et al (1997) and the 20th ATDS (FSANZ 2003). Women, in contrast, consumed similar intakes as estimated by English and colleagues’ study (10.3 v. 10.5 mg/d), but consumed 13% more zinc than Tasmanian women in the NNS and 23% more than estimated in the 20th ATDS. The variations observed may reflect actual eating habit differences, but may also be a result of methodological differences or changing food nutrient content which has been suggested by several researchers with regard to other trace elements (Klevay and Medeiros 1996; Lyons et al. 2004). These possible influencing factors make further comparisons inappropriate.

The lack of difference in serum zinc between genders despite significantly different zinc intakes is most likely due to differences in zinc loss between men and women (Brown et al. 2004), but may also be influenced by variations in zinc absorption caused by different dietary patterns in men and women.

In comparison to the WHO cut-offs for serum zinc, overall 12% of subjects were below the respective serum zinc cut-offs. From this analysis, the sample as a whole would not be considered to be at increased risk of zinc deficiency. Analysis of gender and age sub-groups resulted in low sub-group subjects numbers but nevertheless indicated an interesting trend. Ten percent of all men and 14% of women were below the WHO cut-off for zinc. While the analysis of the male age ranges was certainly hindered by low subject numbers, the distribution of men below the cut-off was relatively even across the age ranges, however in women, where there were greater sub-group sizes, a greater proportion (18%) of women ≥ 55 yrs fell below the respective cut-off level. This is despite a trend of increased zinc intakes with age in
women. This may be explained by the decrease in protein intake observed in these age ranges, a factor which is known to decrease zinc absorption (Brown et al. 2004).

The discrepancy between the proportions of men consuming inadequate zinc and those with low serum zinc concentrations was reasonably large. As discussed earlier, there is certainly potential for inaccuracy, including the possibility of under-reporting, in the FFQ methodology as used for estimating zinc intakes in this study. However, assuming the methodology was sound, it may be that at least for the men in this population, the EAR is higher than required, resulting in a large proportion of men being considered to consume inadequate zinc, with much fewer actually having low serum zinc concentrations. This could explain why the discrepancy was only evident in men, while methodological problems would generally be expected to affect both genders.

**Associations with cardiovascular risk factors**

Copper has previously been associated with cardiovascular risk factors and the development of atherosclerosis and cardiovascular disease. Copper has been found to be inversely associated with cardiovascular risk factors such as BMI, total cholesterol and LDL-cholesterol, directly associated with HDL-cholesterol levels, and considered important for cardiovascular health (Ford 2000; Klevay 2000), however inverse associations with cardiovascular disease risk has been suggested by some studies (Ford 2000; Leone et al. 2006).

In the current study the association between copper indices with cardiovascular risk factors reached statistical significance in only a few cases. The positive association of serum copper with both serum cholesterol and LDL-cholesterol is in contrast to findings of inverse relationships between copper status and such markers (Ghayour-
Mobarhan et al. 2005; Stern et al. 2007), as well as the association of hypocupremia with hypercholesterolemia and decreases in serum HDL-cholesterol observed in copper depleted subjects (Stern et al. 2007). They were in line, however, with findings of positive associations of similar copper levels with LDL-cholesterol and serum cholesterol (Salonen et al. 1991; McMaster et al. 1992; Abiaka et al. 2003). Such findings suggest that lipid metabolism might be disturbed by both low and moderately high copper status, which may be significant in a population such as that in Tasmania which experiences high rates of cardiovascular disease.

To date there have been no consistent associations between zinc status and cardiovascular risk factors and cardiovascular disease incidence. There have been some reports of high zinc intakes resulting in decreased serum HDL-cholesterol (Hughes and Samman 2006), however at this point the significance of any relationship between zinc and serum lipids is uncertain. Although Cu-Zn SOD has been suggested as a potential biomarker for copper status, in this sample the lack of association between other copper indices and SOD suggests SOD activity may not be a useful indicator in this population.

Despite the limitations of a convenience sample, the findings from this study, and particularly given the paucity of Australian data related to this topic, provide some useful indicators in determining groups which may potentially be at risk of inadequate zinc intakes.
5.6 Study C: Effect of increased iron absorption in hereditary haemochromatosis on trace element status

5.6.1 Background

Hereditary haemochromatosis

Hereditary haemochromatosis (HH) is the most common inherited autosomal recessive disease, affecting perhaps 1 in 200 in Caucasian populations (Merryweather-Clarke et al. 1997). The majority of people with HH have a mutation in the HFE gene that prevents correct folding of the protein product which is involved in the regulation of gastrointestinal iron absorption. A single base transition which results in a substitution of an amino acid gives rise to C282Y, the most important mutation. A similar substitution, H63D, is common, but of lesser clinical significance (Pietrangelo 2003). In susceptible people, unregulated iron absorption continues even when iron stores are sufficient and leads to an excessive accumulation of iron and eventually parenchymal cell damage in many organs (Trinder et al. 2002). Typically, high requirements for growth in adolescence and loss through menstruation in women, means that despite abnormal iron absorption, excess iron storage does not become a significant problem until middle age (Franchini and Veneri 2005).

Relationships between iron and other trace elements

There are some limited findings of inverse relationships between tissue selenium and iron levels in animals (Chareonpong-Kawamoto and Yasumoto 1995; Bartfay and Bartfay 2002) and direct relationships between serum selenium and markers of cellular iron depletion (Barany et al. 2005). The possible mechanism of such an association is not clear but would likely be related to regulation of body stores rather
than absorption, as selenium is mostly absorbed as selenomethionine or selenocysteine with other amino acids, whereas iron is predominantly absorbed as the inorganic cation Fe$^{2+}$ via a metal cation transporter. More data exists from studies regarding iron status and other trace elements; however findings to date are inconsistent.

A high rate of iron supplementation is known to result in lowered zinc (Peres et al. 2001) and copper absorption (Wapnir 1998); similar to the effect high zinc intakes have on copper absorption and is likely due to competition during absorption in the intestine. The Divalent Metal Transporter 1 (DMT1) is an important duodenal transporter of divalent metal cations such as Fe$^{2+}$, Zn$^{2+}$, Cu$^{2+}$, Cd$^{2+}$ and Mn$^{2+}$; DMT1 has a greater affinity for ferrous iron compared to other cations which may explain altered copper and zinc absorption in periods of high iron intake.

DMT1 activity is known to be increased in hereditary haemochromatosis (Garrick et al. 2003); this may result in increased absorption of other trace elements possibly explaining the increased tissue levels of non-ferrous metals such as zinc, manganese and lead that have been observed in haemochromatosis (Barton et al. 1998). Studies utilising experimental iron overload (in the absence of molecular defects) have also shown alterations and increases in body stores of zinc, cadmium and manganese suggesting that other regulatory processes for metal absorption may be altered in states of iron overload (Vayenas et al. 1998; Zhang et al. 2009).

The potential deleterious effects of imbalances in trace element status are reasonably well established but may be exacerbated in haemochromatosis given the increased oxidative stress experienced in iron overload conditions. As a study was being undertaken in northern Tasmania on trace element status, this provided the
opportunity to examine whether the gene or iron overload might influence the status of other trace elements such as selenium, copper or zinc
5.6.2 Results

Genotyping using the HFE-ARMS assay resulted in detection of six genotypes (Table 5.3); wild-type \textit{HFE} homozygote, H63D heterozygote, H63D homozygote, C282Y heterozygote, C282Y/H63D compound heterozygote and C282Y homozygote. Study subjects (n = 198) were classified by iron status, as determined by serum ferritin levels, gender and \textit{HFE} genotype producing three gender subgroups; Group 1 (normal \textit{HFE} genotype + normal iron status), Group 2 (abnormal \textit{HFE} genotype + normal iron status) and Group 3 (abnormal \textit{HFE} genotype + abnormal iron status) (Table 5.4). In this classification heterozygote H63D subjects were considered to have a normal \textit{HFE} genotype.

\begin{table}[h]
\centering
\begin{tabular}{lcccccc}
\hline
\textit{HFE} Genotype & Homozygous C282Y & Compound Heterozygous C282Y/H63D & Heterozygous C282Y & Homozygous H63D & Heterozygous H63D & Normal 'wild type' genotype \\
\hline
Male & 10 & 11 & 15 & 1 & 11 & 38 \\
Female & 14 & 13 & 14 & 0 & 23 & 47 \\
All subjects & 24 & 24 & 29 & 1 & 34 & 85 \\
\hline
\end{tabular}
\caption{Distribution of \textit{HFE} genotypes among study subjects.}
\end{table}
Table 5.4: Serum ferritin levels (μg/L) of study subgroups.

<table>
<thead>
<tr>
<th>Iron status/HFE genotype subgroup</th>
<th>Group 1 n = 119</th>
<th>Group 2 n = 42</th>
<th>Group 3 n = 37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>139.3 ± 78.9 (13-280)</td>
<td>155.0 ± 83.7 (12-288)</td>
<td>820.7 ± 547.1 (313-2430)</td>
</tr>
<tr>
<td>Female</td>
<td>71.1 ± 50.5 (7-195)</td>
<td>77.0 ± 54.1 (7-193)</td>
<td>656.9 ± 565.5 (246-2120)</td>
</tr>
</tbody>
</table>

Values are mean ± sd, (range)
Normal ferritin: Men 30-300 μg/L; Women 15-200 μg/L
Group 1 = normal HFE genotype and normal Fe status
Group 2 = abnormal HFE genotype and normal Fe status
Group 3 = abnormal HFE genotype and abnormal Fe status

Measurement of serum ferritin determined 119 subjects with normal HFE genotype had normal iron status; 42 subjects with abnormal HFE genotype with normal iron status and 37 subjects with abnormal HFE genotypes were considered to have abnormally high iron status, or iron overload.

Amongst the male subject subgroups there were no significant differences in age, energy intake or trace element intake. Body mass index did vary significantly among the male subgroups; men in the iron overloaded Group 3 had higher BMI (29.5) compared to both Group 1 (26.6) and Group 2 (26.5) men (P = 0.004 and P = 0.008 respectively). Among the women, BMI was not significantly higher in Group 3 subjects but this group was older on average (63.4 yrs) compared to Group 1 (53.5 yrs) and Group 2 (55.0 yrs) women (P < 0.001 and P = 0.007 respectively).

Although not significantly higher than other groups, the highest mean energy and trace element intakes were observed in the asymptomatic Group 2 men. In women the highest mean energy and trace element intakes were observed in the Group 3 subjects; however the differences between groups were not statistically significant.
Few significant differences were observed in the chosen trace element and antioxidant biomarkers. In men, mean serum levels of selenium were highest in Group 1 but highest mean copper and zinc levels were observed in Group 2 men. Mean total antioxidant status, serum GPx, and RBC SOD were higher also in Group 1 men, but only RBC GPx was significantly so; RBC GPx activity in Group 1 was 25% and 34% higher compared to Group 2 and Group 3 respectively (P = 0.03 and P = 0.001).

In women, only RBC GPx varied significantly; Group 2 activity was 24% lower than RBC GPx activity in Group 1 (P = 0.001) and 15% lower than Group 3 (P = 0.03). In contrast to men, the females in Group 3 (who had iron overload) had the highest mean serum selenium and copper levels, while Group 1 women had the highest mean serum zinc. Women in Group 3 also had the highest mean serum GPx activity, while RBC SOD activity was highest in Group 1 females.

Table 5.5: Age and dietary intakes in male and female subgroup subjects.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Age yrs</th>
<th>Energy MJ</th>
<th>Se intake µg/d</th>
<th>Cu intake mg/d</th>
<th>Zn intake mg/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>49</td>
<td>54.7 ± 13.4</td>
<td>9.43 ± 3.68</td>
<td>77.3 ± 40.1</td>
<td>1.53 ± 0.60</td>
<td>12.8 ± 5.8</td>
</tr>
<tr>
<td>Group 2</td>
<td>15</td>
<td>55.0 ± 12.7</td>
<td>11.27 ± 3.55</td>
<td>85.0 ± 27.1</td>
<td>1.59 ± 0.54</td>
<td>14.7 ± 3.9</td>
</tr>
<tr>
<td>Group 3</td>
<td>23</td>
<td>58.3 ± 10.8</td>
<td>9.72 ± 3.81</td>
<td>71.6 ± 30.7</td>
<td>1.33 ± 0.41</td>
<td>11.9 ± 3.9</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>70</td>
<td>53.5 ± 13.3</td>
<td>6.73 ± 2.08</td>
<td>63.5 ± 19.7</td>
<td>1.27 ± 0.38</td>
<td>10.3 ± 2.8</td>
</tr>
<tr>
<td>Group 2</td>
<td>27</td>
<td>55.0 ± 13.9</td>
<td>6.43 ± 1.89</td>
<td>63.3 ± 35.9</td>
<td>1.22 ± 0.59</td>
<td>10.1 ± 3.1</td>
</tr>
<tr>
<td>Group 3</td>
<td>14</td>
<td>63.4 ± 8.1†</td>
<td>7.52 ± 2.32</td>
<td>66.5 ± 24.7</td>
<td>1.58 ± 0.64</td>
<td>11.5 ± 4.1</td>
</tr>
</tbody>
</table>

Group 1 = normal HFE genotype and normal Fe status
Group 2 = abnormal HFE genotype and normal Fe status
Group 3 = abnormal HFE genotype and abnormal Fe status
†indicates significantly different (p < 0.05) compared to women in other groups
Table 5.6: Serum and red cell trace element and antioxidant indices for male and female subgroup subjects.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Serum Se μmol/L</th>
<th>Serum Cu μmol/L</th>
<th>Serum Zn μmol/L</th>
<th>Serum TAS mmol/L</th>
<th>Serum GPx U/L</th>
<th>Red Cell GPx U/g Hb</th>
<th>Red Cell SOD U/g Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>49</td>
<td>1.11 ± 0.18</td>
<td>15.3 ± 2.7</td>
<td>13.2 ± 2.4</td>
<td>1.31 ± 0.11</td>
<td>877.7 ± 113.6</td>
<td>47.8 ± 17.7†</td>
<td>1250.9 ± 317.7</td>
</tr>
<tr>
<td>Group 2</td>
<td>15</td>
<td>1.09 ± 0.14</td>
<td>16.0 ± 2.1</td>
<td>13.8 ± 2.4</td>
<td>1.31 ± 0.09</td>
<td>791.3 ± 176.5</td>
<td>38.2 ± 13.3</td>
<td>1223.7 ± 287.8</td>
</tr>
<tr>
<td>Group 3</td>
<td>23</td>
<td>1.06 ± 0.16</td>
<td>14.8 ± 2.9</td>
<td>13.5 ± 1.8</td>
<td>1.29 ± 0.17</td>
<td>840.6 ± 162.1</td>
<td>35.7 ± 11.5</td>
<td>1139.8 ± 395.9</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>70</td>
<td>1.08 ± 0.19</td>
<td>19.0 ± 3.9</td>
<td>13.6 ± 3.0</td>
<td>1.24 ± 0.12</td>
<td>861.1 ± 157.3</td>
<td>50.8 ± 19.2</td>
<td>1274.1 ± 305.2</td>
</tr>
<tr>
<td>Group 2</td>
<td>27</td>
<td>1.07 ± 0.16</td>
<td>19.1 ± 4.7</td>
<td>12.9 ± 2.4</td>
<td>1.16 ± 0.17</td>
<td>801.7 ± 146.1</td>
<td>38.6 ± 17.5‡</td>
<td>1180.5 ± 423.5</td>
</tr>
<tr>
<td>Group 3</td>
<td>14</td>
<td>1.12 ± 0.19</td>
<td>20.7 ± 3.8</td>
<td>13.3 ± 2.5</td>
<td>1.24 ± 0.11</td>
<td>963.2 ± 270.8</td>
<td>45.4 ± 18.9</td>
<td>1230.4 ± 328.9</td>
</tr>
</tbody>
</table>

Group 1 = normal *HFE* genotype and normal Fe status
Group 2 = abnormal *HFE* genotype and normal Fe status
Group 3 = abnormal *HFE* genotype and abnormal Fe status
† indicates significantly different (p < 0.05) compared to men in other groups
‡ indicates significantly different (p < 0.05) compared to women in other groups
5.6.3 Discussion

Overall, the results from this sample of subjects provide little evidence to support the hypothesis that either abnormal *HFE* genotype or an associated increase in iron absorption has a significant effect on indicators of trace element or antioxidant status. A lack of significant differences in dietary measures was perhaps surprising given that iron overload subjects (Group 3) might have been expected to have altered diets in order to minimise iron intake and absorption. This appears to be reflected to some degree by the iron overloaded males consuming the lowest mean trace element intakes, but this trend was not evident in the females of this group where the highest mean intakes were observed. Serum levels of selenium, copper and zinc generally followed dietary intake trends for these metals. RBC SOD did not appear to be affected by intake or serum levels of copper or zinc, two trace metals which are components of the enzyme, and probably is not a reliable indicator of copper or zinc status, at least in these subjects.

Serum TAS may have been expected to vary in the group with iron overload due to the potential for increases in oxidative stress in these subjects, but it did not appear to reflect that; either because oxidative stress in the sample was minimal or because the serum components that contribute to the antioxidant status measured using this system were not sensitive to it. That serum TAS results were generally not in line with the antioxidant enzyme activity is perhaps not unexpected as GPx and SOD activity is not directly measured in the TAS assay (Cao and Prior 1998).

The only statistically significant difference observed in trace element or antioxidant measures was in RBC GPx activity, which was highest in Group 1 subjects in both genders. The differences between RBC GPx activities in gender sub-groups appear, as expected, to reflect the trends in serum selenium in each group. Serum GPx also
appeared to reflect serum selenium levels, although sub-group differences were not statistically significant. As red cell enzyme activity is considered an indicator of longer term selenium status, this could suggest that a small, long term difference in selenium status between groups may occur in these subjects. Given these observed GPx trends and the relatively small numbers of subjects in each subgroup it may not be possible to exclude a small effect between the genotype groups; however the likely major determining factor of trace element status measured in these subjects is probably gender and dietary intake.

Although it was interesting to have the opportunity to obtain data on iron overload and hereditary haemochromatosis in relation to other trace elements to complement the small amount of existing data, for subsequent research iron status will not be considered.
5.7 Discussion of preliminary findings

The major limitation of this preliminary research was the non-random nature of the sample recruitment and the bias it introduced. Men were underrepresented, as were subjects in younger age ranges, giving low numbers of male subjects in particular in some age range analyses. This study also did not collect data on socioeconomic status which can be an important factor in nutrition. The small sample size of the studies described in this chapter obviously introduces uncertainty in the estimation of the prevalence of inadequate intakes or status, but overall provided a useful indicator of areas of interest for subsequent research.

In the area of primary interest the study suggested that, as expected, neither overt selenium deficiency nor toxicity was likely in this population. It did however support the hypothesis that many people had marginal selenium status, as indicated by a high prevalence of inadequate intakes (28% <EAR) and by the moderate mean serum selenium level and its significant association with the functional marker GPx. This marginal status did not appear to be limited to particular groups within the sample, indicating further research in the whole population is certainly warranted.

The lack of definitive guidelines on dietary requirements and biomarker levels made the interpretation of copper status difficult; our preliminary research suggests that inadequate copper status is probably not a problem. Due to a lack of Australian data regarding copper status, this data collected from a convenience study may still be considered useful. The findings suggesting that zinc intakes, particularly those in men, were inadequate were also interesting. The prevalence of low zinc intakes in the overall sample met the level of 25% <EAR, suggested by WHO (de Benoist et al. 2007) to indicate increased risk of deficiency. This did not translate into a high prevalence of low serum zinc levels but overall the results indicated that people in
certain age/gender groups in Tasmania may be at risk of inadequate zinc status, and that further study in this area would be valuable.

Findings from the Study C, which investigated the potential effects of increased iron absorption in hereditary haemochromatosis on the trace elements of interest, suggested that it had, at best, a minimal affect on the other trace elements measured. There were some trends for differences between mean serum levels of selenium between the study groups but they did not reach statistical significance. The only index to demonstrate a significant difference was RBC GPx activity which was higher in the normal subjects compared to the iron overloaded subjects. This indicates that perhaps on a long term basis there may be a small difference in selenium status but the significance is unclear.

As a result of this preliminary work, the major study focuses on obtaining a random electoral roll population sample in which to assess selenium status as a primary interest, with assessment of zinc and copper status in the same population sample as the opportunity is available.
Chapter 6

Population study of selenium, copper and zinc status

6.1 Introduction

The findings from the preliminary research of the trace element status of people living in northern Tasmania indicated that the prevalence of marginal selenium status in this population may be quite high. It also suggested that certain sub-sections of this population may be at risk of inadequate zinc status.

These findings may be important if they can be shown to hold true in a representative sample of the northern Tasmanian population, given the importance of these trace elements in many biological processes and the association of selenium in particular with chronic disease such as cancer.

A cross-sectional population study recruited from the electoral roll of the Bass, Lyons and Braddon electoral divisions was proposed. This study would investigate the trace element status of a population sample using dietary and biochemical markers as used in the preliminary research, with particular emphasis on the selenium status of the sample. Recruitment of subjects and collection of biological samples would involve the cooperation of several Pathology departments and doctors surgeries, located between Campbell Town in the south, Burnie in the north west and Scottsdale in the east.
6.2 Study aims

The primary aims for this population study were;

- to estimate selenium intakes; in particular the prevalence of selenium intakes below the EAR.

- to determine the serum selenium and GPx activities; in particular to determine the proportion of the subjects that have serum selenium concentrations below the levels associated with selenoprotein requirements and potential chemopreventative effects.

- to investigate the major contributors to selenium intakes, the factors associated with selenium status, and any specific characteristics of subjects with;
  a) intakes below the EAR
  b) serum selenium concentrations above 1.27 μmol/L
  c) serum selenium concentrations above 1.50 μmol/L

The secondary aims were;

- to assess copper and zinc intakes; in particular estimating the prevalence of low dietary intakes of zinc (<EAR).

- to determine serum copper and zinc; in particular the proportion of subjects with serum zinc below WHO cut-off levels.

- to identify some of the factors associated with copper and zinc status in this population.

Data from the population study addressing the primary and secondary research objectives is presented separately in consecutive sections in this chapter:

Study A: Selenium status in a population study in northern Tasmania

Study B: Copper and zinc status in a population study in northern Tasmania
6.3 Methods

All methods for sample selection, data collection and handling, including all dietary and biochemical analyses, and statistical analysis performed for the population study of selenium, copper and zinc status, and referred to in this chapter, were described in Chapter 2: General methodology.
6.4 Study A: Selenium status in a population study in northern Tasmania

6.4.1 Background

The suggestion that human selenium status in Tasmania may be inadequate can be traced to difficulties experienced previously in this state with selenium deficiency in livestock, particularly sheep. This idea was supported by the report of low selenium levels in an early study by McGlashan et al. (1996) of selenium status in Tasmania, however, subsequent findings have been inconsistent (Daniels et al. 2000; Jacobson et al. 2007). The preliminary finding from the current research of marginal selenium intakes and serum levels also supports this hypothesis and therefore a larger, more representative population based study of selenium status was warranted.

The population study of approximately 500 participants analysing dietary intakes and biochemical measures of selenium status such as serum selenium and GPx activity is the largest and most detailed study of selenium status in Australia.

In addition to determining selenium status, and its associations with various disease risk factors such as smoking and serum lipids, this study has a novel addition of an assessment of the effect of a common GPx gene polymorphism on enzyme activity in this population. The GPx-1 Pro198Leu polymorphism has been associated with decreases in RBC GPx activity (Ravn-Haren et al. 2006) as well as increased risk of lung cancer (Ratnasinghe et al. 2000), bladder cancer (Ichimura et al. 2004) and cardiovascular disease (Hamanishi et al. 2004).
6.4.2 Results

A total of 2545 people were contacted by letter, after selection from the electoral roll, resulting in 520 positive responses; a response rate of 20%. Seventy seven of the letters were returned as "undeliverable" due to the addressee no longer residing at that address, increasing the response rate to 21%; twenty two of the positive responders subsequently withdrew due to family or personal sickness or moving interstate.

Subject characteristics

Of the 498 subjects, 192 were male and 306 were female. The mean (± sd) age of subjects was 57.4 ± 12.3 years; 61% of subjects were between 55 and 74 years of age. Subjects also tended to be over weight; sixty two percent of subjects had a BMI greater than 25 kg/m²; mean BMI was 26.9 ± 5.0 kg/m². Thirty nine subjects were current smokers (male n = 9; female n = 30).

Multivitamin and mineral supplements were regularly taken by 23% and 19% of subjects respectively. Only 8% consumed supplements which contained selenium. The most common self reported health-related conditions were hypertension (25%) and arthritis (19%); the most commonly reported medications used were anti-hypertensives (24%), cholesterol lowering drugs (16%) and anticoagulants (12%).
Table 6.1: Anthropometric, biochemical and dietary indices by gender

<table>
<thead>
<tr>
<th></th>
<th>All Subjects</th>
<th>Male</th>
<th>Female</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 498</td>
<td>n = 192</td>
<td>n = 306</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>57.4 ± 12.3</td>
<td>58.9 ± 12.2</td>
<td>56.5 ± 12.2</td>
<td>0.030</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27.0 ± 5.0</td>
<td>27.7 ± 4.6</td>
<td>26.5 ± 5.2</td>
<td>0.012</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>76.6 ± 16.7</td>
<td>86.2 ± 15.1</td>
<td>70.6 ± 14.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Height, cm</td>
<td>168.5 ± 10.0</td>
<td>176.6 ± 7.9</td>
<td>163.3 ± 7.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Energy, MJ</td>
<td>7.69 ± 2.71</td>
<td>9.11 ± 2.72</td>
<td>6.81 ± 2.29</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Protein, g/d</td>
<td>84.6 ± 31.7</td>
<td>98.2 ± 2.4</td>
<td>76.1 ± 1.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Carbohydrate, g/d</td>
<td>200.1 ± 71.6</td>
<td>233.4 ± 5.3</td>
<td>179.3 ± 3.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fibre, g/d</td>
<td>23.3 ± 9.2</td>
<td>26.7 ± 0.7</td>
<td>21.1 ± 0.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Se intake, µg/d</td>
<td>69.9 ± 27.5</td>
<td>77.4 ± 31.3</td>
<td>65.1 ± 23.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Se intake, µg/kg/d</td>
<td>0.94 ± 0.38</td>
<td>0.92 ± 0.37</td>
<td>0.95 ± 0.39</td>
<td>0.392</td>
</tr>
<tr>
<td>Se intake, µg/MJ/d</td>
<td>9.4 ± 2.9</td>
<td>8.7 ± 2.6</td>
<td>9.9 ± 2.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum Se, µmol/L</td>
<td>1.13 ± 0.19</td>
<td>1.13 ± 0.20</td>
<td>1.13 ± 0.18</td>
<td>0.776</td>
</tr>
<tr>
<td>Serum GPx, U/L</td>
<td>896.3 ± 137.0</td>
<td>901.3 ± 132.7</td>
<td>893.2 ± 139.8</td>
<td>0.515</td>
</tr>
<tr>
<td>RBC GPx, U/g Hb</td>
<td>61.2 ± 16.2</td>
<td>60.3 ± 16.0</td>
<td>61.8 ± 16.3</td>
<td>0.291</td>
</tr>
<tr>
<td>TAS, mmol/L</td>
<td>1.40 ± 0.19</td>
<td>1.45 ± 0.20</td>
<td>1.36 ± 0.17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T-Chol, mmol/L</td>
<td>5.71 ± 1.16</td>
<td>5.51 ± 1.16</td>
<td>5.83 ± 1.14</td>
<td>0.002</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>1.40 ± 0.36</td>
<td>1.22 ± 0.31</td>
<td>1.52 ± 0.35</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-C, mmol/L</td>
<td>2.95 ± 0.86</td>
<td>2.94 ± 0.86</td>
<td>2.96 ± 0.87</td>
<td>0.800</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.99 ± 1.40</td>
<td>2.32 ± 1.73</td>
<td>1.79 ± 1.10</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are mean ± sd, (range)

Effect of gender, smoking and age

Men and women did not differ significantly with respect to serum selenium, serum and RBC GPx and serum LDL cholesterol (P > 0.05); however mean age, weight, height, BMI, serum TAS, serum triglycerides, and absolute intakes of selenium were significantly higher in men (Table 6.1)
Women had significantly higher levels of serum total cholesterol and serum HDL cholesterol. Although they consumed lower absolute intakes of selenium, their diet had significantly higher nutrient density for selenium; intake adjusted for bodyweight compared to men was also higher.

Table 6.2: Anthropometric, biochemical and dietary indices in current smokers and non-smokers.

<table>
<thead>
<tr>
<th></th>
<th>Current Smokers n = 39</th>
<th>Non-smokers n = 459</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>54.6 ± 10.2</td>
<td>57.9 ± 12.3</td>
<td>0.009</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>76.0 ± 25.0</td>
<td>76.9 ± 14.7</td>
<td>0.362</td>
</tr>
<tr>
<td>Height, cm</td>
<td>169.8 ± 8.4</td>
<td>168.4 ± 7.5</td>
<td>0.657</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.1 ± 6.3</td>
<td>27.1 ± 5.0</td>
<td>0.145</td>
</tr>
<tr>
<td>Energy, MJ</td>
<td>6.97 ± 3.03</td>
<td>7.76 ± 2.67</td>
<td>0.383</td>
</tr>
<tr>
<td>Se intake, μg/d</td>
<td>63.8 ± 30.4</td>
<td>70.4 ± 27.3</td>
<td>0.352</td>
</tr>
<tr>
<td>Se intake, μg/kg/d</td>
<td>0.92 ± 0.42</td>
<td>0.94 ± 0.38</td>
<td>0.649</td>
</tr>
<tr>
<td>Se intake, μg/MJ/d</td>
<td>9.4 ± 2.4</td>
<td>9.4 ± 2.9</td>
<td>0.639</td>
</tr>
<tr>
<td>Serum Se, μmol/L</td>
<td>1.07 ± 0.10</td>
<td>1.13 ± 0.19</td>
<td>0.008</td>
</tr>
<tr>
<td>Serum GPx, U/L</td>
<td>847.3 ± 147.1</td>
<td>901.7 ± 136.5</td>
<td>0.004</td>
</tr>
<tr>
<td>RBC GPx, U/g Hb</td>
<td>62.0 ± 17.1</td>
<td>61.2 ± 16.4</td>
<td>0.876</td>
</tr>
<tr>
<td>TAS, mmol/L</td>
<td>1.42 ± 0.26</td>
<td>1.40 ± 0.17</td>
<td>0.612</td>
</tr>
</tbody>
</table>

Adjusted for gender
Values are mean ± sd

Non-smokers were older and also had significantly higher serum selenium and serum GPx levels in comparison to current smokers (Table 6.2).

After adjusting for gender and smoking habit, dietary intake of selenium was not significantly different between age ranges (Table 6.3). The highest absolute and adjusted intakes were in the 35-44 yrs age range. The lowest nutrient density was
observed in the 25-34 yrs age range and the lowest intake by bodyweight was in the 55-64 yrs age range.

Biochemical markers of trace element and antioxidant status demonstrated significant differences between age ranges. The highest mean serum selenium concentration of 1.16 μmol/L was observed in the 45-54 yrs age range; significantly higher than the lowest mean value of 1.03 μmol/L in the oldest subjects (75-84 yrs).

Serum GPx activity was highest in the 55-64 yrs age range, significantly higher than in the 25-34 yrs and 65-74 yrs age range. Lowest RBC GPx activity was also found in the youngest age range (25-34 yrs) and was significantly lower than in the 45-54 yrs age range. The trend for both serum and RBC GPx appeared to be for mean activities to rise to a peak in middle age (45-64 years) and then be decreased with increasing age.

Among the different age ranges, the lowest mean serum TAS was in the 35-44 yrs age range, significantly lower than that of the 55-64 yrs age range. Again, there appeared to be a trend for lower mean TAS in the youngest and oldest age ranges, with peak values found in the middle age ranges.
Table 6.3: Age group differences in dietary and biochemical indices of selenium status.

<table>
<thead>
<tr>
<th>Age range</th>
<th>n</th>
<th>Selenium intake</th>
<th>Serum Se</th>
<th>Serum GPx</th>
<th>RBC GPx</th>
<th>Serum TAS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>µg/d</td>
<td>µg/kg/d</td>
<td>µg/MJ/d</td>
<td>µmol/L</td>
<td>U/L</td>
</tr>
<tr>
<td>25-34</td>
<td>27</td>
<td>70.6 ± 23.8</td>
<td>0.98 ± 0.36</td>
<td>8.91 ± 2.27</td>
<td>1.14 ± 0.18</td>
<td>831.1 ± 166.3</td>
</tr>
<tr>
<td>35-44</td>
<td>57</td>
<td>73.6 ± 29.8</td>
<td>0.98 ± 0.43</td>
<td>9.51 ± 3.80</td>
<td>1.10 ± 0.22</td>
<td>918.2 ± 104.4</td>
</tr>
<tr>
<td>45-54</td>
<td>87</td>
<td>70.1 ± 26.9</td>
<td>0.93 ± 0.38</td>
<td>9.37 ± 2.35</td>
<td>1.16 ± 0.21</td>
<td>897.5 ± 126.2</td>
</tr>
<tr>
<td>55-64</td>
<td>160</td>
<td>69.8 ± 29.6</td>
<td>0.90 ± 0.38</td>
<td>9.41 ± 2.88</td>
<td>1.13 ± 0.18</td>
<td>921.8 ± 129.4</td>
</tr>
<tr>
<td>65-74</td>
<td>144</td>
<td>68.8 ± 23.7</td>
<td>0.97 ± 0.39</td>
<td>9.44 ± 2.47</td>
<td>1.12 ± 0.16</td>
<td>875.3 ± 135.6</td>
</tr>
<tr>
<td>75-84</td>
<td>21</td>
<td>66.4 ± 23.4</td>
<td>0.95 ± 0.31</td>
<td>9.27 ± 2.76</td>
<td>1.03 ± 0.17</td>
<td>856.1 ± 158.6</td>
</tr>
</tbody>
</table>

Values are mean (sd)
Adjusted for gender and smoking
Groups denoted by † are significantly different (P < 0.05)
Groups denoted by ‡ are significantly different (P < 0.02)
Selenium status

The mean selenium intakes of 77.4 and 65.1 µg/d were above estimated average requirement (EAR) values (60 µg/d and 50 µg/d respectively) for males and females; a similar proportion of each gender (28% and 26% respectively) failed to consume the EAR. The age range with the lowest proportion of subjects estimated to consume less than the EAR was the 25-34 yrs age range (18%) while the three oldest age ranges (55-64, 65-74 and 75-84 yrs) had the highest proportions of subjects below this level (Table 6.4)

Figure 6.1: Distribution of estimated dietary selenium intakes.
Table 6.4: Proportions of subjects below EAR intakes and serum selenium threshold associated with maximal GPx activity.

<table>
<thead>
<tr>
<th>&lt;EAR selenium</th>
<th>Age range</th>
<th>Men</th>
<th>Women</th>
<th>All subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>years</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>25-34</td>
<td></td>
<td>2 (22)</td>
<td>3 (16)</td>
<td>5 (18)</td>
</tr>
<tr>
<td>35-44</td>
<td></td>
<td>4 (22)</td>
<td>9 (23)</td>
<td>13 (23)</td>
</tr>
<tr>
<td>45-54</td>
<td></td>
<td>4 (14)</td>
<td>19 (33)</td>
<td>23 (26)</td>
</tr>
<tr>
<td>55-64</td>
<td></td>
<td>20 (33)</td>
<td>26 (26)</td>
<td>46 (29)</td>
</tr>
<tr>
<td>65-74</td>
<td></td>
<td>20 (31)</td>
<td>20 (25)</td>
<td>40 (28)</td>
</tr>
<tr>
<td>75-84</td>
<td></td>
<td>3 (27)</td>
<td>3 (10)</td>
<td>6 (29)</td>
</tr>
<tr>
<td>All</td>
<td></td>
<td>53 (28)</td>
<td>80 (26)</td>
<td>123 (25)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>&lt;1.27 (\mu)mol/L serum selenium</th>
<th>Age range</th>
<th>Men</th>
<th>Women</th>
<th>All subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>years</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>25-34</td>
<td></td>
<td>6 (67)</td>
<td>15 (79)</td>
<td>21 (75)</td>
</tr>
<tr>
<td>35-44</td>
<td></td>
<td>14 (78)</td>
<td>31 (79)</td>
<td>45 (79)</td>
</tr>
<tr>
<td>45-54</td>
<td></td>
<td>19 (66)</td>
<td>47 (81)</td>
<td>66 (76)</td>
</tr>
<tr>
<td>55-64</td>
<td></td>
<td>52 (87)</td>
<td>80 (80)</td>
<td>132 (83)</td>
</tr>
<tr>
<td>65-74</td>
<td></td>
<td>60 (94)</td>
<td>65 (81)</td>
<td>125 (87)</td>
</tr>
<tr>
<td>75-84</td>
<td></td>
<td>10 (91)</td>
<td>10 (100)</td>
<td>20 (95)</td>
</tr>
<tr>
<td>All</td>
<td></td>
<td>161 (84)</td>
<td>248 (81)</td>
<td>409 (82)</td>
</tr>
</tbody>
</table>

The overall mean (± sd) serum selenium was 1.13 ± 0.19 \(\mu\)mol/L. The actual distributions are shown later (Figure 6.2); however, eighty three percent of subjects had serum selenium levels below the level of 1.27 \(\mu\)mol/L that is associated with maximal GPx activity, and hence is indicative of nutritional adequacy (Elsom et al. 2006). Accordingly, serum selenium had a strong, significant positive association with serum GPx activity (\(P < 0.001\)), indicating that enzyme activity had not plateaued in many subjects. In addition, 97% of subjects had serum selenium below the level of 1.50 \(\mu\)mol/L, the target concentration that has been suggested for potential chemopreventative effects of selenium (Combs 2001).
Figure 6.2: Frequency distribution and cumulative proportion of sample and population estimates of serum selenium in northern Tasmania (dashed line indicates GPx requirement).

Due to the variation in response from different age and gender sub-groups in the study sample, a population estimate was made that was weighted for age, gender and socioeconomic status, using data from the 2006 Census for this region. In the adjusted population estimates the prevalence of low selenium intakes and serum selenium levels below threshold values were not significantly different to the study sample (Table 6.5).
Table 6.5: Estimates of population proportions below EAR and serum selenium thresholds associated with maximal GPX activity and putative chemopreventative effects of selenium.

### Dietary selenium intake

<table>
<thead>
<tr>
<th>% with intakes &lt;EAR</th>
<th>Sample estimate</th>
<th>27%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>95% C.I.</td>
<td>(0.23 - 0.32)</td>
</tr>
<tr>
<td>Population estimate</td>
<td>24%</td>
<td></td>
</tr>
<tr>
<td>95% C.I.</td>
<td>(0.23 - 0.24)</td>
<td></td>
</tr>
<tr>
<td>Risk ratio</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>95% C.I.</td>
<td>(0.71 - 1.09)</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.24</td>
<td></td>
</tr>
</tbody>
</table>

### Serum selenium levels

#### Mean serum selenium (μmol/L)

| Sample estimate | 1.13 |
| Population estimate | 1.13 |

#### % with serum selenium <1.27 μmol/L

| Sample estimate | 83% |
| 95% C.I.        | (0.79 - 0.86) |
| Population estimate | 80% |
| 95% C.I.        | (0.76 - 0.83) |
| Risk ratio      | 0.96 |
| 95% C.I.        | (0.91 - 1.02) |
| P value         | 0.26 |

#### % with serum selenium <1.50 μmol/L

| Sample estimate | 97% |
| 95% C.I.        | (0.95 - 0.99) |
| Population estimate | 97% |
| 95% C.I.        | (0.98 - 0.99) |
| Risk ratio      | 1.00 |
| 95% C.I.        | (0.98 - 1.02) |
| P value         | 0.85 |
Dietary contributions

Subjects reporting regular use of supplements containing selenium had serum selenium levels nearly 10% higher than non-users (1.23 v. 1.12 μmol/L; 95%CI of difference 0.04-0.18; P = 0.002). Regular consumers of Brazil nuts (3% of the sample) had 15% higher mean serum selenium levels (1.31 v. 1.12 μmol/L; 95%CI of difference 0.07-0.30; P = 0.002).

Meat products, cereal-based foods, vegetables and dairy based foods (45%, 26%, 8% and 8%) were the major contributors to dietary selenium intakes overall, contributing on average 32 μg, 18 μg, 6 μg and 5 μg of selenium respectively (Figure 6.3). Contributing food groups in men and women were generally similar; however, men consumed proportionally more from mixed dishes (135%), which includes many “takeaway” style foods, and alcoholic beverages (346%) compared to women (both P < 0.001). These were, however, minor contributors to overall intakes. In women, the contribution of dairy foods was 25% greater than in men (P < 0.001). There were no significant differences between age ranges (P > 0.05).
Figure 6.3: Major contributing food groups for selenium intake in population

- Alcohol beverages
- Mixed dishes
- Eggs
- Fruit
- Dairy-based
- Vegetable
- Cereal-based
- All meat

(100%) 0

> d) Significant differences were higher contributions from mixed foods and alcoholic beverages in men compared to women 100’0 > d) Significant differences were higher contributions from mixed subgroups. The only significant differences were higher concentrations from mixed foods and alcoholic beverages in men compared to women.
Other associations

Dietary selenium intakes (µg/d, µg/kg/d and µg/MJ/d) had significant positive associations with serum selenium (P = 0.005, P < 0.001 and P = 0.001 respectively). Also associated with serum selenium were dietary fibre (P = 0.035) and dietary Vitamin E (P = 0.024) (Table 6.6).

Other factors associated with serum selenium were body weight (P = 0.017) and BMI (P = 0.007), as well as serum copper (P = 0.016) and serum TAS (P = 0.022).

Serum selenium had strong positive associations with serum total cholesterol and serum HDL cholesterol (P = 0.015 and P < 0.001). Serum GPx activity was positively associated with serum HDL cholesterol and serum LDL cholesterol (P < 0.001 and P = 0.047) and negatively associated with serum triglycerides (P = 0.013). RBC GPx activity was also associated with serum HDL cholesterol (P = 0.004).

Adjusted dietary intakes of selenium (µg/kg/d) were positively associated with serum HDL (P = 0.008), but negatively with serum LDL and serum triglycerides (P = 0.022 and P = 0.014).

There was no association between serum selenium concentrations and socioeconomic status, as indicated by the SEIFA index. The SEIFA (socioeconomic indexes for areas) index used is derived from Census variables related to both advantage and disadvantage, including households with low or high income, unemployment rates and proportions of people with limited or higher education. A lower SEIFA score in a given Census district indicates that district is relatively disadvantaged compared to one with a higher score.
Table 6.6: Univariate associations of anthropometric, demographic, dietary and biochemical factors with serum selenium.

<table>
<thead>
<tr>
<th>Dietary factors</th>
<th>β</th>
<th>95% C.I.</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>zscore - selenium consumed</td>
<td>µg/d</td>
<td>0.03</td>
<td>0.01, 0.04</td>
</tr>
<tr>
<td>zscore - selenium/ kg/d consumed</td>
<td>µg/kg/d</td>
<td>0.03</td>
<td>0.02, 0.05</td>
</tr>
<tr>
<td>zscore - selenium/MJ/d consumed</td>
<td>µg/MJ/d</td>
<td>0.03</td>
<td>0.01, 0.04</td>
</tr>
<tr>
<td>zscore - energy consumed</td>
<td>MJ</td>
<td>0.01</td>
<td>-0.01, 0.02</td>
</tr>
<tr>
<td>zscore - saturated fat consumed</td>
<td>g/d</td>
<td>-0.01</td>
<td>-0.03, 0.01</td>
</tr>
<tr>
<td>zscore - polyunsaturated fat consumed</td>
<td>g/d</td>
<td>0.02</td>
<td>-0.01, 0.04</td>
</tr>
<tr>
<td>zscore - monounsaturated fat consumed</td>
<td>g/d</td>
<td>0.00</td>
<td>-0.02, 0.02</td>
</tr>
<tr>
<td>zscore - protein consumed</td>
<td>g/d</td>
<td>0.01</td>
<td>-0.01, 0.03</td>
</tr>
<tr>
<td>zscore - carbohydrate consumed</td>
<td>g/d</td>
<td>-0.0</td>
<td>-0.02, 0.02</td>
</tr>
<tr>
<td>zscore - fibre consumed</td>
<td>g/d</td>
<td>0.02</td>
<td>0.01, 0.03</td>
</tr>
<tr>
<td>zscore - alcohol consumed</td>
<td>g/d</td>
<td>0.02</td>
<td>-0.01, 0.04</td>
</tr>
<tr>
<td>zscore - vitamin E consumed</td>
<td>mg/d</td>
<td>0.02</td>
<td>0.01, 0.04</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Anthropometric, demographic and biochemical factors</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>zscore - age</td>
<td>years</td>
<td>-0.01</td>
<td>-0.03, 0.01</td>
</tr>
<tr>
<td>zscore - body weight</td>
<td>kg</td>
<td>-0.02</td>
<td>-0.04, 0.00</td>
</tr>
<tr>
<td>zscore - height</td>
<td>cm</td>
<td>-0.01</td>
<td>-0.03, 0.02</td>
</tr>
<tr>
<td>zscore - BMI</td>
<td>kg/m²</td>
<td>-0.02</td>
<td>-0.03, -0.01</td>
</tr>
<tr>
<td>zscore - SEIFA ranking</td>
<td>µmol/L</td>
<td>0.01</td>
<td>-0.01, 0.03</td>
</tr>
<tr>
<td>zscore - serum copper</td>
<td>µmol/L</td>
<td>-0.02</td>
<td>-0.04, -0.01</td>
</tr>
<tr>
<td>zscore - serum zinc</td>
<td>µmol/L</td>
<td>0.01</td>
<td>-0.01, 0.03</td>
</tr>
<tr>
<td>zscore - serum TAS</td>
<td>mmol/L</td>
<td>0.02</td>
<td>0.01, 0.04</td>
</tr>
</tbody>
</table>

zscore: standardised normal value = (subject variable value - group mean)/standard deviation). Coefficient for demographic, anthropometric, dietary and biochemical factors is the effect of a 1 sd increase in the covariant on serum selenium (µmol/L). Each separate variable, adjusted for gender and smoking, was modelled using general linear modelling with robust standard error estimation. β = regression coefficient.
Table 6.7: Univariate associations of selenium status indices with serum lipids.

<table>
<thead>
<tr>
<th>Biochemical factors</th>
<th>ΔTotal Cholesterol mmol/L</th>
<th>ΔHDL Cholesterol mmol/L</th>
<th>ΔLDL Cholesterol mmol/L</th>
<th>ΔTriglycerides mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β</td>
<td>95% CI</td>
<td>P</td>
<td>β</td>
</tr>
<tr>
<td>zscore - serum selenium</td>
<td>0.12</td>
<td>0.02, 0.20</td>
<td>0.015</td>
<td>0.06</td>
</tr>
<tr>
<td>zscore - serum GPx</td>
<td>-0.11</td>
<td>-10.67, 10.45</td>
<td>0.984</td>
<td>0.06</td>
</tr>
<tr>
<td>zscore - RBC GPx</td>
<td>2.31</td>
<td>-7.84, 12.47</td>
<td>0.655</td>
<td>0.04</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dietary factors</th>
<th>ΔTotal Cholesterol mmol/L</th>
<th>ΔHDL Cholesterol mmol/L</th>
<th>ΔLDL Cholesterol mmol/L</th>
<th>ΔTriglycerides mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β</td>
<td>95% CI</td>
<td>P</td>
<td>β</td>
</tr>
<tr>
<td>zscore - selenium</td>
<td>-0.04</td>
<td>-0.13, 0.06</td>
<td>0.431</td>
<td>0.01</td>
</tr>
<tr>
<td>zscore - selenium/kg bodyweight</td>
<td>-0.03</td>
<td>-0.13, 0.06</td>
<td>0.494</td>
<td>0.05</td>
</tr>
<tr>
<td>zscore - selenium/MJ energy</td>
<td>-0.04</td>
<td>-0.14, 0.05</td>
<td>0.353</td>
<td>-0.02</td>
</tr>
</tbody>
</table>

zscore: standardised normal value = (subject variable value – group mean)/standard deviation. Coefficient for dietary and biochemical factors is the effect of a 1 sd increase in the covariant on serum total cholesterol (mmol/L), serum HDL (mmol/L), serum LDL (mmol/L), serum triglycerides (mmol/L). Each separate variable, adjusted for gender and smoking, was modelled using general linear modelling with robust standard error estimation. β = regression coefficient.
Characteristics of selenium status groups

Dietary and lifestyle characteristics of the various selenium status groups within the population sample are summarised in Table 6.8.

Subjects with intakes <EAR values

The main distinguishing feature of subjects with low intakes appeared to be lower food consumption overall. They also had differences in some major contributing food groups; a lower contribution proportionally from meat sources but a greater contribution from vegetable foods. The lower intake subjects had lower selenium levels.

Subjects with serum selenium >1.27 μmol/L

Major defining features of subjects in this higher selenium group were a diet with greater nutrient density and intake by body weight, greater supplement usage and younger average age.

Subjects with serum selenium >1.50 μmol/L

Intakes (particularly absolute intakes and intakes per kg bodyweight) tended to be higher in the high selenium group. Socio-economic status and BMI did not appear different between groups; multivitamin use and consumption of Brazil nuts was much more common in subjects with higher selenium levels; none of the subjects in the high selenium level group were current smokers.
<table>
<thead>
<tr>
<th>Subjects consuming &lt;EAR selenium (n = 134)</th>
<th>Subjects with serum selenium &gt; 1.27 μmol/L (n = 89)</th>
<th>Subjects with serum selenium &gt; 1.50 μmol/L (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenium intakes and contributors</td>
<td>Absolute intake of selenium not significantly different (P = 0.051). Adjusted intakes (μg/MJ energy and μg/kg bodyweight) were both higher (7%; P = 0.015 and 11%; P = 0.009 respectively).</td>
<td>Mean energy consumption and selenium intakes (absolute, adjusted for bodyweight and energy) were higher (4%, 11%, 23% and 4% higher respectively) but not significantly.</td>
</tr>
<tr>
<td>• 26% lower mean energy intake in men (7.26 v. 9.85 MJ) (P &lt; 0.001); 32% lower energy intake in women (5.03 v. 7.44 MJ) (P &lt; 0.001).</td>
<td>• Only slightly more subjects reported regular consumption of Brazil nuts (6% v. 2%).</td>
<td>• 25% reported regular consumption of Brazil nuts compared to 2% of others.</td>
</tr>
<tr>
<td>• Meat contributed 7% less to intakes (P = 0.028); vegetables contributed nearly a third more (29%) than in other subjects (P &lt; 0.001).</td>
<td>• Absolute intake of selenium not significantly different (P = 0.051). Adjusted intakes (μg/MJ energy and μg/kg bodyweight) were both higher (7%; P = 0.015 and 11%; P = 0.009 respectively).</td>
<td>Regular use of multivitamin supplements was more common in this group compared to the lower selenium group (42% v. 23%). Use of selenium containing supplements was however similar (27% v. 25%).</td>
</tr>
<tr>
<td>Supplement use</td>
<td>Multivitamin use was higher (31% v. 22%). A greater proportion of these subjects regularly consumed selenium containing supplements (17% v. 4%)</td>
<td>Multivitamin use was higher (31% v. 22%). A greater proportion of these subjects regularly consumed selenium containing supplements (17% v. 4%)</td>
</tr>
<tr>
<td>• Multivitamin and mineral supplement use was similar compared to other subjects (26% v. 22%); including supplements containing selenium (8% v. 6%).</td>
<td>• No significant difference in age, smoking habit, gender distribution or economic status.</td>
<td>Regular use of multivitamin supplements was more common in this group compared to the lower selenium group (42% v. 23%). Use of selenium containing supplements was however similar (27% v. 25%).</td>
</tr>
<tr>
<td>Other</td>
<td>No significant difference in smoking, gender distribution or socioeconomic status.</td>
<td>No significant difference in age or socioeconomic status.</td>
</tr>
<tr>
<td>• No significant difference in age, smoking habit, gender distribution or economic status.</td>
<td>• Mean serum selenium levels were 8% lower (1.08 v. 1.15 μmol/L) compared to other subjects.</td>
<td>• There were no current smokers compared to 8% of all other subjects.</td>
</tr>
<tr>
<td>• Mean serum selenium levels were 8% lower (1.08 v. 1.15 μmol/L) compared to other subjects.</td>
<td>• Mean age of subjects was 3.5 years younger than all other subjects (54.5 v. 58.0 yrs; P = 0.014).</td>
<td>• Mean age of subjects was 3.5 years younger than all other subjects (54.5 v. 58.0 yrs; P = 0.014).</td>
</tr>
</tbody>
</table>
GPx-1 Pro198Leu genotype

Distribution of GPx-1 Pro198Leu genotype was not in Hardy-Weinberg equilibrium ($X^2 = 53.18; P < 0.001$); frequencies of the Pro and Leu alleles were 0.742 and 0.258 respectively. The Leu/Leu genotype group had lower mean age, RBC GPx activity and selenium intakes compared to other genotypes, but there were only two subjects with this genotype. When grouped together, genotypes containing the mutant Leu allele were not significantly different for any biochemical measure, including serum selenium, RBC GPx, serum TAS and serum lipids, compared to the homozygous Pro genotype ($P > 0.05$).

Table 6.9: Characteristics of GPx-1 Pro198Leu genotypes in study sample.

<table>
<thead>
<tr>
<th></th>
<th>Pro/Pro</th>
<th>Pro/Leu</th>
<th>Leu/Leu</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (male/female)</td>
<td>243 (96/147)</td>
<td>253 (94/159)</td>
<td>2 (2/0)</td>
</tr>
<tr>
<td>Age, years</td>
<td>57.5 ± 12.3</td>
<td>57.5 ± 12.2</td>
<td>42.5 ± 20.5</td>
</tr>
<tr>
<td>Selenium intake, µg/d</td>
<td>69.4 ± 26.9</td>
<td>70.5 ± 27.2</td>
<td>46.1 ± 0.17*</td>
</tr>
<tr>
<td>Serum Se, µmol/L</td>
<td>1.12 ± 0.21</td>
<td>1.13 ± 0.17</td>
<td>1.13 ± 0.08</td>
</tr>
<tr>
<td>Serum GPx, U/L</td>
<td>900.7 ± 130.5</td>
<td>891.8 ± 142.0</td>
<td>956.7 ± 170.1</td>
</tr>
<tr>
<td>RBC GPx, U/g Hb</td>
<td>61.4 ± 15.8</td>
<td>61.2 ± 16.5</td>
<td>52.6 ± 4.7**</td>
</tr>
<tr>
<td>Serum TAS, mmol/L</td>
<td>1.40 ± 0.19</td>
<td>1.40 ± 0.18</td>
<td>1.45 ± 0.06</td>
</tr>
</tbody>
</table>

Dietary and biochemical indices adjusted for age and gender
Values are mean ± sd
*P < 0.05 compared to Pro/Pro and Leu/Leu
**P < 0.001 compared to Pro/Pro and Pro/Leu
6.4.3 Discussion

Selenium intakes

Mean dietary intakes of selenium were above the EAR; however over one quarter of the study subjects were estimated to consume less than this amount, exceeding considerably the 3% level that FSANZ and the US Food and Nutrition Board/Institute of Medicine currently use as an indicator of nutritional inadequacy in a population (Food and Nutrition Board 2000c). The observation that similar proportions of both males and females were below the EAR indicates that gender does not appear to significantly influence the risk of consuming inadequate amounts of selenium in this population. Even though men and women had significantly different intakes of selenium, the major food groups contributed mostly similar proportions to total selenium intake in both genders; the major significant difference being an increased contribution from dairy-based foods in women, the discrepancy appearing to be accounted for in men by proportionally higher intakes from mixed-dish foods and alcoholic beverages.

Although age range intake differences were not significant, the older age groups in this study had lower mean selenium intakes and hence a greater proportion of older subjects consumed less than the EAR. The decreases in selenium intakes with increasing age appear to be due mostly to a reduction in overall food consumption rather than changes in selenium nutrient density of the diets. This was also the case in smokers who had lower, although not significantly, mean intakes, which were in line with lower energy intakes in comparison to the non-smoking subjects.

Selenium intakes in men were lower than in the few previous Australian estimates. The early studies by Fardy and co-workers (1989) and Reilly (1992) both reported higher mean intakes for men of 96 and 89 μg/d respectively, using direct analysis of
representative foods. More recently, the 20\textsuperscript{th} Australian Total Diet survey (FSANZ 2003) also estimated higher intakes in men (range: 1.2 - 1.7 μg/kg/d) compared to our study (mean: 0.92 μg/kg/d). Intakes for women were similar to those in the early studies. The estimates of Fardy \textit{et al} (63 μg/d) and Reilly (59 μg/d) compare favourably with the estimate from the current study of 65.1 μg/d. In comparison to FSANZ's Total Diet survey however, the female subjects' mean adjusted intake (0.95 μg/kg/d) was just below the range of 0.96 - 1.18 μg/kg/d estimated by that study.

Due to the age and methodological differences in the dietary estimates between these studies, any further comparison is probably imprudent.

\textbf{Serum selenium}

The overall mean serum selenium was below 1.27 μmol/L; a level that has been suggested to coincide with maximal GPx activity (Elsom \textit{et al.} 2006) and hence nutritional adequacy. Consequently a large proportion of subjects (83\%) may be considered to have marginal selenium status. The strong positive association of serum selenium with serum GPx activity in this sample, suggesting GPx activity has not plateaued in many subjects, supports this finding, though the significance of this is still uncertain.

Most subjects (97\%) had serum selenium concentrations below 1.50 μmol/L; considered as a level where the potential chemopreventative effects of selenium may be achieved (Combs 2001). There have been many promising findings from geographical, animal, \textit{in vitro}, case control and prospective studies regarding the association of low selenium status with cancer incidence and the protective effects of selenium supplementation (Rayman 2005). At this time, however, results from the only large randomized controlled trial are inconclusive; the recently ended SELECT trial in the US (Lippman \textit{et al.} 2009) found no significant protective effect of
selenium supplementation on prostate or other cancers in 35,533 men. Unfortunately, this trial did not test supplementation in a low selenium population where an effect could be expected to be observed; most subjects in this trial, where the median baseline serum selenium was 1.71 μmol/L, were probably selenium replete.

A population estimate (Table 6.5 and Figure 6.1) was weighted for age, gender and socioeconomic status in the study sample compared to northern Tasmanian census data, due to the variation in response rates in the different study age and gender subgroups. This analysis was not significantly different to the study sample estimates and hence it may be estimated that almost one quarter of the 126,958 residents from the northern Tasmanian region surveyed may consume inadequate selenium. Eighty per cent of this population was estimated to have serum selenium below the level associated with nutritional adequacy; while 97% were estimated to have selenium levels below the level that has been suggested to potentially offer chemopreventative benefits.

Selenium levels in the current study were similar, but possibly slightly higher than in the earliest reported Tasmanian data. The studies by McGlashan et al (1996) (1.02 μmol/L; n = 171) and Daniels et al (2000) (0.98 μmol/L; n = 25) of adult blood donors, did not, however collect dietary data or publish their demographic data. In addition, the blood donors used in these studies may not be representative of the population, as donors would be considered a healthy sample due to the exclusion of individuals with infectious diseases.

Serum selenium was considerably lower than in Jacobson and co-workers’ (2007) recent study of selenium status in people from southern Tasmania (n = 335). Their mean plasma selenium concentration of 1.39 μmol/L is the highest reported from an Australian population and suggests that the majority of subjects were probably selenium replete. There were differences in the analytical methods used (ICP-MS v.
GFAAS) as well as in sample type (plasma v. serum), but these are not expected to have caused major differences in results. Although this group encountered analytical difficulties when using biological matrices with this newer ICP-MS method (A. Townsend, personal communication, September 25, 2004), the method appeared to perform well; analysis of an external commercial reference material with a certified concentration of $1.05 \pm 0.8 \, \mu\text{mol/L}$ gave a mean of $1.05 \pm 0.6 \, \mu\text{mol/L}$. On this basis, the ICP-MS method was arguably a more accurate method than the GF-AAS method utilised in the current study, and this may explain some of the difference in selenium concentration between the two studies. Unfortunately, this group did not report dietary data; and hence it is not possible to detect additional dietary differences which may also explain the apparent substantial difference in selenium levels between these two studies.

Although significant differences in selenium status have been observed between close geographical regions of the same country, such as in New Zealand (Thomson 2004b; Thomson et al. 2007) and France (Arnaud et al. 2006), further research is required to determine if this is also the case in Tasmania.

In comparison to other Australian states, selenium levels were similar to South Australian (Daniels et al. 2000) and NSW cohorts (Dhindsa et al. 1998) (1.11 and 1.16 $\mu\text{mol/L}$ respectively), but lower than in more recent Queensland (Lymbury et al. 2008) and South Australian (Lyons et al. 2004) studies (1.27 and 1.30 $\mu\text{mol/L}$ respectively).

The mean selenium concentration was higher than those reported from the South Island of New Zealand (0.84 - 0.98 $\mu\text{mol/L}$) (Thomson 2004b); a region with well described low selenium status. It was similar to many European countries where selenium status is marginal, and much lower than countries with high selenium intakes such as the US, Canada, Japan and Venezuela (Combs 2001).
Interestingly, even in studies reporting very similar selenium levels to the current study, reported dietary intakes appear to be much lower in comparison. As an example, the recent small British study of Sunde and co-workers (2008) reported a mean plasma selenium of 1.13 μmol/L (n = 39), but estimated dietary intake, calculated using diet diaries, was only 48 μg/d. The nutrient density was approximately 60% of that calculated in the current study. This trend can be observed when reported dietary intakes and serum and/or plasma selenium from various other countries are compared (Combs 2001), and suggests that the dietary intakes may be over-estimated in the current study. If this was in fact the case it could explain the significant discrepancy observed between the proportions of subjects consuming inadequate selenium (27% < EAR) and those with marginal serum selenium concentrations (83% < 1.27 μmol/L). An inaccuracy in estimated intakes in this case may result from the design of the FFQ, or the use of food content data that is not representative of Tasmanian foods. The food content database used in this study, NUTTAB2006, does not provide data specific to Tasmanian foods, but rather data derived from a variety of Australian sources collected since the 1980s. While this data is mostly determined from direct food analysis, it may also be borrowed from overseas tables, estimated from “like” foods, provided by food manufacturers or calculated by FSANZ where needed (for examples of various data sources see Appendix 1).

**Associations with age, smoking and lipids**

Mean serum selenium concentrations were lowest in the oldest age group, significantly lower than the group with highest mean levels; roughly in line with the trend for lower mean intakes in older subjects. A trend for decreasing selenium status with age has been observed in other studies (Brooks *et al*. 2001; De Jong *et al*. 2001;
Bates et al. 2002a) and is of concern in populations with already marginal selenium status.

Smoking and alcohol consumption have been associated with lower and higher selenium status respectively (Ellingsen et al. 1997; Kafai and Ganji 2003; Galan et al. 2005). Smokers in this study had significantly lower serum selenium and serum GPx activity compared to non-smokers. This appeared to be related to lower selenium intakes, even though differences were not significant, rather than increased turnover or some other unknown mechanism related to smoking. Although serum TAS was associated with serum selenium, there was no significant difference in antioxidant status as measured by TAS between smokers and non-smokers, supporting the suggestion that the lower selenium and GPx levels in smokers were probably not the result of increased oxidative stress due to smoking.

Findings regarding the relationship between the various indices of selenium status with cardiovascular risk factors have been inconsistent. Several studies have reported positive associations between selenium and serum cholesterol (Suadicani et al. 1992; Gamez et al. 1997; Hercberg et al. 2005; Bleys et al. 2008); one study has reported positive associations with each of serum cholesterol, serum HDL, serum LDL and serum triglycerides (Bleys et al. 2008), while other studies have found no association with any serum lipid component (Ghayour-Mobarhan et al. 2005). In this study, serum selenium and serum GPx were significantly associated with several lipid measures as well as bodyweight and BMI. The mechanisms for such associations with serum lipids are unknown at this time; they may be due to the effects of selenoproteins such as GPx or iodothyronine deiodinase on lipid metabolism or the result of interrelated dietary exposures as proposed by Bleys and colleagues (2008); which could explain the significant associations found in this study between adjusted intakes and serum lipid components.
Characteristics of subjects with low or high selenium status

Subjects of this study were classified by selenium intake (< EAR) and by serum selenium concentrations (> 1.27 μmol/L and > 1.50 μmol/L) in an attempt to identify factors that influenced selenium status at these levels (Table 6.8).

For all classifications, socioeconomic status (as measured by SEIFA index) did not appear to be a significant factor influencing selenium status in these groups and was not associated with serum selenium overall (Table 6.6), despite the association between socioeconomic status and healthy diets in Australia and elsewhere (Giskes et al. 2002; Beydoun and Wang 2008). The lack of association is consistent with the findings in the recent Tasmanian study of Jacobson et al. (2007) and in France (Arnaud et al. 2006), but in contrast to some findings in Britain (Bates et al. 2002a; Bates et al. 2002b).

Subjects who consumed less than the EAR for selenium had an 8% lower mean serum selenium than those who consumed adequate selenium. The major influence on intakes of these subjects was a significantly lower total energy intake compared to subjects of the same gender who consumed more than the EAR. There was lower dietary selenium contribution from meat products and a higher contribution from vegetables; the nutrient density of diets in this group was also significantly lower, indicating they ate a smaller and perhaps also a less nutritious diet. As well as socioeconomic status, age, smoking habit and gender did not appear to be significant influences in this comparison.

Dietary differences were also significant when a comparison was made between groups of subjects with serum selenium above and below 1.27 μmol/L; the level associated with selenoprotein requirements. Adjusted intakes (μg/MJ and μg/kg) were higher in subjects with serum selenium above 1.27 μmol/L, while a similar difference in absolute intakes did not reach statistical significance. As well as a higher regular
dietary intake, this group also consumed multivitamin and mineral supplements, including selenium containing supplements, more commonly than the group with lower selenium status. The rate of reported consumption of Brazil nuts, the richest dietary source of selenium, was slightly higher in the group with higher selenium levels. Socioeconomic status, gender distribution and smoking habit were not significantly different between these groups; however subjects in the higher selenium group were significantly younger than those with low selenium. This is consistent with the negative, though non-significant, association between age and serum selenium overall, and the significant differences observed in age range comparisons. Dietary intakes, both absolute and adjusted, were non-significantly higher in subjects with serum selenium concentrations greater than 1.50 μmol/L; the level associated with the potential chemopreventative effect of selenium, although these differences could be expected to have become significant with greater numbers of subjects. In addition to potentially higher intakes from their normal diet in this group, the rate of multivitamin and mineral supplement use was higher, although the use of selenium containing supplements was similar.

Brazil nut consumption was particularly common in the high selenium group, with one quarter of these subjects reporting regular consumption compared to only 2% of all other subjects. Brazil nuts have been suggested as an effective method for increasing selenium status in marginal populations. A study by Thomson and colleagues (2008) in subjects with baseline selenium status similar to that observed in the current study, found that despite significant variability in the selenium content of Brazil nuts, consumption of just two per day resulted in a 64% increase in plasma selenium after 12 weeks of supplementation. Their potent effect and ready supply in most major supermarkets year-round therefore make them an ideal candidate food for increasing selenium status in a population, without the need for fortification of foods
or use of commercial supplements. One caveat is that Brazil nuts are known to also contain high amounts of toxic metals such as radium and barium (Parekh et al. 2008).

The only other apparent difference between the high selenium group and others was the absence of smokers amongst the high selenium subjects, while 8% of all other subjects were current smokers. There appears, therefore, to be a trend for subjects with high selenium status (serum selenium >1.50 μmol/L) to be non-smokers and to consume a more nutritious diet including multivitamin and mineral supplements; while this may be the result of a more conscientious approach to dietary and health related aspects of their lifestyle, it may have been affected by the low numbers of subjects (14) in this group.

**Effect of GPx-1 Pro198Leu genotype**

The genotype distribution of the GPx-1 Pro198Leu polymorphism was not in Hardy-Weinberg equilibrium. There was a greater representation of heterozygotes and a lower representation of both homozygote genotypes than expected; this deviation was most likely a function of the sample size which was probably too small to allow an accurate estimate of the true genotype distribution in this population.

The Leu/Leu genotype had a lower mean RBC GPx activity than other genotypes even though serum selenium was not significantly different. Unfortunately there were only two subjects with the Leu/Leu genotype, which limits the power of such analyses; however several selenoprotein gene polymorphisms have been shown to influence the response of their protein products to selenium levels including polymorphisms in GPx-1 (Hu and Diamond 2003; Ravn-Haren et al. 2006; Jablonska et al. 2009), GPx-4 (Meplan et al. 2008), SelP (Meplan et al. 2007) and Sep15 (Hu et al. 2001).
There were no significant differences in any dietary or biochemical variable when Pro/Pro subjects were compared with those with at least one copy of the Leu allele; however, sample size calculations indicate that detection of a 5% difference in RBC GPx, such as that suggested by Ravn-Haren and colleagues (2006) to result from 1 copy of a mutant Leu allele in GPX1, would require a sample size of 1160, more than twice that of the current study.

6.4.4 Conclusion

The major finding of this study is that dietary intakes in a significant proportion of the population appear likely to be inadequate to meet the physiological requirement for selenium. For the most part, this inadequacy had a similar prevalence across age and gender subgroups and appeared independent of socioeconomic status. As observed in other studies, however, the prevalence of inadequacy was increased in the elderly (75+ yrs) in this study and this may be a factor that could contribute to the decline in health associated with aging. Smokers were the only other population subgroup to have significantly lower selenium levels. This effect appeared to be related to lower overall dietary intakes and adds to health concerns for these subjects given the oxidative stress caused by smoking and the importance of selenium in antioxidant defence.

Important contributing foods groups were as expected; meat products and cereal-based foods, and their contribution to diets across population subgroups did not vary significantly. The use of selenium containing multivitamin and mineral supplements, which are becoming more common on the supermarket shelf, were associated with higher selenium status, but because of their generally quite low selenium content (often approximately 25 μg/dose) are not a realistic method of increasing selenium status more than a few percent. Brazil nuts, the richest natural source of selenium for
humans, were infrequently consumed by most subjects, but were a part of the diet for many who had a higher selenium status and, as has been suggested, could provide a reasonably convenient and cost effective mode for increasing selenium intakes in the population.

The analysis of subjects by $GPx-1$ genotype was unfortunately hindered by the sample size and genotype distribution, and did not appear to be a significant factor in this population. Despite this finding, such polymorphisms will remain of interest due to the increasing evidence of associations between selenoprotein polymorphisms and chronic disease (Meplan et al. 2007; Cooper et al. 2008; Jablonska et al. 2008; Meplan et al. 2008).

At this time, the relationships and the mechanism of any relationship between selenium status and serum lipids remain unclear, and the association of selenium with cardiovascular disease is also uncertain. However, it is noted that Tasmania has high rates of chronic disease, particularly cardiovascular disease, and also some cancers (A.B.S. 2006a).
6.5 Study B: Copper and zinc status in a population study in northern Tasmania

6.5.1 Background

Copper and zinc are important components of numerous proteins with wide ranging roles including antioxidant defence, gene expression and cellular respiration. Mild copper deficiency is associated with impaired immune response, altered lipid metabolism and haematological abnormalities while mild zinc deficiency can cause delayed wound healing, altered immune function and stunted growth and development. Like selenium, overt deficiency of copper or zinc is rare. Marginal copper status is thought to be much more common, and it has been suggested that perhaps 20% of the world’s population may be affected by mild zinc deficiency (Sanghvi et al. 2007). Populations in developing countries are thought to be most at risk, but experience in developed countries with other trace elements such as selenium and iodine suggests that populations in these countries are not immune to such problems.

Despite the importance of these trace metals, data regarding copper or zinc status in Australian populations is limited. Our preliminary research has suggested that some sub-sections of the Tasmanian population may be at risk of inadequate zinc status. This population study of approximately 500 participants which analyses dietary intakes as well as biochemical markers of copper and zinc status is the largest and most detailed study of its type in Australia to date.
6.5.2 Results

Subject characteristics

Sample demographics and anthropometric data are described in Chapter 6 - Study A: Selenium status in a population study in northern Tasmania (pg. 162).
Effects of gender, smoking and age

Gender

Mean dietary energy intake and absolute intakes of copper and zinc were significantly higher in men ($P < 0.001$, $P = 0.002$ and $P < 0.001$ respectively) (Table 6.10). Men and women did not differ with respect to serum zinc or RBC SOD ($P > 0.05$). Women had a significantly higher serum copper concentration ($P < 0.001$), and although women consumed lower absolute intakes of copper and zinc, their diet had significantly higher nutrient density for these minerals (both $P < 0.001$); they also consumed more copper and zinc per kg bodyweight compared to men ($P = 0.003$ and $P = 0.045$ respectively).

Table 6.10: Dietary and biochemical indices of copper and zinc status

<table>
<thead>
<tr>
<th></th>
<th>All Subjects</th>
<th>Male</th>
<th>Female</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 498</td>
<td>n = 192</td>
<td>n = 306</td>
<td></td>
</tr>
<tr>
<td>Energy, MJ</td>
<td>7.69 ± 2.71</td>
<td>(2.05 - 20.90)</td>
<td>9.11 ± 2.72</td>
<td>6.81 ± 2.29</td>
</tr>
<tr>
<td>Cu intake, mg/d</td>
<td>1.5 ± 0.5</td>
<td>(0.2 - 4.2)</td>
<td>1.6 ± 0.6</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>Cu intake, µg/kg/d</td>
<td>19.9 ± 8.8</td>
<td>(3.0 - 60.0)</td>
<td>18.5 ± 8.0</td>
<td>20.9 ± 9.2</td>
</tr>
<tr>
<td>Cu intake, µg/MJ/d</td>
<td>0.20 ± 0.60</td>
<td>(0.08 - 0.41)</td>
<td>0.18 ± 0.05</td>
<td>0.21 ± 0.06</td>
</tr>
<tr>
<td>Zn intake, mg/d</td>
<td>11.6 ± 4.0</td>
<td>(3.5 - 33.4)</td>
<td>12.6 ± 4.4</td>
<td>10.9 ± 3.6</td>
</tr>
<tr>
<td>Zn intake, µg/kg/d</td>
<td>156.4 ± 58.4</td>
<td>(50.0 - 510.0)</td>
<td>149.8 ± 56.1</td>
<td>160.6 ± 59.5</td>
</tr>
<tr>
<td>Zn intake, µg/MJ/d</td>
<td>1.56 ± 0.39</td>
<td>(0.68 - 3.51)</td>
<td>1.41 ± 0.36</td>
<td>1.65 ± 0.38</td>
</tr>
<tr>
<td>Serum Cu, µmol/L</td>
<td>17.6 ± 3.8</td>
<td>(9.5 - 44.8)</td>
<td>15.5 ± 2.7</td>
<td>18.9 ± 3.9</td>
</tr>
<tr>
<td>Serum Zn, µmol/L</td>
<td>13.0 ± 2.4</td>
<td>(7.5 - 24.5)</td>
<td>13.0 ± 2.4</td>
<td>13.0 ± 2.5</td>
</tr>
<tr>
<td>RBC SOD, U/g Hb</td>
<td>1306 ± 253</td>
<td>(500 - 2363)</td>
<td>1323 ± 237</td>
<td>1295 ± 261</td>
</tr>
</tbody>
</table>

Values are mean ± sd
Smoking

When adjusted for gender, mean energy intakes and intakes of copper and zinc, both absolute and adjusted, appeared higher in non-smokers (Table 6.11), however only the difference in nutrient density of copper reached statistical significance ($P = 0.023$).

A gender comparison of copper and zinc data was made for smokers versus non-smokers (Appendix 2). The only significant difference observed was the higher serum copper in the 9 male smokers (17.6 v. 15.4 $\mu$mol/L; $P = 0.001$) compared to the non-smokers.

Table 6.11: Dietary and biochemical indices of smokers and non-smokers

<table>
<thead>
<tr>
<th></th>
<th>Current Smokers</th>
<th>Non-smokers</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 39</td>
<td>n = 459</td>
<td></td>
</tr>
<tr>
<td>Energy, MJ</td>
<td>6.97 ± 3.03</td>
<td>7.76 ± 2.67</td>
<td>0.383</td>
</tr>
<tr>
<td>Cu intake, mg/d</td>
<td>1.3 ± 0.7</td>
<td>1.5 ± 0.5</td>
<td>0.081</td>
</tr>
<tr>
<td>Cu intake, $\mu$g/kg/d</td>
<td>18.6 ± 9.7</td>
<td>20.1 ± 8.7</td>
<td>0.453</td>
</tr>
<tr>
<td>Cu intake, $\mu$g/MJ/d</td>
<td>0.18 ± 0.05</td>
<td>0.20 ± 0.06</td>
<td>0.023</td>
</tr>
<tr>
<td>Zn intake, mg/d</td>
<td>10.9 ± 6.1</td>
<td>11.6 ± 3.7</td>
<td>0.521</td>
</tr>
<tr>
<td>Zn intake, $\mu$g/kg/d</td>
<td>150.7 ± 65.7</td>
<td>156.7 ± 56.5</td>
<td>0.809</td>
</tr>
<tr>
<td>Zn intake, $\mu$g/MJ/d</td>
<td>1.50 ± 0.34</td>
<td>1.56 ± 0.38</td>
<td>0.528</td>
</tr>
<tr>
<td>Serum Cu, $\mu$mol/L</td>
<td>18.4 ± 2.8</td>
<td>17.5 ± 3.4</td>
<td>0.245</td>
</tr>
<tr>
<td>Serum Zn, $\mu$mol/L</td>
<td>13.4 ± 2.5</td>
<td>13.0 ± 2.4</td>
<td>0.170</td>
</tr>
<tr>
<td>RBC SOD, U/g Hb</td>
<td>1329.8 ± 225.7</td>
<td>1305.2 ± 255.4</td>
<td>0.632</td>
</tr>
</tbody>
</table>

Adjusted for gender
Values are mean ± sd
Age

Copper

The lowest mean copper intakes for men (1.3 mg/d) were observed in the 25-34 year age range; significantly lower than intakes of men in the 45-54 yrs (P = 0.027) and 65-74 yrs (P = 0.011) age ranges. Women in the 45-54 yrs age range apparently had the lowest mean copper intakes (1.3 mg/d) but it was not significantly lower than in other age ranges. Mean serum copper was highest in the 75-84 yrs range in men (16.2 μmol/L) and in the 25-34 yrs range women (21.6 μmol/L) but differences between age ranges were not statistically significant.

Figure 6.4: Dietary copper intakes and serum copper concentrations in age range and gender sub-groups. *Copper intake in men aged 25-34 yrs significantly lower than in 45-54 yrs (P = 0.027) and 65-74 yrs age range (P = 0.011). Values are mean ± sd.
Zinc

Lowest mean zinc intakes were observed in men aged 75-84 yrs (12.2 mg/d) and in women aged 45-54 yrs (10.1 mg/d), however differences between age ranges for each gender were not significantly different. The lowest mean serum zinc for men (11.5 μmol/L) and women (11.6 μmol/L) was observed in the 75-84 yrs age group, however only in men were there significant differences between age ranges. Men aged 35-44 yrs (13.6 v. 11.5 μmol/L; 95%CI of difference 0.7-3.5; P = 0.011) and 45-54 yrs (14.0 v. 11.5 μmol/L; 95%CI of difference 1.1-3.7; P = 0.001) both had higher serum zinc than the oldest age range.

Figure 6.5: Dietary zinc intakes and serum zinc concentrations in age range and gender sub-groups. *Serum zinc in men aged 75-84 yrs significantly lower than men in 35-44 yrs (P = 0.011) and 45-54 yrs age range (P = 0.001). Values are mean ± sd.
Copper status

The mean dietary copper intake for men (1.6 mg/d) was below the AI of 1.7 mg/d; 68% of men consumed less than the AI. The mean intake in women (1.4 mg/d) was above the AI of 1.2 mg/d; however 36% did not consume the AI. In men, subjects in the 25-34 yrs age range were least likely to consume the AI; 100% of male subjects did not consume the AI in this age range compared to 78%, 61%, 68%, 67% and 73% for the subsequent age ranges 35-44 yrs, 45-54 yrs, 55-64 yrs, 65-74 yrs and 75-84 yrs respectively. In women, 50% of subjects aged 45-54 yrs consumed less than 1.2 mg/d, compared to 32%, 41%, 35%, 30% and 20% in the 25-34 yrs, 35-44 yrs, 55-64 yrs, 65-74 yrs and 75-84 yrs age ranges respectively (Table 6.12).

Figure 6.6: Distribution of estimated copper intakes of all subjects.
Only 3 subjects had serum copper concentrations below the lower end of the clinical reference range of 11 \( \mu \text{mol/L} \); 2 males and 1 female.

**Figure 6.7:** Frequency distribution and cumulative proportion of population and samples estimates of serum copper concentrations.
Zinc status

Mean zinc intake for men was 12.6 ± 4.4 mg/d. Over half of the male subjects (52%) consumed less than the zinc EAR of 12 mg/d. The mean intake for women was 10.9 ± 3.6 mg/d and only 9% of females failed to consume the EAR of 6.5 mg/day. The proportion of men who consumed less than the zinc EAR increased in each successive age range (Table 6.12). In the 25-34 yrs age range 22% of men consumed less than the EAR but this rose to 73% in the 75-84 yrs age range. A similar trend was not observed in women; no subjects in the 75-84 yrs age range consumed less than the EAR and the greatest proportion of subjects to do so were in the 45-54 yrs age range (14%).

Figure 6.8: Distribution of estimated zinc intakes of all subjects.
Table 6.12: Prevalence in age ranges of <AI intakes of copper and <EAR intakes of zinc.

<table>
<thead>
<tr>
<th>Age range years</th>
<th>&lt;AI Copper</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Men</td>
<td>Women</td>
<td>All subjects</td>
<td></td>
</tr>
<tr>
<td>25-34</td>
<td>9 (100)</td>
<td>6 (32)</td>
<td>15 (54)</td>
<td></td>
</tr>
<tr>
<td>35-44</td>
<td>14 (78)</td>
<td>15 (41)</td>
<td>29 (53)</td>
<td></td>
</tr>
<tr>
<td>45-54</td>
<td>17 (61)</td>
<td>29 (50)</td>
<td>46 (53)</td>
<td></td>
</tr>
<tr>
<td>55-64</td>
<td>41 (68)</td>
<td>35 (55)</td>
<td>76 (48)</td>
<td></td>
</tr>
<tr>
<td>65-74</td>
<td>42 (67)</td>
<td>24 (30)</td>
<td>66 (46)</td>
<td></td>
</tr>
<tr>
<td>75-84</td>
<td>8 (73)</td>
<td>2 (20)</td>
<td>10 (48)</td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>131 (68)</td>
<td>111 (36)</td>
<td>242 (49)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age range years</th>
<th>&lt;EAR Zinc</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Men</td>
<td>Women</td>
<td>All subjects</td>
<td></td>
</tr>
<tr>
<td>25-34</td>
<td>2 (22)</td>
<td>1 (5)</td>
<td>3 (11)</td>
<td></td>
</tr>
<tr>
<td>35-44</td>
<td>6 (33)</td>
<td>3 (8)</td>
<td>9 (16)</td>
<td></td>
</tr>
<tr>
<td>45-54</td>
<td>13 (46)</td>
<td>8 (14)</td>
<td>21 (24)</td>
<td></td>
</tr>
<tr>
<td>55-64</td>
<td>33 (55)</td>
<td>7 (7)</td>
<td>40 (25)</td>
<td></td>
</tr>
<tr>
<td>65-74</td>
<td>38 (60)</td>
<td>8 (10)</td>
<td>46 (32)</td>
<td></td>
</tr>
<tr>
<td>75-84</td>
<td>8 (73)</td>
<td>0 (0)</td>
<td>8 (38)</td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>100 (52)</td>
<td>27 (9)</td>
<td>127 (26)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age range years</th>
<th>&lt;WHO Serum zinc cut-off</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Men</td>
<td>Women</td>
<td>All subjects</td>
<td></td>
</tr>
<tr>
<td>25-34</td>
<td>0 (0)</td>
<td>3 (16)</td>
<td>3 (11)</td>
<td></td>
</tr>
<tr>
<td>35-44</td>
<td>0 (0)</td>
<td>2 (5)</td>
<td>2 (4)</td>
<td></td>
</tr>
<tr>
<td>45-54</td>
<td>1 (3)</td>
<td>6 (11)</td>
<td>7 (8)</td>
<td></td>
</tr>
<tr>
<td>55-64</td>
<td>10 (17)</td>
<td>4 (4)</td>
<td>14 (9)</td>
<td></td>
</tr>
<tr>
<td>65-74</td>
<td>13 (21)</td>
<td>3 (4)</td>
<td>16 (11)</td>
<td></td>
</tr>
<tr>
<td>75-84</td>
<td>4 (36)</td>
<td>3 (30)</td>
<td>7 (33)</td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>28 (15)</td>
<td>21 (7)</td>
<td>49 (10)</td>
<td></td>
</tr>
</tbody>
</table>

AI - adequate intake: men 1.7 mg/d, women 1.2 mg/d
EAR - estimated average requirement: men 12 mg/d, women 6.5 mg/d
WHO serum zinc cut-off: men 10.7 μmol/L, women 10.1 μmol/L.
Compared to WHO serum zinc cut-off values, 15% of males and 7% of females had low serum zinc (Table 6.12). In men <45 yrs there were no incidences of serum zinc below the WHO cut-off, however the incidence increased with age. In the 45-54 yrs age range only 3% had serum zinc below this value, but this increased to peak at 36% in the 75-84 yrs age range. In women the greatest proportion of serum zinc concentrations below the cut-off also occurred in the oldest age range (30%), but the next greatest proportion was seen in young females (25-34 yrs) with 16%.

![Cumulative proportion vs Serum zinc graph](image)

**Figure 6.9:** Frequency distribution and cumulative proportion of population and sample estimates of serum zinc concentrations.

**Adjusted population estimates**

In population estimates weighted for age, gender and socioeconomic status, the proportion of sub-AI copper intakes was estimated to be significantly lower (51 v. 44%; \( P = 0.048 \)), but the prevalence of low zinc intakes and serum levels were not significantly different (Table 6.13).
Table 6.13: Population estimates of prevalence of <AI copper intakes, <EAR zinc intakes and <WHO serum zinc cut-off values.

### Dietary copper intake

<table>
<thead>
<tr>
<th></th>
<th>Sample estimate</th>
<th>95% C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>% with intakes &lt;AI</td>
<td>51%</td>
<td>(0.46 - 0.55)</td>
</tr>
<tr>
<td>Population estimate</td>
<td>44%</td>
<td>(0.40 - 0.49)</td>
</tr>
<tr>
<td>Risk ratio</td>
<td>0.88</td>
<td>(0.77 - 1.00)</td>
</tr>
<tr>
<td>P value</td>
<td>0.048</td>
<td></td>
</tr>
</tbody>
</table>

### Dietary zinc intake

<table>
<thead>
<tr>
<th></th>
<th>Sample estimate</th>
<th>95% C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>% with intakes &lt;EAR</td>
<td>26%</td>
<td>(0.22 - 0.30)</td>
</tr>
<tr>
<td>Population estimate</td>
<td>25%</td>
<td>(0.21 - 0.29)</td>
</tr>
<tr>
<td>Risk ratio</td>
<td>0.96</td>
<td>(0.78 - 1.19)</td>
</tr>
<tr>
<td>P value</td>
<td>0.720</td>
<td></td>
</tr>
</tbody>
</table>

### Serum zinc levels

<table>
<thead>
<tr>
<th></th>
<th>Sample estimate</th>
<th>Population estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean serum zinc (μmol/L)</td>
<td>13.0</td>
<td>13.2</td>
</tr>
</tbody>
</table>

### % with serum zinc <WHO cut-off

<table>
<thead>
<tr>
<th></th>
<th>Sample estimate</th>
<th>95% C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population estimate</td>
<td>9%</td>
<td>(0.07 - 0.12)</td>
</tr>
<tr>
<td>Risk ratio</td>
<td>0.94</td>
<td>(0.64 - 1.38)</td>
</tr>
<tr>
<td>P value</td>
<td>0.750</td>
<td></td>
</tr>
</tbody>
</table>
The major contributing food groups to copper intake were cereal-based foods.

**Figure 6.10:** Major contributing food groups to copper intake in population subgroups.
consumed proportionally more copper than women from meat (8.1 v. 6.9%; P < 0.001), mixed dishes (3.4 v. 2.3 %; P < 0.001) and alcoholic beverages (3.6 v. 1.5%; P < 0.001). In women, significantly greater contributions were made from vegetables (34.3 v. 32.3%; P = 0.039), dairy-based foods (5.5 v. 4.4%; P < 0.001) and fruit (16.4 v. 13.6%; P < 0.001).

Age group comparisons revealed significant differences in the contribution of meat between the 35-44 and the 65-74 yrs range (8.2 v. 6.7%; P = 0.024). Vegetables were greater contributors proportionally for the 55-64 yrs age range (35.4%) compared to the 25-34 yrs range (31%; P = 0.024) and 45-54 yrs range (31%; P = 0.011). Mixed dishes were a greater contributor in the 25-34 yrs age range (5%) compared to the 45-54 yrs (3.5%; P = 0.023), 55-64 yrs (2.4%), 65-74 yrs (1.9%) and 75-84 yrs (1.4%) age ranges (all P < 0.001).

Zinc

The major contributors to zinc intakes were meat products, cereal-based foods, vegetables and dairy foods (Figure 6.11). In men, meat (31.6 v. 26.3%) and mixed dishes (5.7 v. 4.0%) made significantly higher contributions to zinc intakes compared to women (both P < 0.001), while vegetables (19.4 v. 16.8%), dairy-based foods (20.1 v. 16.3%) and fruit (6.7 v. 5.7%) made greater contributions (all P < 0.001) to zinc intakes in women.

There were also several small but significant differences amongst age groups. Vegetables were significantly lower contributors to zinc intakes in the 25-34 yrs group when compared with the 65-74yrs age group (16.1 v. 19.5%; P = 0.038). Fruit was a greater contributor in the 75-84 yrs age group compared to the 35-44 yrs age group (6.7 v. 5.0%; P = 0.048), while mixed dish foods made their highest contribution in the 25-34 yrs age group (7.9%); significantly higher than in the 35-44
Figure 6.11: Major contributing food groups to zinc intake in population subgroups.

- Mixed dishes
- Fruit
- Dairy-based
- Vegetable
- Cereal-based
- All meat

% Contribution to dietary intake

(100'0 > $d_{III}$) (8%) (2') (%)

(%) 75-84 yrs (3.6%) 65-74 yrs (4.3%) 55-64 yrs (5.5%; $P = 0.008$), 55-64 yrs (4.3%) 65-74 yrs (3.6%) and 75-84 yrs (2.8%)
Other associations

Dietary intake of copper was not significantly associated with serum concentrations of copper (P = 0.500). Serum copper had a significant positive association with RBC SOD activity (P = 0.024), bodyweight (P = 0.013), BMI (P = 0.003) and dietary carbohydrate (P < 0.001). Serum copper was negatively associated with serum selenium (P = 0.013), but was not significantly associated with serum RBC GPx (P > 0.05). A positive association was observed between serum copper and serum total cholesterol (P = 0.032) and serum LDL-cholesterol (P = 0.049).

Dietary zinc was also not significantly associated with serum concentrations of zinc (P = 0.348). Serum zinc concentration had a strong positive association with serum TAS (P < 0.001) but not RBC SOD (P = 0.178), nor serum or RBC GPX (both P > 0.05).

Like copper, serum zinc was positively associated with serum total cholesterol (P = 0.001) and serum LDL-cholesterol (P < 0.001) but negatively with serum HDL-cholesterol (P = 0.005). Serum zinc concentration was also positively associated with BMI (P = 0.011) but negatively with socioeconomic status (P = 0.003).
Characteristics of copper and zinc status groups

Dietary and lifestyle characteristics of the various copper and zinc status groups within the population sample are summarised in Table 6.14

Subjects with copper intakes <AI

Of all the variables measured, the major commonality of subjects with low intakes appeared to be lower food consumption overall as indicated by lower energy intakes. There were small differences in the contribution of various food types, increased multivitamin and mineral usage and rates of smoking.

Subjects with zinc intakes <EAR

Subjects consuming <EAR of zinc were mostly older males. The lower intakes appeared to be related mainly to significantly lower food intake overall.

Subjects with serum zinc <WHO cut-off

Subjects with low serum zinc were mostly older males who consumed a diet with lower zinc nutrient density and a considerable reduction in zinc from meat sources. Men in this group had higher socioeconomic status and lower body mass index.
### Table 6.14: Copper and zinc status group characteristics in comparison to other study subjects.

<table>
<thead>
<tr>
<th>Subjects consuming &lt;AI copper (n = 242)</th>
<th>Subjects consuming &lt;EAR zinc (n = 127)</th>
<th>Subjects with serum zinc &lt;WHO cut-off (n = 49)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dietary intakes and contributors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- 24% lower mean energy intake in men (8.31 v. 10.94 MJ) (P &lt; 0.001); 29% lower energy intake in women (5.40 v. 7.62 MJ) (P &lt; 0.001).</td>
<td>- 24% lower energy intake in men (7.91 v. 10.47 MJ) (P &lt; 0.001); 41% lower energy intake in women (4.18 v. 7.06 MJ) (P &lt; 0.001).</td>
<td>- Energy intakes not significantly different. Nutrient density was 8% lower (P = 0.017) in subjects with low serum zinc.</td>
</tr>
<tr>
<td>- Contributions were higher from meat (8 v. 6%) and dairy products (6 v. 5%), but lower from cereal-based foods (26 v. 29%) and fruit (14 v. 17%) (all P &lt; 0.001).</td>
<td></td>
<td>- Meat sources provided 28% less zinc in men (P &lt; 0.001).</td>
</tr>
<tr>
<td><strong>Supplement use</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Compared to others, subjects consuming &lt;AI used multivitamin/mineral (18% v. 28%) and mineral (13% v. 24%) supplements less frequently</td>
<td>- Use of combined multivitamin &amp; mineral supplements was similar (24% v. 19%). Only 13% used individual mineral supplements (eg calcium) compared to 21% of other subjects.</td>
<td>- Use of combined multivitamin &amp; mineral supplements was similar compared to other subjects (30 v. 23%), as was use of individual mineral supplements (25 v. 18%).</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- No significant difference in age, gender distribution or socioeconomic status.</td>
<td>- No significant difference in smoking or socioeconomic status.</td>
<td>- Men had 9% lower BMI than other subjects (P &lt;0.001), similar trend in women did not reach significance (P =0.063)</td>
</tr>
<tr>
<td>- 12% of this group were current smokers compared to 4% of all other subjects</td>
<td>- Mean age of male subjects was 5.6 years older than other subjects (56.0 v. 61.6 yrs; P = 0.001).</td>
<td>- No significant difference in smoking rates.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- In men, socioeconomic status was significantly higher (P = 0.003).</td>
</tr>
</tbody>
</table>
6.5.3 Discussion

Copper

The assessment of nutritional adequacy for copper is hindered by the lack of an Australian EAR value for copper intakes, and uncertainty over target levels of biochemical indices such as serum copper. Copper intakes are also likely to be underestimated when the copper content of drinking water is not considered in daily intakes. The major source of copper in drinking water is the copper pipes and fittings commonly used in household plumbing which, while relatively stable, can contribute significant amounts of copper to water with low pH or low hardness levels. The NHMRC Australian Drinking Water Guidelines (2004) suggest that copper content in drinking water should not exceed 2 mg/L and that while copper in major Australian reticulated supplies copper may range as high as 0.8 mg/L, typical levels are approximately 0.05 mg/L.

Dietary intakes

A large proportion of the study subjects were estimated to consume copper at levels below the AI. Such an estimate however cannot be used to suggest an inadequacy in a population in the same way as one can assess intakes using the EAR cut-point, as the AI is simply a value which is expected to exceed the requirements of almost all members of a given age or gender group. An AI value will be above the true nutrient EAR or RDI value, so when a group has a mean intake above the AI, it suggests that the prevalence of inadequacy is likely to be low. Because of the uncertainty of the specific nutrient requirements, mean intakes below the AI cannot be used in the same way to indicate the prevalence of inadequacy.
In the current study, men had mean intakes below the AI, while women’s intakes were above the AI. Given the limitations in using an AI for nutritional assessments, the only conclusion that may be made is that in the women in this study, an inadequacy of copper intake is probably unlikely.

Comparison of copper intakes from the current study with the US EAR of 0.7 mg/d produces an interesting result. In this case 20 of 498 subjects (4%) had intakes that fell below the US EAR which just exceeds the 3% level which has been suggested by the US Food and Nutrition Board/Institute of Medicine to indicate increased risk of inadequacy (Food and Nutrition Board 2000c). Of further interest is that 19 of the 20 were females which equates to 6% of the female subjects in this study. While this EAR is a US value and the Australian AI is based on Australian intake data, the discrepancy between the findings based on AI and EAR values highlights the uncertainty that exists in copper requirements and hence the current difficulty associated with assessing nutritional adequacy in populations.

Despite men consuming significantly more copper, and women eating a more copper dense diet, there were only minor differences in the contribution of the various food groups to copper intakes. As with selenium, men consumed more copper from mixed dishes (which includes many takeaway style foods) and alcoholic beverages, as well as meat products, and women having increased intakes from vegetables, fruit and dairy foods. The contribution of meat to copper intakes appeared to decrease slightly with increases in age, while intakes from fruit and vegetable sources rose, a trend which may be due to some of the older subjects having difficulty consuming meat meals. Unsurprisingly, the youngest age range consumed significantly more copper from mixed dishes, but what was perhaps surprising was that this group consumed the lowest amount from alcoholic beverages. This was not due to differences in the type
of alcoholic beverages consumed, but rather due to lower reported total consumption of alcohol in these younger subjects.

There were few significant differences across the age ranges in either gender, however copper intakes and serum copper concentrations generally followed the same trend; the age ranges that consumed the lowest and highest copper intakes also had the lowest and highest serum copper concentrations respectively.

Mean adjusted copper intakes (μg/MJ/d) were significantly lower in smokers compared to the non-smoking subjects, which may be explained by a greater contribution from meat and a reduction from some cereal-based foods and fruit, foods which have comparatively higher copper-energy densities.

Copper intakes in the current study were lower than in most of the previously reported Australian data. Mean intakes of women were 10% lower than the reported intakes in a study of Victorian women estimated using food frequency questionnaires (Cleverdon 2003). Only the intakes in the 25-34 yrs (1.56 mg/d) and 75-84 yrs age range (1.50 mg/d) approached the mean intake of 1.56 mg/d reported in the Victorian study. This study also reported lower intakes in young women in contrast to the current study where young women consumed the highest mean intakes. The author suggested the lower intakes in young women were related to higher consumption of takeaway style foods, a trend that, though not statistically significant, was evident in this study. Despite this similarity, the young women in the current study consumed relatively high copper intakes, which appeared to be due to greater total energy intakes in this age range.

The international Total Diet Study (Parr et al. 2006) estimated copper intakes from different Australian cities by analyzing six daily diet samples by neutron activation analysis. This study's median intake of 1.83 mg/d was far above the current study
median intake of 1.4 mg/d, however a limited sample was used, the methodology was direct measurement rather than diet recording and a lack of demographic data hinders any comparison. The Total Diet Study involved over 20 countries, and while its estimate of 1.82 mg/d placed Australia towards the top of the range for intakes among these countries, the median intake of the current study appears moderate on this scale, being of a similar level as the reported intakes from Italy, China and Thailand.

The 20th Australian Total Diet Survey undertaken by FSANZ (2003) only reported intakes per kg bodyweight for men and women aged 25-34 yrs. The estimate of 16 μg/kg/d for men was higher than the current study estimate of 14.8 μg/kg/d for men of this age. However, women in the current study consumed considerably more compared to the Total Diet Survey estimate (19.5 v. 14 μg/kg/d).

**Serum copper**

An assessment of nutritional adequacy based on serum copper is equally problematic as there is currently no accepted serum concentration cut-off like that adopted by the WHO for zinc (Gibson *et al.* 2008), or the selenium level widely associated with maximal GPX activity (Elsom *et al.* 2006). In the absence of such cut-off target values, the only comparison that can be made for serum copper concentrations is with the clinical reference range, and in this study less than 1% of subjects had serum copper concentrations below the lower reference range of 11 μmol/L, indicating that the prevalence of clinically low serum copper in this sample was particularly low.

Men consumed more copper than women; however, across all age ranges women had higher mean copper concentrations. This is not unexpected due to the influence that female hormones and particularly the use of oral contraceptives are known to have on serum copper concentrations (Johnson *et al.* 1992; Louro *et al.* 2001). The influence
of hormones may explain the highest serum copper in women occurring in the peak childbearing and oral contraceptive usage age range of 25-34 yrs and then tapering towards old age where hormone levels may be expected to fall. Serum copper in men, conversely, was relatively stable across the various age ranges.

There is no Australian data on serum copper from comparable subjects with which the data from the current study can be compared. There is also limited published data from elsewhere in the world; however serum copper concentrations from the current study are similar to these. The mean serum copper in men of 15.5 μmol/L was slightly higher than that reported from an English study (14.8 μmol/L; n = 95) (Ghayour-Mobarhan et al. 2005), and slightly lower than in Northern Ireland (17.9 μmol/L; n = 1144) (McMaster et al. 1992) and the US (17.5 μmol/L; n = 2113) (Ford 2000). The mean serum copper in women of 18.9 μmol/L compared to the same studies was also higher than the English data (17.5 μmol/L; n = 94), but lower than the Northern Ireland (19.0 μmol/L; n = 1055) and US values (20.9 μmol/L; n = 2461).
Zinc

**Dietary intake**

Dietary analysis in this study indicated that 26% of subjects consumed inadequate zinc intakes; with 52% of men and 9% of women consuming less than the EAR. The WHO has determined that the risk of zinc deficiency is elevated when the prevalence of inadequate intakes (<EAR) in a population is >25% (de Benoist et al. 2007). The estimate of 26% of subjects from this sample who consumed inadequate zinc did not vary significantly when an adjusted population estimate weighted for gender, age and socioeconomic status was made. So, while it appears females may not be considered to be at risk of deficiency by the WHO definition, men in this population are.

While there were no significant differences in mean zinc intakes between various age ranges, in men, the prevalence of sub-EAR intakes increased with each successive age range, until nearly three quarters of men aged 75+ years consumed inadequate zinc. A similar pattern was not observed in women where peak incidences of low zinc intakes occurred in the middle age ranges. In both men and women, sub-EAR intakes generally coincided with significantly lower energy intakes, suggesting that rather than a change in the types of foods eaten, and a subsequent decrease in nutrient density, the major difference was simply a reduction in the overall amount of food eaten in these subjects.

The large discrepancy between the proportion of men consuming inadequate zinc (52%) and those with low zinc status (15%) was not evident in women; a pattern that was also observed in the pilot study (Chapter 5). This perhaps suggests that for men in this population the EAR may be higher than required and hence sub-EAR intakes may not be as accurate as a predictor of the risk of zinc inadequacy. Determining the
possible cause of this is outside of the scope of this study, but may result from a greater bioavailability of zinc in the foods consumed or a lower zinc turnover/requirement in this population.

Major contributing food types were meat, cereal-based foods, vegetables and dairy foods. Meat was a significantly greater contributor to zinc intakes in men compared to women, and the higher bioavailability zinc from such sources (Lonnerdal 2000; Brown et al. 2004), is perhaps important for men in this sample to maximize the absorption from seemingly inadequate intakes.

Men in this study consumed slightly less zinc than estimated in the only other published Tasmanian data. The National Nutrition Survey (NNS) estimated zinc intakes of 13,858 Australian participants aged 19+ years (McLennan and Podger 1995). Tasmanian men in this study were estimated to consume 13.5 mg/d, which was lower than the national average for men of 14.4 mg/d. Intakes in men were also lower than those reported in a study by English and colleagues (1997) (17% lower) and in the 20th ATDS (FSANZ 2003) (13% lower). Women, however, consumed a similar zinc intake as estimated by English and colleagues (10.9 v. 10.5 mg/d), 20% more than Tasmanian women from the NNS and 30% more than estimated in the 20th ATDS. Whether these differences are a reflection of actual variation between the different populations sampled, or a product of the different methodologies, ranging from 24hr diet records to direct food analysis, is uncertain. There has also been some suggestion of changes in trace element contents in the food supply (Klevay and Medeiros 1996; Lyons et al. 2004). The comparison of intake data is therefore difficult between studies employing differing methodologies and those separated by considerable periods of time.
Serum zinc

Overall, 10% of the study subjects had serum zinc concentrations below the WHO cut-off. The WHO has determined that a population subgroup is considered at risk of zinc deficiency when >20% have serum zinc levels below the respective cut-off. By this definition this study cohort as a whole is not considered at increased risk. However, when the serum zinc of each gender and age range subgroup is compared with the cut-off, this finding is altered. Whilst overall only 15% of men and 7% of women have low serum zinc, the proportion of subjects with low zinc levels rises substantially in particular age ranges. In men over 55 years, 20% had low serum zinc, while 23% of those over 65 years and 57% over 75 years had low serum zinc. In women 30% of those over 75 years were also below the cut-off level.

This finding ties in with the dietary analysis, at least in the men, which estimates greater proportions of male subjects in the older age groups were likely to consume inadequate dietary zinc. In addition, a considerable decrease in protein intake in these men, particularly the oldest age group (17% decrease in protein compared to 25-34 yrs and a 23% decrease compared to 35-44 yrs) and not only is zinc intake decreased in these subjects but bioavailability of the zinc consumed may well be reduced compared to other subjects.

Men with low serum zinc were found to have a significantly lower BMI, but higher socioeconomic status, reflecting the associations both variables have with serum zinc over the whole sample. Because of the design of this study it is not possible to determine whether either have a causal relationship on serum zinc. BMI has previously been found to be inversely associated with serum zinc (Ghayour-Mobarhan et al. 2005), which may be because people with higher body mass have higher zinc requirements. In this study, however, the lower BMI of subjects with low serum zinc
most likely results from the direct relationship between energy intakes and zinc intakes.

The inverse relationship of socioeconomic status with serum zinc appears to be in contrast with an association that is commonly observed between higher socioeconomic status and higher quality diets with increased diet nutrient density (Giskes et al. 2002; Beydoun and Wang 2008). In this study, zinc intakes, both absolute and adjusted, were not associated with socioeconomic status. The contribution of meat to zinc intakes appeared to be lower with increasing socioeconomic status. However, when analysed on a gender basis, this association was only observed in men, and was only statistically significant in the older age range, suggesting this effect was probably more related to age than socioeconomic status.

In Australia, data on serum zinc concentrations is very limited. The only study to report serum zinc was a small study of 42 participants (Ball and Ackland 2000). Men (13 μmol/L; n = 21) and women (13.9 μmol/L; n = 21) in this study had mean serum zinc similar to the current study.

Comparable data from healthy adult populations in other countries is also limited. A small study in England (Ghayour-Mobarhan et al. 2005) reported similar serum zinc levels in a sample of slightly younger men (13.9 μmol/L) and women (13.3 μmol/L) (mean age 45 and 49 years respectively). Reported serum zinc levels were also similar in a large Northern Ireland study (McMaster et al. 1992) for both men (13.2 μmol/L; n = 1142) and women (12.7 μmol/L; n = 1034). This study reported a significant decline in serum zinc concentrations with age in men, but not in women. In a New Zealand study (De Jong et al. 2001) of elderly women (mean age 74.9 years; n = 102), mean serum zinc (12.4 μmol/L) was higher than women of a similar age range in the
current study (11.6 µmol/L). All of the aforementioned studies utilized similar FAAS methodology for serum zinc analysis.

**Associations with cardiovascular risk factors**

In this study both serum copper and serum zinc were observed to have significant associations with cardiovascular risk factors such as serum total cholesterol, serum LDL-cholesterol and BMI. Such findings are not unexpected, particularly for copper, and have been reported in a number of other studies (Salonen et al. 1991; McMaster et al. 1992; Abiaka et al. 2003; Ghayour-Mobarhan et al. 2005).

**Copper**

Copper has been associated with various cardiovascular risk factors, atherogenesis (Salonen et al. 1991) and cardiovascular disease (Ford 2000; Leone et al. 2006).

In this study, copper was positively associated with serum total cholesterol, serum LDL-cholesterol and BMI in concordance with several studies (Salonen et al. 1991; McMaster et al. 1992; Abiaka et al. 2003), but is in contrast to others that have observed negative relationships between copper and such risk factors (Stern et al. 2007). Such disagreement suggests that the relationship between copper and various cardiovascular risk factors is likely complicated by many factors, such as dietary intake levels, lifestyle factors and possibly genetic variability.

**Zinc**

In this study serum zinc was also observed to be positively related to serum total cholesterol and serum LDL-cholesterol but negatively with serum HDL-cholesterol. In contrast to copper, where there have been numerous studies indicating links
between copper status and various risk factors for cardiovascular disease, including serum lipids, findings of similar relationships with zinc are limited. A decline in serum HDL-cholesterol during high dose zinc supplementation has been observed in several studies (Hughes and Samman 2006), which is concordant with the inverse relationship in the current study between zinc and HDL-cholesterol. Given the wide range of roles in which zinc is involved through the numerous zinc proteins, it may be involved in lipid metabolism but at this point the basis for any such relationships is unknown, and hence any relationship has unknown significance in this population.

6.5.4 Conclusion

The assessment of copper status in this sample is limited by the constraints of our current incomplete understanding of copper requirements and their relationship to biomarkers such as serum copper. With this in mind, using the current Australian dietary recommendations as the basis of assessment, the copper status of women in this population does not appear to be inadequate; however the results are inconclusive for men at this time. If in the future, advancements in our knowledge of copper requirements can occur, the data from this study or the stored samples may be revisited or reanalysed and provide a more useful indication of copper status in this population.

The assessment of zinc status has indicated that a significant proportion of the population may consume inadequate zinc. Dietary intake data indicated that the prevalence of low zinc intakes exceeded the criteria used by FSANZ (>3% consuming <EAR) to indicate an increased risk of inadequate zinc intake (Food and Nutrition Board 2000c) and that used by the WHO to suggest an elevated risk of zinc deficiency (de Benoist et al. 2007). Older subjects and older men in particular, were at increased
risk of zinc deficiency. Interestingly, subjects with lower BMI also appeared to be at greater risk of low zinc status, which maybe simply due to a lower overall dietary intake in these subjects.
Chapter 7

General discussion and conclusions

The finding of selenium deficiency in the past in livestock across Tasmania has led to the development of a hypothesis that the human population of Tasmania may be at risk of inadequate selenium intakes. Previous small studies in the Tasmanian population have been limited and provided conflicting results.

The research encompassed by this thesis focused on assessing the selenium status in a population sample from the north of the state, on which there was no previous data. The work toward this primary objective presented an opportunity to also assess the status of two other important trace elements, copper and zinc, for which published data is very limited in Australian populations. The study included concurrent dietary, lifestyle and blood analysis. A novel aspect was an initial assessment of the influence of some genetic factors, such as the \textit{HFE} polymorphisms involved in hereditary haemochromatosis, and \textit{GPx-1} polymorphisms.

This thesis therefore contributes significantly to the field of trace element nutrition.

7.1 Preliminary research

The findings from preliminary research (Chapter 5) suggested that a significant proportion of people in a convenience sample from northern Tasmania may have selenium intakes and serum selenium concentrations below that associated with maximal selenoprotein activity, and well below levels where the chemopreventative effects of selenium are postulated to be achieved. Copper intakes did not appear inadequate but, although subject numbers were low, there appeared a potential for a subset of men to have clinically low serum copper levels. There was also an indication
that a substantial proportion of men, especially older men, may be at risk of inadequate zinc status.

An opportunity was also taken to investigate whether hereditary haemochromatosis, a genetic disorder of iron metabolism common in Tasmania, affected the status of other trace elements such as selenium, copper and zinc, and there appeared to be no major effect.

7.2 Population study

Selenium

The large cross-sectional population study, based on the electoral roll, was conducted primarily to investigate the status of selenium, but also copper and zinc, in a more representative sample from this region of Tasmania (Chapter 6). The assessment of selenium status in a sample of nearly 500 subjects further supported the hypothesis that selenium intakes are inadequate in northern Tasmania. Approximately one quarter of both men and women consumed less than the EAR for selenium and eight out of every ten participants had a serum selenium concentration below that associated with selenoprotein requirements. Prevalence of this marginal selenium status was generally similar across gender and age ranges and independent of socioeconomic status; however the elderly appeared to be at increased risk possibly due to an overall lower food intake. Smokers were also at increased risk of reduced selenium status; having approximately 10% lower serum selenium than non-smoking subjects.

Due to the variations in the response from different gender and age subgroups, a population estimate weighted for age, gender and socioeconomic status, using data from the 2006 Census, was made. This was not significantly different to the sample estimate and therefore suggests that the prevalence of marginal intakes and serum
levels in the population of northern Tasmania is likely to be similar to that estimated in the sample.

Serum selenium levels were considerably lower than those in a recent study of selenium status in the south of the state (Greater Hobart area), and while there were methodological differences, it raises a question of whether there may be variations in selenium status between different regions in the state. While the geographical area is small, significant regional variations in selenium status have been observed elsewhere in relatively small countries. A demonstration of regional variation in nutrient intakes in Tasmania may be significant and might be associated with the particularly high rates of chronic disease in certain areas in the north of the state.

While further evidence from large randomised controlled trials is still required, a body of evidence suggests that people with marginal selenium status, as observed in this sample, could obtain possible chemopreventative benefit by increasing their selenium intakes. The chemopreventative action of selenium is currently thought to coincide with serum selenium levels >1.50 μmol/L; in this sample, almost all subjects (97%) were below this level. If, in the future, selenium supplementation in people with marginal selenium status can be shown to have real benefits, there could be significant implications for this population. These findings, however, must be considered in light of some observations that selenium status in the higher range of normal may be associated with adverse health effects.

Recent reports suggest that higher selenium status (Bleys et al. 2007), or selenium supplementation in subjects with already adequate selenium status, (Stranges et al. 2007; Lippman et al. 2009) may be associated with increased risk of diabetes. Positive associations between selenium and fasting glucose levels have also been reported (Czernichow et al. 2006), while high levels of selenoproteins such as GPx have
previously been associated with insulin resistance in mice (McClung et al. 2004) and humans (Chen et al. 2003).

Positive associations between selenium status and higher levels of serum lipids have also been reported recently, both in high selenium status populations such as the US (Bleys et al. 2008) as well as a marginal selenium status population in the UK (Stranges et al. 2009). As these were cross-sectional studies, further work in this area is required to determine the basis of such associations.

Although these effects were mostly observed in populations with relatively high selenium status, and in some cases, in studies where secondary endpoints were used, it appears caution must be taken prior to recommending supplementation for populations which are perhaps marginally selenium replete. It seems apparent that from emerging evidence regarding the influence of genetic variation among selenoproteins on the response to dietary selenium and associations with chronic disease (Meplan et al. 2007; Voetsch et al. 2007; Cooper et al. 2008; Jablonska et al. 2008; Meplan et al. 2008), it may be inappropriate to suggest supplementation for all members of such a population. In selenium replete populations even greater caution may be required. In reality, the optimal selenium status probably varies from person to person based on genetics, gender, age and lifestyle factors such as smoking, and as such determining ideal intakes may be difficult without in-depth investigation of individuals.

Due to the influence of genetic variation in selenoproteins, subjects in this population sample were genotyped for the GPx-1 Pro198Leu polymorphism. Although a number of studies have indicated a significant effect of some selenoprotein gene SNPs on protein product activities, there has been no previous report on the influence of the GPx-1 Pro198Leu polymorphism, an SNP associated with decreased GPx activity, on
selenium status indicators in an Australian population. This study was unable to detect a significant effect of GPx-1 Pro198Leu genotype on selenium status indicators, although the sample size may have been inadequate. Growing evidence relating to the importance of such polymorphisms should ensure they remain of interest because of the potential association with decreased selenoprotein activity, some cancers and cardiovascular disease.

Copper and zinc
The population study of copper and zinc status was a first in Australia; previous research on these trace minerals has been much smaller in scale, in specific population sub-groups and collected less detailed data. Internationally, large studies are also relatively scarce, despite growing awareness of the importance of copper and zinc, and suggestions that a large proportion of the world's population has micronutrient deficiencies. While the copper data from this study was inconclusive, mostly due to the lack of definitive nutritional assessment criteria for this nutrient, the findings from the assessment of zinc status indicated that certain subsections of the population, particularly older men, may be at risk of mild zinc deficiency.

The proportion of subjects who consumed inadequate zinc exceeded the threshold of 25% used by the WHO to define populations it considers to have an elevated risk of deficiency. When compared to the same organisations' cut-offs for serum zinc, the overall proportion of subjects with low serum zinc did not exceed the level (20%) used to identify populations considered at risk of zinc deficiency. However, 20% of the sub-group of men older than 55 years had low serum zinc, and the prevalence rose further with age. Over 20% of women aged 75+ years also had low serum zinc. A weighted population estimate suggested that the prevalence of low zinc intakes and
serum levels in the northern Tasmanian population would not vary significantly from this sample.

7.3 Study limitations

Limitations of this research include the general limitations of a cross sectional study design. That is, the data collected is a snapshot of trace element status at a single point in time for each subject. Various personal factors, such as short term health and lifestyle could affect status over time. It is not possible to determine causality for the associations detected between the variables measured.

The main limitations specific to this study are associated with the sample composition and the methodology for estimating dietary trace element intakes.

Despite using a randomly selected database extracted from the electoral roll in the target regions, the variable response meant the final sample composition had a greater representation of females and older subjects. In key data analyses, weighted population estimates using Census age, gender and socioeconomic distribution data were thus used to correct for these sampling imbalances. Differences in the sample and population estimates were not significant for any of the selenium or zinc analyses, and only just reached statistical significance for the dietary copper analysis.

Dietary intake estimates have some general limitations and some specific to this study. Although they may be validated for specific nutrients, research using doubly labelled water has demonstrated that the FFQ can underestimate energy intakes significantly. The FFQ relies on a subject's memory as it requires subjects to recall both frequency and the portion sizes of foods eaten. Less educated, elderly subjects or those with poor eyesight may have difficulty completing the questionnaire accurately, leading to under or over reporting of food and nutrient intakes. A desire for social acceptability may also lead subjects to report consumption of various foods or
beverages, such as alcohol, differently. Perhaps most importantly, the FFQ is reliant on food content data tables to determine nutrient intakes. This is particularly important as the source of food content data varies; for specific nutrients and food types there may be very few measurements or the results may be calculated or borrowed from overseas tables. While such variation in data sources may not result in significant differences for some macronutrients, they may introduce significant error for nutrients that could be expected to vary geographically, as selenium was in this study. Should the reported values for such a nutrient not be representative of the foods commonly consumed in the geographical region being studied, intake estimates generated could well be inaccurate. In this case it is probably likely given the various sources of food content data for selenium that are currently used in Australia (see Appendix 1). Comparison of estimated nutrient intakes with other reported intakes determined using direct food analysis is also problematic for the same reason. Finally, the assessment of copper status in this population was compromised by the current lack of clear dietary requirements for copper in Australia, or any accepted nutritional assessment criteria using biomarkers such as serum copper.

7.4 Future research

The current research has indicated that the prevalence of marginal selenium was similar across age range and gender sub-groups and appeared to be independent of socioeconomic status. Future research on this topic should include assessment of selenium status of a large electoral roll sample from across the whole of Tasmania using the same methodology, to determine if the prevalence of marginal selenium status is similar in different regions. A survey of Tasmanian foods eaten, plus the analysis of local foods, would be useful to determine what differences there may be in
the major contributors to intakes, compared to the values in current Australian data tables. Studies of selenium status in other states may also be warranted.

This research also indicates that men and older subjects in Tasmania may be at risk of mild zinc deficiency. Given the importance of zinc in many areas of human physiology, the findings in northern Tasmania suggest further investigation is warranted in Australia as a whole.
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## Appendix 1

Examples of NUTTAB06 food content table derivation codes (FSANZ 2006).

<table>
<thead>
<tr>
<th>Food</th>
<th>Derivation code</th>
</tr>
</thead>
<tbody>
<tr>
<td>12C10166 Chocolate, Milk</td>
<td>A</td>
</tr>
<tr>
<td>09D10130 Ice Cream, Vanilla, Reduced Fat</td>
<td>LAB</td>
</tr>
<tr>
<td>09A10077 Milk, Skim</td>
<td>A</td>
</tr>
<tr>
<td>09C10024 Yoghurt, Vanilla</td>
<td>A</td>
</tr>
<tr>
<td>12D10022 Jelly, Prepared, Sugar Sweetened</td>
<td>R</td>
</tr>
<tr>
<td>02A20040 Flour, White, Breadmaking</td>
<td>NNS</td>
</tr>
<tr>
<td>02A10148 Rice Bran, Processed</td>
<td>IND</td>
</tr>
<tr>
<td>02A10146 Oat Bran, Unprocessed</td>
<td>US</td>
</tr>
<tr>
<td>02D10108 Muesli, Toasted</td>
<td>A</td>
</tr>
<tr>
<td>02B10126 Bread, White</td>
<td>A</td>
</tr>
<tr>
<td>06B10036 Orange, Navel, Leng, Raw, Peeled</td>
<td>A</td>
</tr>
<tr>
<td>13A10793 Carrot, Mature, Peeled</td>
<td>A</td>
</tr>
<tr>
<td>13A10979 Bean, Butter, Fresh, Boiled</td>
<td>R</td>
</tr>
<tr>
<td>13A10901 Zucchini, Green Skin</td>
<td>A</td>
</tr>
<tr>
<td>13A10912 Tomato, Common</td>
<td>A</td>
</tr>
<tr>
<td>05A10282 Tuna, Canned In Water, Drained</td>
<td>IMP</td>
</tr>
<tr>
<td>08A10338 Beef, Round Steak, Grilled, Semi Trimmed</td>
<td>A</td>
</tr>
<tr>
<td>05A10319 Sardine, Canned In Tomato Sauce</td>
<td>B</td>
</tr>
<tr>
<td>05A10276 Sardine, Canned In Water, Unsalted, Drained</td>
<td>UK</td>
</tr>
<tr>
<td>08E30153 Ham, Leg, Non Canned, Lean</td>
<td>A</td>
</tr>
<tr>
<td>08C10166 Chicken, Breast, Baked, Lean</td>
<td>A</td>
</tr>
<tr>
<td>08E20065 Sausage, Beef, Fried, Home Prepared</td>
<td>A</td>
</tr>
</tbody>
</table>

A – **Analysed**: derived from food analysis

B – **Borrowed**: taken from overseas food composition table (country of origin denoted as US, UK or NZ)

C – **Calculated**: calculated by FSANZ due to lack of suitable data

IND – **Industry**: Provided by Australian food manufacturer; generated by various means

IMP – **Imputed**: nutrient value assumed from similar food type

LAB – **Label**: derived from food label information

NNS – **National Nutrition Survey**: derived from data generated from the 1995 National Nutrition Survey

R – **Recipe**: derived from individual ingredient proportion of common recipe
Appendix 2
Table: Gender comparison of copper and zinc data in currently smoking and non-smoking population study subjects.

<table>
<thead>
<tr>
<th></th>
<th>Current smokers</th>
<th>Non-smokers</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Men</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy, MJ</td>
<td>9.05 ± 0.90</td>
<td>9.12 ± 0.93</td>
<td>0.935</td>
</tr>
<tr>
<td>Cu intake, mg/d</td>
<td>1.35 ± 0.17</td>
<td>1.58 ± 0.17</td>
<td>0.179</td>
</tr>
<tr>
<td>Cu intake, µg/kg/d</td>
<td>16.3 ± 2.5</td>
<td>18.6 ± 2.5</td>
<td>0.346</td>
</tr>
<tr>
<td>Cu intake, µg/MJ/d</td>
<td>0.15 ± 0.01</td>
<td>0.18 ± 0.13</td>
<td>0.059</td>
</tr>
<tr>
<td>Zn intake, mg/d</td>
<td>11.8 ± 1.9</td>
<td>12.6 ± 1.9</td>
<td>0.670</td>
</tr>
<tr>
<td>Zn intake, µg/kg/d</td>
<td>133.8 ± 13.0</td>
<td>150.3 ± 13.7</td>
<td>0.226</td>
</tr>
<tr>
<td>Zn intake, µg/MJ/d</td>
<td>1.28 ± 0.09</td>
<td>1.42 ± 0.10</td>
<td>0.168</td>
</tr>
<tr>
<td>Serum Cu, µmol/L</td>
<td>17.6 ± 0.6</td>
<td>15.4 ± 0.7</td>
<td>0.001</td>
</tr>
<tr>
<td>Serum Zn, µmol/L</td>
<td>12.5 ± 0.7</td>
<td>13.0 ± 0.8</td>
<td>0.455</td>
</tr>
<tr>
<td>RBC SOD, U/g Hb</td>
<td>1377.3 ± 70.6</td>
<td>1321.4 ± 72.8</td>
<td>0.443</td>
</tr>
<tr>
<td><strong>Women</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy, MJ</td>
<td>6.35 ± 0.51</td>
<td>6.86 ± 0.53</td>
<td>0.339</td>
</tr>
<tr>
<td>Cu intake, mg/d</td>
<td>1.24 ± 0.14</td>
<td>1.43 ± 0.14</td>
<td>0.177</td>
</tr>
<tr>
<td>Cu intake, µg/kg/d</td>
<td>20.0 ± 2.0</td>
<td>21.0 ± 2.1</td>
<td>0.640</td>
</tr>
<tr>
<td>Cu intake, µg/MJ/d</td>
<td>0.19 ± 0.01</td>
<td>0.22 ± 0.01</td>
<td>0.088</td>
</tr>
<tr>
<td>Zn intake, mg/d</td>
<td>10.5 ± 1.0</td>
<td>10.9 ± 1.0</td>
<td>0.623</td>
</tr>
<tr>
<td>Zn intake, µg/kg/d</td>
<td>161.3 ± 15.1</td>
<td>160.5 ± 15.4</td>
<td>0.956</td>
</tr>
<tr>
<td>Zn intake, µg/MJ/d</td>
<td>1.65 ± 0.06</td>
<td>1.65 ± 0.07</td>
<td>0.982</td>
</tr>
<tr>
<td>Serum Cu, µmol/L</td>
<td>18.9 ± 0.6</td>
<td>18.8 ± 0.7</td>
<td>0.880</td>
</tr>
<tr>
<td>Serum Zn, µmol/L</td>
<td>13.9 ± 0.5</td>
<td>13.0 ± 0.5</td>
<td>0.050</td>
</tr>
<tr>
<td>RBC SOD, U/g Hb</td>
<td>1299.9 ± 37.7</td>
<td>1295.0 ± 41.0</td>
<td>0.905</td>
</tr>
</tbody>
</table>