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ABSTRACT

Iodine deficiency was not considered a major public health problem in Australia in the 1990s. However, the Australian National Iodine Nutrition Study in the early 2000s provided evidence of the resurgence of iodine deficiency in the Australian population. As a result, mandatory iodine of bread flour was introduced in Australia in late 2009. However, while several studies had assessed the iodine status of Australian children post fortification there were limited data regarding the impact of fortification on the iodine status of pregnant and lactating women and their infants.

The results of this thesis demonstrate that the urinary iodine concentrations (UIC) of lactating, pregnant women and their infants in South Australia post iodine fortification are consistent with an iodine sufficient status, independent of the intake of iodine supplements. However, iodine status of women who did not consume iodine supplements during pregnancy may be suboptimal as indicated by a borderline UIC level.

Breast milk is a sole source of iodine for exclusively breastfed infants, making the measurement of iodine concentration in breast milk clinically relevant. However, there had been limited previous attempts to assess breast milk iodine concentrations (BMICs), largely due to the lack of robust methods for routine analysis. This thesis describes the development and validation of a new method for assessing iodine concentrations in human breast milk. This method was subsequently applied to measure BMIC in samples collected from women from the same region of South Australia before and after the introduction of mandatory iodine fortification. Median BMICs post fortification was well above the suggested cut-off for providing a sufficient iodine supply for full-term infants. Importantly, the median BMICs in the post fortification samples were significantly higher than those of the women before mandatory iodine fortification, independent of iodine supplements, while the proportion of women in the sample with BMICs below 100µg/l was reduced by 28%. These data suggest
that mandatory iodine fortification and recommendations regarding iodine supplements in pregnancy and lactation have been effective in increasing the iodine supplied to the average South Australian infants.

Obesity and insulin resistance are currently major public health issues worldwide, and there is increasing evidence that the nutritional environment experienced in early life is an important determinant of long-term metabolic health. Chapter 6 of this thesis assessed relationships between markers of neonatal and current thyroid function and metabolic health of young children. Fasting glucose concentrations, HOMA-IR and height z-score in male children at 5 years of age were inversely related to neonatal TSH level at birth, however there was no evidence to suggest that current TSH or Tg concentrations were associated with measures of growth or insulin resistance at 5 years of age, in either males or females.

In conclusion, this thesis presents the first data regarding the iodine status of pregnant and lactating women and their infants after the introduction of mandatory iodine fortification, from a large and representative population, and has provided evidence that BMICs have been significantly improved since the introduction of iodine fortification in Australia. This adds important new information regarding the current iodine status of pregnant, lactating women and their infants in Australia, and provides insights into the potential role of neonatal iodine nutrition/thyroid status for long-term metabolic health.
DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Dao Hoa Anh Huynh and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Dao Hoa Anh Huynh
ACKNOWLEDGEMENTS

At the moment of accomplishment, it is my great pleasure to express my sincere thanks to all those who contributed to the completion of this thesis.

First and foremost, I am greatly indebted to my wonderful principle supervisor, Dr Beverly Muhlhausler for her support, encouragement and patience at every stage of my PhD. I am also thankful for her constructive criticism and fruitful discussion which helped me in achieving a good grasp of knowledge and improving my writing skill. This work would not have been possible without her guidance. I really appreciate for her support to help me overcome difficulties in my personal life.

I would like to express my sincere gratitude to co-supervisor, Dr Shao Jia Zhou for her invaluable advice and terrific support during my PhD journey. I also want to thank my co-supervisor, Prof Robert Gibson for providing me with the opportunity to complete my PhD thesis at FOODplus Research Centre.

I would also deeply like to thanks Mr Lyndon Palmer in Waite Analytical Service for his patience and guidance in laboratory technical skills; Dr John Carragher, for willing to help and inspire me with his best suggestion.

I want to thank present and past members of FOODplus Research Centre: Pamela Sim, Wei-Chun, and Zhi Yi Ong for sharing knowledge and laboratory technical skills. Thanks to Jing Zhou for collecting and pre-processing DOMInO blood samples. Also thanks to Ela Zielinski and David Apps for helping me with the sample collection at Waite. Thank you very much to Mini Vithayathil, Jessica Gugusheff, Liu Ge and Yichao for their support and encouragement. Thank you very much to Dominique Condo for supporting me in recruitments and sample collection. I would like to thank all colleagues and friends in FOODplus Research Centre and Waite Analytical Service their support and assistants that helped me with all aspects of my PhD study.
I also give my acknowledgement to staffs of DOMInO and PINK study in Women’s and Children’s Hospital and Flinders Medical Centre who helped me collect samples and data for my project. I really appreciate all participants of DOMInO and PINK study who kindly donated blood, urine and breast milk samples.
I dedicate this thesis....

...To my parents and my aunt, Le Nguyen, for always believing and supporting me throughout my life

...To my husband, Trung Nguyen, and my son, Khoa Nguyen, for bringing me so much love and happiness

...To my younger sisters, Ruby Huynh, Du Nguyen and Alanna Pham, for always supporting and consulting me in whatever I have been doing

COMMONLY USED ABBREVIATIONS

µg/l  microgram per litter
µIU  micro international unit
AI  Adequate Intake
BFB  body fat percentage
BFM  body fat mass
BIA  bioelectrical impedance analysis
BMI  body mass index
BMIC  breast milk iodine concentration
BMR  basal metabolic rate
BW  body weight
CI  confidence interval
CV  coefficient of variation
DEXA  dual-energy X-ray absorptiometry
DHA  Docosahexaenoic acid
DIT  Diiodotyrosine
DOMInO  DHA to Optimise Mother Infant Outcome
EAR  estimated average requirement
ELISA  enzyme-linked immunosorbent assay
HOMA-IR  homeostatic model assessment-insulin resistance
HPLC  high performance liquid chromatography
I/I0  Iodide/Iodine
ICCIDDD  International Council for the Control of Iodine Deficiency Disorders
ICPMS  inductively coupled plasma mass spectrometry
IMVS  Institute of Medical and Veterinary Sciences
IQR  interquartile range
ISI  insulin sensitivity index
ISIS  integrated sample introduction system
IUPAC  International Union of Pure and Applied Chemistry
LCPUFA  long chain polyunsaturated fatty acid
LOD  limit of detection
mg/kg  milligram per kilogram
min  minute
MIT  Monoiodotyrosine

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<td>SF</td>
<td>abdominal subcutaneous fat</td>
</tr>
<tr>
<td>T3/fT3</td>
<td>Triiodothyronine/free Triiodothyronine</td>
</tr>
<tr>
<td>T4/fT4</td>
<td>Thyroxine/free Thyroxine</td>
</tr>
<tr>
<td>Te</td>
<td>Tellurium</td>
</tr>
<tr>
<td>TFM</td>
<td>total fat mass</td>
</tr>
<tr>
<td>Tg</td>
<td>Thyroglobulin</td>
</tr>
<tr>
<td>TMAH</td>
<td>Tetramethylammonium hydroxide</td>
</tr>
<tr>
<td>TPO</td>
<td>Thyroperoxidase</td>
</tr>
<tr>
<td>TRH</td>
<td>Thyrotropin releasing hormone</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid stimulating hormone</td>
</tr>
<tr>
<td>UIC</td>
<td>urinary iodine concentration</td>
</tr>
<tr>
<td>UL</td>
<td>Upper Level</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
</tbody>
</table>
CHAPTER 1
CHAPTER 1 LITERATURE REVIEW

1.1 GENERAL OVERVIEW OF IODINE’S PHYSIOLOGICAL FUNCTION

Iodine is an essential trace element known to be important for thyroid function because it is required for the synthesis of thyroid hormones. Thyroid hormones have numerous roles in the human body, activating the transcription of a large number of genes and thereby regulating important biochemical processes, including protein synthesis and enzyme activity [1]. Thyroid hormones also play an important role in regulating metabolic processes in the body, including fat, carbohydrate and protein metabolism [2-4]. In addition, thyroid hormones are essential for the appropriate development of the central nervous system before birth and in early infancy.

1.1.1 Iodine and thyroid hormone synthesis

Iodine in food is reduced to iodide (I⁻) in the stomach, absorbed through the intestine into the blood and transported to the thyroid gland via the circulation. Iodine is taken up into the thyroid follicular (outer) cell by the action of a Na⁺/I⁻ symporter (NIS). Iodine then leaves the follicular cell and enters the lumen of follicle [5].

In parallel with the uptake of I⁻ into the lumen of the follicle, the follicular cell secretes thyroglobulin (Tg), which is the protein precursor of thyroid hormones and produced by the follicular cells of thyroid gland, into the lumen. The secretory vesicles that contain Tg also carry thyroperoxidase (TPO), which is an enzyme in the thyroid gland that releases iodine from thyroglobulin to produce triiodothyronine (T3) and thyroxine (T4). When the secretory vesicles reach the cell membrane this enzyme faces the follicular lumen and catalyses the oxidation of I⁻ to I⁰ [6].
TPO also catalyses a reaction in which iodine is bound to tyrosine residues in the Tg molecule to form moniodotyrosine (MIT) and diiodotyrosine (DIT). Under the catalysis of TPO, there is a coupling of either two DIT to form thyroxine (T4) or one DIT and one MIT to form triiodothyronine (T3). Tg molecules loaded with T3 and T4 are then transported back to the follicular cell where T3 and T4 are separated from Tg by intracellular enzymes and free T3 and T4 are released into the blood. A summary of this process is depicted in Figure 1.1.

![Figure 1.1: A schematic diagram of the key steps involved in thyroid hormone synthesis](image)

1.1.2 The regulation of thyroid hormone synthesis in the human body

1.1.2.1 The regulation of thyroid hormone synthesis in the general population

The thyroid gland is a part of the hypothalamic-pituitary-thyroid axis, and the synthesis of thyroid hormones is regulated by thyrotropin releasing hormone (TRH) in the hypothalamus and thyroid stimulating hormone (TSH) in the pituitary gland [7]. When circulating thyroid
hormone levels are low, a signal is sent to hypothalamus to stimulate TRH production. TRH then acts on the pituitary to increase TSH production which, in turn, acts on the thyroid gland to upregulate thyroid hormone production. Once thyroid hormone concentrations increase above basal levels, they exert a negative feedback effect on the hypothalamus to downregulate TRH production, which in turn reduces the release of TSH from the pituitary and thus switches off thyroid hormone synthesis. The interaction between thyroid hormones, TRH and TSH serves to maintain thyroid hormone levels in blood within a relatively narrow range in healthy individuals [7]. A schematic representation of the negative feedback regulation of thyroid hormone synthesis is shown in Figure 1.2.
Figure 1.2: Feedback regulation of thyroid hormone synthesis (adapted from [7])
Iodine plays a crucial role in neurodevelopment and physical growth during fetal life and early infancy, and iodine deficiency during the critical period from before birth until the end of the first year of life causes permanent impairments to brain function [8]. In early pregnancy, the fetus receives thyroid hormones solely from the mother because the fetal thyroid is not yet capable of synthesising thyroid hormones [9]. However, even after the fetal thyroid acquires the capacity for thyroid hormone synthesis, which occurs at around 10-12 weeks of gestation, the fetus continues to rely on iodine supply from the maternal circulation to achieve optimal growth and development, including development of the brain and nervous system [10].

The regulation of thyroid hormone synthesis during pregnancy

Pregnancy is a complex metabolic state involving significant alterations in the hormonal environment, including the increased levels of estrogen, progesterone and human chorionic gonadotropin (hCG) [11]. Hormonal changes and the modification of metabolic processes during pregnancy require an increase in thyroid hormone production [12]. Maintaining an adequate iodine intake is important for ensuring that the maternal physiological adaptations associated with these modifications in thyroid function can occur normally. From the outset of pregnancy, thyroid function is altered as a result of four key physiological changes that stimulate the maternal thyroid gland to produce more free thyroid hormones (Figure 1.3):

1. Increased thyroxine binding globulin (TBG) levels: TBG production in pregnancy is stimulated by the presence of the elevated levels of estrogen. TBG binds to thyroid hormones in the circulation and thereby decreases the level of circulating free thyroid hormone. This decrease is sensed by the pituitary, which therefore stimulates the thyroid to increase thyroid hormone production.
(2) Elevated hCG levels: hCG is an analogue of TSH and thus acts directly on the thyroid gland to stimulate thyroid hormone production.

(3) Increased renal iodine clearance: this decreases the availability of iodine for thyroid hormone production, which reduces circulating thyroid hormone levels and consequently stimulates thyroid hormone synthesis.

(4) Activity of type 2 and type 3 iodothyronine deiodinase in the placenta: this increases placental uptake of thyroid hormones and maternal T4 turnover which in turn upregulates maternal production of thyroid hormone.

These events occur at different stages of pregnancy, and are together responsible for driving the increased rate of thyroid hormone synthesis, and thus the increased demand of the thyroid gland for iodine, during pregnancy. This, in turn, is responsible for the increased iodine requirements of pregnant women compared to the non-pregnant female population.
High estrogen levels

Increased TBG levels

Decreased free thyroid hormone levels

Increased TSH level

Elevated hCG level during the first trimester

hCG-thyrotrophic activity stimulates thyroid gland

Changes in peripheral metabolism of thyroid hormones by the placenta during the second half of gestation

Increased glomerular filtration rate

Increased iodide clearance from the circulation

Fall in plasma iodine level

Stimulation of maternal thyroid gland to increase thyroid hormone synthesis

Increased maternal iodine requirements

Figure 1.3: Physiological adaption of the thyroid function during pregnancy (adapted from [13])
The regulation of thyroid hormone synthesis in the fetus

The fetal thyroid gland is able to accumulate iodide and synthesise thyroid hormones by about week 10-12 of gestation in humans [14]. Iodide is supplied to fetal thyroid gland from both the maternal circulation and the deiodination of iodothyronines in the placenta [13] and becomes an important source of substrate for fetal thyroid hormone production following maturation of thyroid synthetic capacity in the fetus. However, even after the onset of thyroid hormone synthesis in fetus, thyroid hormones derived from the maternal circulation continue to make an important contribution to fetal T4 concentrations and are important throughout pregnancy to ensure normal neurodevelopment [15]. In the case of fetal thyroid failure, maternal T4 transfer plays an even more critical role in protecting the fetus against cerebral T3 deficiency, since cerebral T3 levels during fetal and early postnatal development depend on its local generation from T4 by the activity of type 2 5’ iodothyronine deiodinase [16]. Cerebral T3 deficiency can lead to abnormal neurodevelopment outcomes [17].

The sodium/iodide symporter (NIS) is an iodide pump that actively transports iodide across cell membranes and iodine is transported across the placenta via a similar mechanism [18]. The presence of the NIS in the cytotrophoblast layer [19] has led to the view that the NIS may actively transport iodide from the maternal blood to the fetus [20], however, the precise mechanism remains unclear. In addition, placental deiodination of iodothyronine removes iodide moieties from T4 to form T3 and rT3. These iodides are also transferred to the fetus, where they can be utilised as substrates for the synthesis of thyroid hormones [13].

The physiology of iodine secretion into human breast milk

The mammary gland is capable of accumulating iodine during late pregnancy and lactation, and the iodine concentration of human milk is typically 20-50 times higher than that of maternal plasma [21]. The accumulation of iodine in the mammary gland and release of iodine into the breast milk occurs via the same mechanism as in the thyroid gland; iodine is
taken up into the mammary gland by a Na⁺/I⁻ symporter (mgNIS) and secreted into breast milk by the mammary gland pendrin transporter (mgPT) [22]. This active transfer of iodine from the maternal circulation into the breast milk means that around 40-45% of the iodine in the diet of breast feeding women is delivered to their infant via breast milk [23].

1.2 RECOMMENDED DIETARY IODINE INTAKE

1.2.1 Iodine requirements in pregnancy, lactation and infancy

As discussed earlier, iodine requirements during pregnancy, lactation and infancy are greater than those of the general population. This, coupled with recent evidence suggesting a resurgence of iodine deficiency in Australia, has led to the recommendations by the National Health and Medical Research Council of Australia (NHMRC) that all women considering pregnancy or who are pregnant or breast feeding should take iodine supplements providing 150µg/day of iodine, in addition to their normal diet [24, 25].
Table 1.1: Australia and New Zealand Iodine Reference Value

<table>
<thead>
<tr>
<th>Age</th>
<th>AI&lt;sup&gt;a&lt;/sup&gt; (µg/day)</th>
<th>EAR&lt;sup&gt;b&lt;/sup&gt; (µg/day)</th>
<th>RDI&lt;sup&gt;c&lt;/sup&gt; (µg/day)</th>
<th>UL&lt;sup&gt;d&lt;/sup&gt; (µg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnancy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-18 years</td>
<td>-</td>
<td>160</td>
<td>220</td>
<td>900</td>
</tr>
<tr>
<td>19-50 years</td>
<td>-</td>
<td>160</td>
<td>220</td>
<td>1100</td>
</tr>
<tr>
<td>Lactation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-18 years</td>
<td>-</td>
<td>190</td>
<td>270</td>
<td>900</td>
</tr>
<tr>
<td>19-50 years</td>
<td>-</td>
<td>190</td>
<td>270</td>
<td>1100</td>
</tr>
<tr>
<td>Infants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-7 months</td>
<td>90</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7-12 months</td>
<td>110</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Adequate intake (AI): the recommended average dietary intake level based on observed or experimentally determined approximations of nutrient intake by group(s) of apparently healthy people that are assumed to be adequate.

<sup>b</sup> Estimated average requirement (EAR) refers to a daily nutrient level estimated to meet the requirements for half of the healthy individuals in a particular life stage and gender.

<sup>c</sup> Recommended dietary intake (RDI) is the average daily intake level that is sufficient to meet the nutrient requirements of the vast majority (97-98%) of healthy individuals of a given sex and at a particular life stage.

<sup>d</sup> Upper level (UL) refers to the highest average daily nutrient intake likely to eliminate adverse health effects associated with inadequate intake of the specific nutrients in virtually all individual in the general population.

The EAR for pregnancy was based on data of the thyroid content of newborns, iodine balance studies and studies involving iodine supplementation during pregnancy [26]. The EAR for lactation was based on the iodine requirement for non-pregnant adult females (100µg/day) with the addition of estimated replacement needs for iodine secreted into breast milk (90µg/day). The RDI in both pregnancy and lactation was established based on assuming a CV of 20% for the EAR [26]. The UL of iodine intake for pregnant and lactating women was recommended as same as those established for adult.

The AI for infants aged from 0-6 months has been estimated based on multiplying the average daily intake of breast milk in breast fed infants in Australia (0.78l/day) by the estimated average concentration of iodine in breast milk (115µg/l) [26]. The AI for iodine for infants aged between 7-12 months was extrapolated from that of younger infants by applying a metabolic weight ratio [26].
Many current recommended values are derived using a number of assumptions and extrapolations. The current EAR and RDI values for pregnant and lactating women and infants in Australia have been adapted from the US:Canadian Dietary Reference Intake (DRI), which are based on data collected from studies investigating thyroid iodine content of the newborns, iodine balance and iodine supplementation during pregnancy [27]. The assumption to establish EAR of pregnant women in this approach has been based on the average iodine retention of fetus which was extrapolated from the average iodine content of newborn thyroid gland. However, this extrapolation may result in the overestimation of EAR because iodine content in newborn thyroid gland only reflect iodine retention in fetus at late gestation. Furthermore, this approach was deemed appropriate to establish the AI of infants because the iodine statuses of USA/Canadian vulnerable populations were assumed to be sufficient [27]. However, there has been a limited data about the breast milk iodine concentration supplying an adequate iodine intake for breastfed infants in USA/Canada. Therefore, the use of US/Canadian data may impact the accuracy of current Australian recommendations for this population. Zimmermann and colleagues have recently developed a method to estimate the EAR for iodine of a population based on spot UIC [28]. However, this method cannot be used to estimate iodine intake of pregnant and lactating women because it does not include iodine content transferred to fetus during pregnancy and to the breast milk during lactation [24]. Furthermore, UL for non-pregnant women is used for pregnant women because the assumption that no altered susceptibility of pregnant women to excess iodine has been addressed. However, there is a lack of data on the effect of this upper level on both pregnant women and their fetus. Therefore, further studies on the estimation of iodine intake of these populations are still required in order to provide more reliable/accurate estimates of the levels of dietary iodine intake required to achieve an iodine sufficient status in pregnant and lactating women and infants in Australia.
1.2.2 Natural sources of dietary iodine

1.2.2.1 Drinking water

Drinking water without iodine fortification contains iodine in the form of iodide and consequently its iodine content is easier to analyse than soils and vegetation. A wide range of studies have reported the iodine levels in drinking water in several countries. These studies have indicated that the median iodine content in drinking water varies considerably between countries. Several countries have been reported to have only low iodine concentrations (<10µg/l) in their drinking water, for example 4.55µg/l in US [29] and 3.3µg/l in Greenland (Denmark) [30]. In contrast, other countries, including the Shandong Province (China), have iodine levels in their drinking water in excess of 150µg/l [31]. Substantial variation in the iodine content of drinking water is also found between different geographical regions of the same country. A National Survey in China into the geographic distribution of iodine levels in drinking water for example, reported that of 1978 towns included, 488 had iodine levels in their drinking water of between 150µg/l and 300µg/l, while 248 had iodine levels in excess of 300µg/l [32].

According to a report by Nutrition Australia [33], the iodine content in drinking water in Australia and New Zealand ranges from 5-200µg/l depending on the region examined. Notably, one study from Gippsland (Victoria, Australia) reported a iodine level of only 0.38µg/l in tap water [34]. Although not the focus of this particular study, the authors also suggested that the iodine concentration in the drinking water could potentially serve as an important surrogate marker of iodine deficiency in a given region, and hence should be taken into account when estimating the contribution of iodine from dietary sources in a particular region.
1.2.2.2 *Food*

Iodine is present in a variety of foods; however most of the foods which form the basis of the typical Australia and New Zealand diets contain relatively low levels. Marine foods generally have the highest iodine content while fruits and vegetables contain the lowest amounts. In addition, the iodine content of plant-based foods depends on iodine content of the soil in which they are grown [35]. Several studies have also emphasised the importance of cooking methods for the iodine content of foods; these studies have suggested that almost 50% of the iodine present in food is lost if it is boiled during preparation [36-38]. On the other hand, approximately 90% of iodine amount in food is retained during roasting; suggesting that this is more efficient method for ensuring that the iodine content is retained during food preparation [36, 38].

Between the 1960s and 1990s, milk and dairy foods played an important role in supplying dietary iodine in Australia because of the use of iodine-based sanitisers in the dairy industry [39]. Although iodine levels in milk have declined since the replacement of these sanitisers with non-iodine based disinfectants, milk is still the dominant dietary source of iodine for the majority of Australians and contributes >40% of iodine intake for all age groups from 2 to 70+ years old [40].

1.3 **IODINE DEFICIENCY**

1.3.1 *Etiology*

Mannar & Dunn [41] suggested that the main causes of iodine deficiency in humans was iodine depletion of the soils in which staple foods were grown, rather than social or economic problems. Soil erosion [42] and deforestation for agriculture production [43] leads to the loss of iodine from the soil. Food grown in areas with iodine-deficient soil has a low iodine content which limits the supply of iodine for humans [41]. Iodine deficiency can also result
from dietary patterns/food choices which are deficient in iodine-rich foods, in particular limited intakes of marine-derived food [44]. Since marine-based foods are the richest dietary source of iodine, those populations which consume high amounts of foods such as seaweed and fish have significantly higher iodine intakes and urinary iodine concentration (UIC) on a population level than other countries. For example, Lee and colleagues [45] reported that the UIC of preschool Korean children was 438.8µg/l; with only 3.9% having a UIC<100µg/l (indicative of insufficient iodine intake) and 66.4% having a UIC>300µg/l. This high UIC is most likely a result of the high seaweed consumption of the Korean population [46]. High iodine intake (117.6µg/day) was similarly found in preschool children in Japan who consumed relatively high amounts of seaweed [47]. Iodine intakes in both these countries are much higher than in Australia, where the average UIC of preschool children was reported to be 129 µg/l (35% with UIC<100µg/l and 11% with UIC >300µg/l) prior to iodine fortification [48]. Population groups that eat no fish/seafood, such as vegetarians and vegans, are at particular risk of iodine deficiency [49, 50].

1.3.2 Prevalence

The number of countries classified as iodine deficient has decreased over the past decade from 54 in 2003 and 47 in 2007 [51] to only 30 in 2013 [52]. Furthermore, the prevalence of low iodine intake in school age children globally also decreased from 285 million in 2005 to 246 million in 2012 [53].

In spite of these declines, iodine deficiency is still a major global health issue. Iodine deficiency is estimated to affect approximately 2 billion people [54]. Furthermore, approximately 38 million newborns in developing countries are classified as iodine deficient every year [55].
1.3.3 Consequences of iodine deficiency

The physical manifestations of iodine deficiency are due to the presence of inadequate levels of thyroid hormones over extended periods of time. The spectrum of iodine deficiency disorders is listed in Table 1.2. [43]. The most visible consequence of iodine deficiency is a goiter, which results from the enlargement of thyroid gland. Iodine deficiency disorders occur at every stage of life. However, the most serious consequences of iodine deficiency occur when it is encountered during pregnancy, fetal life or early infancy [56].

Table 1.2: The spectrum of iodine deficiency disorders [43]

<table>
<thead>
<tr>
<th>Physiological group</th>
<th>Health consequences of iodine deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>All ages</td>
<td>Goiter</td>
</tr>
<tr>
<td></td>
<td>Hypothyroidism</td>
</tr>
<tr>
<td></td>
<td>Increased susceptibility to nuclear radiation</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>Spontaneous abortion</td>
</tr>
<tr>
<td></td>
<td>Stillbirth</td>
</tr>
<tr>
<td></td>
<td>Congenital anomalies</td>
</tr>
<tr>
<td></td>
<td>Perinatal mortality</td>
</tr>
<tr>
<td>Infancy</td>
<td>Endemic cretinism including mental deficiency with a mixture of mutism, spastic dysplegia, squint, hypothyroidism and short stature</td>
</tr>
<tr>
<td></td>
<td>Infant mortality</td>
</tr>
<tr>
<td>Childhood and adolescence</td>
<td>Impaired mental function</td>
</tr>
<tr>
<td></td>
<td>Delayed physical development</td>
</tr>
<tr>
<td>Adulthood</td>
<td>Impaired mental function</td>
</tr>
</tbody>
</table>

1.3.4 Specific risks of iodine deficiency in pregnant and lactating women

The importance of adequate iodine intake has been highlighted in the wide range of studies which have investigated the effects of iodine supplementation during pregnancy on neurodevelopmental outcomes in children. Recently, Zhou and colleagues [57] conducted a systematic review of iodine supplementation in pregnancy. Two of the 8 included randomized
controlled trials assessed developmental outcomes in the children and demonstrated that a reduced risk of cretinism and improved motor functions were found in children of mothers who used an iodised oil injection during pregnancy compared with children whose mothers did not [58-62]. Another review of 6 European prospective cohort studies by Trumpff and colleagues reported that mild iodine deficient status of pregnant women was found to be associated with negative effects on the neurodevelopmental outcomes of children from 3 to 10 years of age [63]. Trumpff’s review also indicated that the consumption of iodine supplements providing of 300µg of iodine/day in pregnant women living in mild to moderate iodine deficient regions resulted in improved measures of psychomotor development in their infants [63].

In addition to the effects of iodine on neurodevelopmental outcomes, there has been emerging interest in the potential impact of iodine status/thyroid function during pregnancy on metabolic health outcomes of the children. A population-based study was conducted in 5646 pregnant women in the Netherlands to explore the relationship between fT4/TSH levels in early pregnancy and markers of metabolic health of children [64]. The outcomes of this previous study demonstrated that fT4 levels were negatively correlated to both BMI and fat mass of children. However, maternal TSH levels were found to be associated with body fat mass, but not with BMI. This is the only study to date to evaluate the relationship between maternal and/or fetal thyroid hormone levels and later body weight and fat mass in the child, and further studies are needed to confirm these results.

Numerous epidemiological and experimental studies have emphasised the potential negative impacts of iodine deficiency/impaired thyroid function during pregnancy on the health of children, particularly in relation the cognitive function and, more recently, metabolic health. Knowledge and assessment of iodine status of pregnant and lactating women is therefore important for controlling or eliminating the adverse consequences of iodine deficiency on infants and children.
1.4 ASSESSMENT OF IODINE STATUS

Historically, a number of approaches have been utilised for the assessment of iodine status, the most widely used include: assessment of thyroid size, urinary iodine concentration (UIC) and the measurement of Tg and TSH concentration in serum or plasma [65]. Assessment of thyroid size involves palpation and ultrasonography. Palpation is a traditional and time-honoured method, however, this method is not sensitive to acute changes in iodine intake and requires highly skilled personnel [43]. The introduction of ultrasonography represented a significant advance in thyroid size assessment since it made the method more sensitive and less subjective, but it still requires appropriate machinery and electricity. In addition, even with highly trained operators, this method still has a high inter-observer variability [43, 66]. Thus, this method is not practical for studying large populations or for use in the field and does not appear to be particularly accurate.

1.4.1 Circulating Tg, TSH, T3 and T4 concentrations as indicators of iodine nutrition status

1.4.1.1 Thyroid hormone concentration (T3 and T4 levels)

A systematic review which used random-effects meta-analysis to assess the reliability of T3 and T4 concentrations as biomarkers of iodine status in humans suggested that serum T4, but not serum T3, provides an appropriate biomarker of iodine status in children, adolescents and adults [67]. Zimmermann, however, suggested that neither T3 or T4 concentrations in the serum provided a particularly sensitive indicator of iodine status [68]. This was based on his observations that T3 and T4 concentrations in iodine deficient populations often fell within the normal range and that there was a significant overlap in these concentrations with those found in iodine sufficient populations [68]. Furthermore, the methods for measuring T3 and T4 in plasma/serum are more cumbersome and expensive than those for measuring Tg and TSH [43].
1.4.1.2 TSH concentration

TSH is released from the pituitary gland in response to low thyroid hormone concentrations in the circulation. Thus, TSH levels increase when thyroid hormone levels in the circulation are low and decrease when thyroid hormone levels are high. Consequently, high serum TSH is used as indicator of iodine nutrition status in the absence of thyroid disease [65].

However, although TSH levels are elevated slightly in iodine deficient compared to iodine sufficient groups of adults and older children, the concentrations often still fall within the normal range [65]. This suggests that plasma/serum TSH is unlikely to be a useful marker of iodine deficiency in these population groups.

While TSH does not appear to be a reliable indicator of iodine deficiency in adults, TSH level in neonates is considered to be a sensitive indicator of iodine status. This is due to the fact that the thyroid gland in neonates has a low iodine content but high rate of iodine turnover, meaning that TSH increases much more rapidly in response to reduced iodine supply/reduced thyroid hormone production than in adults or older children [43]. Serum TSH concentration in newborns in iodine deficient areas is reported to be higher than levels seen in iodine-sufficient areas for the first few weeks of life, a phenomenon referred to as transient hyperthyrotopinemia [43]. The proportion of neonates within the given population who have elevated TSH levels is therefore considered to be a useful marker of the iodine nutrition status of that population. Specially, WHO guidelines suggests that the percentage of newborns with whole-blood TSH concentrations over 5mIU/l provides a reliable indicator of the iodine status in a population [43]. According to these guidelines, a population can be considered to be iodine sufficient if less than 3% of neonates older than 72 hours have TSH levels over 5mIU/l. The measurement of TSH levels is also part of routine neonatal screening to detect congenital hypothyroidism in newborns. However, a number of more recent studies have identified some potential issues with using neonatal TSH as the indicator of iodine status. Li
and colleagues [69] showed that babies born by vaginal delivery had higher TSH level at birth than babies born by caesarean section. In addition, maternal or neonatal exposure to iodine-containing antiseptics can also cause transient hyperthyrotropinemia and/or hypothyroidism in newborns and thus provide misleading results [70].

In addition to providing a measure of iodine status in the infants, neonatal TSH level is also considered to be a sensitive indicator of iodine status of the mother during late pregnancy [65]. Ladenson and colleagues [71] suggested that serum TSH measurement is the most reliable test to diagnose thyroid hormone deficiency or iodine deficiency in the neonatal/infant period due to the accuracy, safety and relatively low cost of this diagnostic test.

1.4.1.3 Tg concentration

Tg is a scaffold protein in the synthesis of thyroid hormones and a small amount of Tg is secreted into the circulation, along with thyroid hormones, following thyroid hormone synthesis and secretion. In individuals who are iodine deficient, however, the greater thyroid cell mass and TSH stimulation is associated with an increase in serum Tg concentration [68]. Therefore, serum Tg concentrations reflect iodine nutrition status over a period of months to years [43]. Serum Tg appears to be a good indicator of iodine status in school-age children [43] and adults (women aged 18-65 years and men aged from 60-65 years), based on the association between serum Tg levels and thyroid size, TSH level and urinary iodine concentration (UIC) [72-74]. While useful as an indicator of iodine status in adults and children, Tg does not appear to be a reliable method for assessing iodine status during pregnancy and lactation, based on the conclusion of systematic review of 4 studies assessing the usefulness of biomarkers of iodine status in pregnant and lactating women [67].

Tg is also difficult to assay reliably and reproducibly, which has made it difficult to establish normal ranges and cut-offs to identify the severity of iodine deficient status based on Tg
concentrations. The WHO [75] suggested that a median Tg level of less than 10µg/l indicated iodine sufficiency in both children and adults. More recently, Zimmermann and colleagues conducted a cross-sectional study in primary schools in 12 countries with included a total of 2512 children aged between 6 and 12 years [76]. The findings of this study suggested that a median Tg of <13µg/l and/or <3% of the population having a Tg value >40µg/l was indicative of iodine sufficiency in a population. However, there has been no validation of this cut-off in pregnant/lactating women. In a review assessing the reliability of Tg as a marker of iodine deficiency, which included 8 observational studies measuring Tg level in pregnant women, almost all studies reported a median Tg of ≥13µg/l in iodine-deficient pregnant women [77]. However, the studies included in the review had small sample sizes, different study designs, and different forms of iodine supplementation as well as varied gestational ages at the time of Tg measurements. Therefore, it remains unclear whether there is a specific threshold of Tg level for defining iodine deficiency in pregnancy.

Although the level of TSH and Tg are good indicators of iodine deficiency in neonates and children, testing the levels of these factors requires blood samples to be taken, which is invasive and can cause discomfort for the infant/child. In addition, the cost of these assessments is too high to allow for routine use in developing countries. These methods also require use of a standard and sensitive assay and access to the appropriate laboratory infrastructure.

1.4.2 Urinary iodine concentration (UIC) as an indicator of iodine nutrition status

1.4.2.1 Background

Due to the limitations of other assessment methods, the measurement of UIC is the most widely used method of assessing the iodine status of a population. This is because it is easy to obtain the samples and the test is relatively inexpensive. Since the bioavailability of iodine in
food appears to be relatively high [78] and more than 90% of iodine is excreted into urine [79, 80], the UIC is a good indicator of iodine intake over the previous few days. The UIC test is a valuable tool for assessing the iodine status of a population consisting of at least 40-50 individuals, but it is not suitable for diagnosing iodine deficiency in an individual due to the significant day-to-day variations in iodine intake and in urine volume, both of which influence the urinary iodine concentration [81]. Thus, the use of casual urine samples for the determination of UIC in this study would have been provided less reliable information on the iodine status of individuals than assessing 24 hour urinary iodine excretion [82, 83]. In support of this, Condo and colleagues have recently reported that UIC obtained from 24 hour urine samples was correlated with iodine intake of Australian pregnant women while of the UIC obtained from a casual urine sample was not [84]. However, in large studies, such as the one reported in this thesis, it is not logistically possible to collect repeated or 24 hour urine samples from all participants, therefore reliance on casual urine samples is necessary.

For population-based surveys, the WHO and International Council for the Control of Iodine Deficiency Disorders (ICCIDD) suggest that school-aged children are the most suitable group for providing an indication of the iodine status of the general population [85]. This relates to vulnerability of children to iodine deficiency and relative ease of access to samples for UIC assessment through school-based surveys [43]. Furthermore, pre-school children (from 0 to 59 months) also appear vulnerable to iodine deficiency, and may therefore be another appropriate sample in which to assess iodine status. This is because young children consume limited amounts of iodine-rich food, e.g. fish and seafood, as well as the fact that the addition of salt into home-prepared and manufactured foods is discouraged for children in this age group, meaning they are unlikely to be exposed to iodized salt in their diets [48].

For pre-school, school-age children, adults, non-pregnant and non-lactating women, a median UIC in the population of between 100 and 199μg/l with no more than 20% of population having a median UIC of below 50μg/l is considered to be an indication that the iodine status
of a population is sufficient [43]. In pregnant women, the range of median UIC considered to indicate iodine sufficiency in the population is slightly higher, between 150 and 249μg/l, reflecting the higher iodine requirements of this population group (Table 1.3).

Table 1.3: Epidemiological criteria for assessing iodine nutrition of population based on median urinary iodine concentration of school-age children (≥6 years)⁴³ [43]

<table>
<thead>
<tr>
<th>Median UIC (μg/l)</th>
<th>Iodine intake</th>
<th>Iodine status</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;20</td>
<td>Insufficient</td>
<td>Severe iodine deficiency</td>
</tr>
<tr>
<td>20-49</td>
<td>Insufficient</td>
<td>Moderate iodine deficiency</td>
</tr>
<tr>
<td>50-99</td>
<td>Insufficient</td>
<td>Mild iodine deficiency</td>
</tr>
<tr>
<td>100-199</td>
<td>Adequate</td>
<td>Adequate iodine nutrition</td>
</tr>
<tr>
<td>200-299</td>
<td>Above requirements</td>
<td>Likely to be adequate for pregnant/lactating women, but may pose a slight risk of more than adequate intake in the general population</td>
</tr>
<tr>
<td>≥300</td>
<td>Excessive</td>
<td>Risk of adverse health consequences (hyperthyroidism, autoimmune thyroid diseases)</td>
</tr>
</tbody>
</table>

⁴³ Applies to adults, but not to pregnant and lactating women.

Table 1.4: The median or range of UIC used to categorise the iodine intake of pregnant women, lactating women and children less than 2 years of age [43]

<table>
<thead>
<tr>
<th>Population group</th>
<th>Median UIC (μg/l)</th>
<th>Category of iodine intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant women</td>
<td>&lt;150</td>
<td>Insufficient</td>
</tr>
<tr>
<td></td>
<td>150-249</td>
<td>Adequate</td>
</tr>
<tr>
<td></td>
<td>250-499</td>
<td>More than adequate</td>
</tr>
<tr>
<td></td>
<td>≥500</td>
<td>No added health benefit expected</td>
</tr>
<tr>
<td>Lactating women ⁣³⁴</td>
<td>&lt;100</td>
<td>Insufficient</td>
</tr>
<tr>
<td></td>
<td>≥100</td>
<td>Adequate</td>
</tr>
<tr>
<td>Children less than 2 years-old</td>
<td>&lt;100</td>
<td>Insufficient</td>
</tr>
<tr>
<td></td>
<td>≥100</td>
<td>Adequate</td>
</tr>
</tbody>
</table>

³⁴ In lactating women, the figures for median UIC are lower than the iodine requirements because of the iodine excreted in breast milk
1.4.2.2 Methods of UIC measurement

Numerous analytical methods, from highly complicated to simple, are used to measure UIC. The common methods in routine use are based on the Sandell-Kolthoff (S-K) reaction [86]. These methods consist of 2 stages: digestion and analysis. The digestion stage is used to eliminate the interference of other substances in the sample that could affect the performance of the assay and to convert all iodate in the samples to the iodide form, which is the catalyst for the S-K reaction. Either chloric acid or ammonium persulphate is employed in the digestion [87]. In the reading stage, the rate of the colour disappearance is measured by spectrophotometric or colorimetric instruments. Inductively coupled plasma mass spectrometry (ICPMS) is the gold standard method for UIC analysis [88]. It is a technically advanced method with high accuracy and precision, but also with high cost, so is not widely used for large scale screening programs. In addition, several less common methods are also employed for UIC measurement such as pair-ion reversed-phase high performance liquid chromatography (HPLC) [89], a Technicon Autoanalyzer [90] and ultraviolet digestion methods [91], but these are used relatively infrequently.

1.4.3 Breast milk iodine level as an indicator of iodine intake of breastfed infants

Iodine concentration in breast milk is affected by the dietary intake of mothers during lactation, and is correlated with the UIC of breastfed infants [92, 93]. The measurement of breast milk iodine level is therefore a good indicator of iodine status of exclusively breastfed infants. In iodine sufficient areas, iodine levels in breast milk range from 100-150µg/l, whereas in deficient areas the level is typically lower than 100µg/l [94]. There is disagreement between studies as to which values should be considered as a cut-off for indicating a sufficient iodine level in breast milk. Bazrafshan et al [93] used a cut-off of 50µg/l, but did not provide a strong justification for selecting this value. Azizi & Smyth [94], on the other hand, suggested that breast milk iodine levels above 75µg/l was an appropriate
indicator of sufficient iodine intake in a review of studies focused on iodine secretion in human milk and infant iodine nutrition during breast feeding; this review, however, did not indicate how the cut-off was determined. Another study suggested that positive iodine balance in infants is only achieved when iodine intake is 15µg/kg/day in full-term infants and 30 µg/kg/day for pre-term infants, which requires breast milk iodine concentrations in the range of 100-200µg/l [21].

Breast milk iodine measurement is generally more difficult than UIC measurement because breast milk is a complex matrix containing a variety of substances which all have the potential to interfere with the assay. Sample preparation is considered to be the most critical step in the analysis of iodine in breast milk [95]. Numerous methods have been set up in an attempt to measure iodine concentrations in breast milk, including methods based on the S-K reaction [96, 97], a kinetic-photometric method using mixed acid digestion [98], and ICPMS-based methods [99-101]. These methods are based on approaches used to assess iodine concentrations in foods, but to date have lacked level of validation required to confirm their appropriateness for assessing iodine concentration in human breast milk. Therefore, new approaches to reliably measure iodine breast milk level are required. This formed the basis of Chapter 3 of this thesis.

1.5 IODINE FORTIFICATION IN AUSTRALIA

In the early 1990s, the iodine status of the Australian population was classified as sufficient (UIC≥200µg/l) [102]. However, more recent reports of iodine status in Australian children carried out between 2001 and 2004 have indicated the presence of iodine deficiency in several states/cities of Australia, including Tasmania [103], New South Wales [104], Melbourne [105] and Sydney [39]. The WHO [106] also reported the presence of mild iodine deficiency in school-age children in Australia in 2000-2001 (median UIC 77µg/l). Moreover, a national iodine nutrition survey conducted in 2004 in five Australian states provided evidence that the
median UIC of the school-age children was only 104μg/l, indicating a borderline-deficient iodine status [107].

In Australia, a number of suggestions have been put forward to explain the resurgence of iodine deficiency, including limited use of iodized salt in commercially produced food and in household cooking/seasoning [108]. Widespread replacement of iodophors by other cleaning agents in dairy industry also reduced the content of iodine in milk and other dairy products which are a major source of iodine in Australian diets [109]. Poor awareness of the importance of iodine for health outcomes has also contributed to decreased iodine intake, for example, despite iodized salt being readily available in Australian supermarkets for many years, it only makes up 15-20% of the total salt purchases [108].

In response to the resurgence of iodine deficiency, the Australian Government introduced voluntary iodine fortification of salt in bread making in 2001 [110], which was subsequently replaced by mandatory fortification in 2009. Since September 2009, all salt used in bread making in Australia and New Zealand, with an exception of organic bread, is required to contain iodine. This is achieved by adding iodized salt (containing 25-65 mg iodine/kg) to bread flour [85]. The level of fortification selected was based on that required to meet the iodine requirements of the general population. However, as described earlier, the iodine requirements of pregnant and lactating women are substantially higher than of the general population. Therefore, it is not yet clear whether the introduction of mandatory iodine fortification in Australia has been successful in restoring the iodine status of pregnant and lactating women to iodine sufficiency.

1.6 IODINE STATUS OF PREGNANT AND LACTATING WOMEN AND INFANTS IN AUSTRALIA

Several studies have evaluated the iodine status of pregnant women prior to the introduction of iodine fortification (Table 1.5). The majority of these studies were conducted between
2001 and 2006 and all reported iodine deficiency in pregnancy; the median UIC was within the range of 50-100µg/l [39, 111-114]. Two out of the seven studies, namely Rahman et al. [111] and Travers et al. [115], were cross-sectional studies, the remaining studies used participants who were recruited from specific hospitals with particular medical conditions (convenience samples). Therefore, it is not clear whether these results are truly representative of the iodine status in the general pregnant population.

Relatively few studies have assessed the iodine status of pregnant women after the introduction of iodine fortification and the results of these studies have been inconsistent between regions of Australia (Table 1.5). Rahman and colleagues [34] found no significant difference in UIC levels before and after the introduction of fortification in Victoria. However, a more recent study conducted in Sydney reported a significant improvement in the UIC level of pregnant women after fortification [116]. In spite of this improvement, the UIC levels in both studies were still at the borderline of sufficiency. Furthermore, in a small study of pregnant women in South Australia, Clifton and colleagues reported that iodine status of the women was still classified as deficient with UIC level of 84µg/l in 2010 [117], after the introduction of mandatory iodine fortification. It is however important to note that this study was conducted in an area of relatively low socioeconomic status so may not be representative of the general population. In addition, all studies to date which have assessed iodine status in pregnant women after fortification have only included a small number of women. Therefore, further studies which have a sufficiently large sample size and which are representative of the general population are required to determine whether fortification has had any impact on iodine status of pregnant women in Australia.

There are also limited data on the iodine status of lactating women in Australia (Table 1.5). Only two studies by Chan and colleagues [118] and Gunton and colleagues [114], have specifically determined iodine status pre fortification in lactating women in Australia, and both of these studies reported that this population was iodine deficient. The only study to
assess the iodine status of lactating women in Australia after iodine fortification suggested that these women had an iodine sufficient status [119]. Although this finding is the first to indicate the impact of the fortification on iodine nutrition status of lactating women, the small sample size (n=60) and the fact that was conducted in Sydney means that the results may not be generalised. Furthermore, there are no studies which have assessed iodine status in infants both before and after the introduction of mandatory iodine fortification. Therefore, the aim of Chapter 4 of this thesis was to assess the current iodine status of pregnant and lactating women post iodine fortification. In addition, iodine content of breast milk in women from the same region of South Australia before and after the introduction of mandatory iodine fortification were compared to determine the impact of mandatory iodine fortification/iodine supplement recommendations for pregnant/lactating women on breast milk iodine content (Chapter 5).
Table 1.5: Summary of studies of iodine status in pregnant women and lactating women in Australia

<table>
<thead>
<tr>
<th>Reference</th>
<th>Data collection period</th>
<th>Setting &amp; Location</th>
<th>Study population</th>
<th>Median Urinary Iodine Concentration (UIC) (µg/l) (IQR)</th>
<th>Other indicator of iodine status</th>
<th>Fortification program</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pregnancy</strong></td>
<td></td>
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</tr>
<tr>
<td>Blumenthal, Byth &amp; Eastman [120]</td>
<td>11/2007-2/2009</td>
<td>Private obstetric practice (North Western Sydney)</td>
<td>Pregnant women at 7-11 weeks gestation (n=367)</td>
<td>81 µg/l (41-169)</td>
<td>Median TSH: 0.98mIU/l</td>
<td>None</td>
<td>26% with UIC&lt;50-100 µg/L, 20%&lt;20-49 µg/l, 12% &lt;20 µg/l</td>
</tr>
<tr>
<td>Burgess et al. [121]</td>
<td>Stage 11/10/2000-30/9/2001 Stage 2: 2003-2006</td>
<td>Royal Hobart Hospital (RHH) and primary health care centres (RHCCs) (Tasmania)</td>
<td>Pregnant women in all trimesters in RHH (285 for pre and 299 for post fortification) and in RHCC (288 for post fortification)</td>
<td>In RHH Pre fortification : 76µg/L (43-189) with 30.9% &lt;50µg/l Post fortification: 86µg/L (57-160) with 19.2% &lt;50µg/l In RHCC, post fortification: 81µg/l (63-115) with 18.8% &lt;50µg/l</td>
<td></td>
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</tbody>
</table>

28
<table>
<thead>
<tr>
<th>Reference</th>
<th>Data collection period</th>
<th>Setting &amp; Location</th>
<th>Study population</th>
<th>Median Urinary Iodine Concentration (UIC) (µg/l) (IQR)</th>
<th>Other indicator of iodine status</th>
<th>Fortification program</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charlton et al. [112]</td>
<td>8/2008-9/2008</td>
<td>Public antenatal clinic (New South Wales)</td>
<td>110 pregnant women (across all 3 trimesters)</td>
<td>87.5 µg/l (no IQR reported)</td>
<td>No</td>
<td>None</td>
<td>35% of the women using iodine supplementation during pregnancy 41% of women reported duration of iodine supplementation over 6 months.</td>
</tr>
<tr>
<td>Clifton et al [117]</td>
<td>1/2009-7/2010</td>
<td>Queen Elizabeth and Lyell McEwin Hospitals (Northern Adelaide South Australia)</td>
<td>Pregnant women at 12, 18, 30, 36 weeks (n=196)</td>
<td>Pre: 68µg/l (no IQR reported) Post: 84 µg/l (no IQR reported)</td>
<td>No</td>
<td>Mandatory fortification of all bread</td>
<td>Women who took iodine supplements had significantly higher UIC</td>
</tr>
<tr>
<td>Reference</td>
<td>Data collection period</td>
<td>Setting &amp; Location</td>
<td>Study population</td>
<td>Median Urinary Iodine Concentration (UIC) (µg/l) (IQR)</td>
<td>Other indicator of iodine status</td>
<td>Fortification program</td>
<td>Comments</td>
</tr>
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</tr>
<tr>
<td>Gunton et al. [114]</td>
<td>8/1998-4/1999</td>
<td>a tertiary referral hospital (Sydney, New South Wales)</td>
<td>81 pregnant women (weeks of gestation not reported)</td>
<td>104 µg/l (89-129) with 19.8% &lt;50 µg/l, 29.8% from 50-100µg/l</td>
<td>159 µg iodine/creatinine</td>
<td>None</td>
<td>Median TSH/Tg levels of women with UIC&gt;100µg/l: 1.56±0.80 µIU/ml; 12.9±3.7 pmol/l</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Median TSH/Tg levels of women with UIC of 50-100µg/l: 1.67±0.9 µIU/ml; 12.5±3.00 pmol/l</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Median TSH/Tg levels of women with UIC&lt;50µg/l: 1.56±0.77 µIU/ml; 12.1±2.25 pmol/l</td>
</tr>
<tr>
<td>Hamrosi, Wallace &amp; Riley [113]</td>
<td>1998-early 2002</td>
<td>Monash Medical Centre (Melbourne, Victoria)</td>
<td>263 Vietnamese, 262 India/ Sri Lanka, 277 Caucasian women at 14-20 weeks of gestation</td>
<td>Vietnamese: 58µg/L (38-92) (38.4% &lt;50µg/L), Sri Lankan/Indian: 61 µg/L (30-95) (40.8% &lt;50µg/L), Caucasian: 52µg/L (36-80) (48.4&lt;50µg/L)</td>
<td>No</td>
<td>None</td>
<td>UIC of Caucasian women significantly lower than Vietnamese and Indian/Sri Lankan women</td>
</tr>
<tr>
<td>Joseph et al. [122]</td>
<td>7-8/2009</td>
<td>Queen Elizabeth II Medical Centre (West Australia)</td>
<td>Pregnant women in 1st trimester (n=327)</td>
<td>181 µg/l (no IQR reported)</td>
<td>No</td>
<td>None</td>
<td>11% had s UIC &lt;50µg/l, 4% &lt;25 µg/l, 7% from 25-50 µg/l, 57% from 51-100 µg/l</td>
</tr>
</tbody>
</table>
Continued

<table>
<thead>
<tr>
<th>Reference</th>
<th>Data collection period</th>
<th>Setting &amp; Location</th>
<th>Study population</th>
<th>Median Urinary Iodine Concentration (UIC) (µg/l) (IQR)</th>
<th>Other indicator of iodine status</th>
<th>Fortification program</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li et al.[39]</td>
<td>Late 1998-early 1999</td>
<td>Westmead Hospital (Sydney, New South Wales)</td>
<td>101 pregnant women</td>
<td>88 µg/l (no IQR reported)</td>
<td>No</td>
<td>None</td>
<td>-</td>
</tr>
<tr>
<td>Nguyen [123]</td>
<td>2/2009-5/2009</td>
<td>Canberra Hospital (Australian Capital Territory)</td>
<td>100 pregnant women (across all 3 trimesters)</td>
<td>62 µg/l (no IQR reported)</td>
<td>No</td>
<td>None</td>
<td>Women taking iodine supplements had higher UIC than women who did not.</td>
</tr>
<tr>
<td>Rahman et al. [111]</td>
<td>1/2009-2/2010</td>
<td>Hospital antenatal care services and private obstetrician clinics (Gippsland, Victoria)</td>
<td>86 Pregnant Women (≥28 weeks gestation): 24 before fortification and 62 after fortification</td>
<td>Before fortification: 96µg/l(45-153), after fortification: 95.5µg/l(60-156)</td>
<td>No</td>
<td>Mandatory fortification of all bread</td>
<td>54% of women pre fortification and 50% of women post fortification were taking mineral and vitamin supplements containing 25-300µg of iodine.</td>
</tr>
<tr>
<td>Travers et al. [115]</td>
<td>3-6/2004</td>
<td>Hospital antenatal care services and private obstetrician clinics (Central Coast areas of New South Wales)</td>
<td>796 pregnant women (≥28 weeks gestation)</td>
<td>85 µg/L(58) (with 16.6% UIC &lt;50µg/l)</td>
<td>No</td>
<td>None</td>
<td>Negative association between neonatal TSH level and maternal UIC</td>
</tr>
<tr>
<td>Reference</td>
<td>Data collection period</td>
<td>Setting &amp; Location</td>
<td>Study population</td>
<td>Median Urinary Iodine Concentration (UIC) (µg/l) (IQR)</td>
<td>Other indicator of iodine status</td>
<td>Fortification program</td>
<td>Comments</td>
</tr>
<tr>
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</tr>
<tr>
<td><strong>Lactation</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chan et al. [118]</td>
<td>3/2000-12/2000</td>
<td>A tertiary referral hospital (Sydney, New South Wales)</td>
<td>50 women in week 1-2 of lactation</td>
<td>46 µg/l (no IQR reported) (58% &lt;50µg/l)</td>
<td>81.4 µg iodine/g of creatinine (15.4-195.4), neonatal TSH of 1.5 mIU/l (0.2-21)</td>
<td>None</td>
<td>Median breast milk iodine level was 84 µg/l(25-234)</td>
</tr>
<tr>
<td>Gunton et al. [114]</td>
<td>1/8/1998-1/4/1999</td>
<td>A tertiary referral hospital (Sydney, New South Wales)</td>
<td>26 lactating women at 3 months postpartum</td>
<td>79 µg/l(44-229) 19.2%&lt;50µg/l, 34.6% from 51-100µg/l</td>
<td>131 µg iodine/g of creatinine (106-218)</td>
<td>None</td>
<td>-</td>
</tr>
</tbody>
</table>

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1.7 THYROID HORMONES AND METABOLIC HEALTH

Thyroid hormones play an important role in the development, growth and function of almost all cells in the body. One of the key roles attributed to thyroid hormones is the regulation of metabolic rate and a number of human studies have reported the existence of relationships between circulating thyroid hormone concentrations and measures related to metabolic health, including weight, body mass index (BMI) and insulin sensitivity, in adults [124]. Since iodine is a major constituent of thyroid hormones, it has been proposed that iodine nutritional status may also impact on the regulation of energy balance, weight gain and insulin sensitivity in both children and adults.

1.7.1 Thyroid function and body composition

Studies in both humans and animal model have shown that thyroid hormones increase basal metabolic rate (BMR) by stimulating carbohydrate, protein and lipid metabolism [124]. Thus, excess thyroid hormone levels raise the BMR, while thyroid hormone deficiency is associated with a decreased BMR [125]. As a result of the actions of thyroid hormones on BMR, and thus the basal rate of heat production and oxygen consumed by the body, these hormones play a critical role in regulating energy balance.

1.7.1.1 Association between thyroid status and body weight

The important role of thyroid hormones in the regulation of BMR prompted studies investigating the relationship between thyroid function and body weight, weight gain and body fat mass. Studies in animals provided initial evidence that increases or decreases in thyroid hormone concentrations could result in weight loss/weight gain [126-128]. More recently, in line with the increase in obesity prevalence in humans, there have been a number of population-based studies which have aimed to determine the relationship between thyroid function and body weight/weight gain and BMI in human studies.
Thus far, 18 population-based studies have examined the relationship between thyroid function and weight status, 14 in adults and 4 in children/adolescents. A detailed summary of these studies is presented in Table 1.6. The majority of these studies have been conducted in euthyroid populations, or have only included participants with a defined range of TSH values. Most have had relatively large sample sizes (over 300 participants), and have assessed thyroid function by assessing TSH, fT4 or fT3 concentration with many assessing more than one of these parameters.

**Adults**

The studies conducted to date have provided inconsistent evidence regarding the association between thyroid function and body mass index (BMI) and/or body weight in adult humans. Of the 13 cross-sectional studies that included measures of circulating TSH levels (while one study only measured fT3 and fT4 levels), 8 studies reported a positive correlation between TSH and BMI and/or body weight while the remaining 5 studies showed no relationship.

Similarly, several recent studies reported that fT3, but not fT4, levels, were positively correlated with BMI and/or body weight in adult humans [129, 130]. However, this was not the case in all studies, with a study conducted in Korea by Shon and colleagues [131] actually reporting a negative correlation between fT4 levels and BMI in females. In a separate study, Dvorakova and colleagues [132] also found a negative correlation between circulating fT4 and BMI in females, but no correlation in males. This same study also found that fT3 levels were positively correlated with BMI in males, and this relationship has since been replicated in a more recent study [133]. These results therefore suggest that the relationship between thyroid function and BMI/body weight may be sex-specific and that this is a possible cause of conflicting results between studies which have included mixed populations of male and females. This implies that the sex of subjects should be taken into account in any further
studies investigating the relationship between thyroid function and measures of BMI/body weight.

The inconsistent findings may also be related in part to the fact that the vast majority of these studies only measured thyroid hormones/TSH levels at a single time point. These levels are not necessarily representative of past thyroid hormone status, which would be more likely to correlate with body weight gain over a period of time. Investigating the relationship between thyroid hormone status at a given time-period and body weight gain over the subsequent months/years may be more informative. Two studies, both with large sample sizes, have shown that changes in TSH concentration were directly related to the change in body weight over the same 3.5 year [134] or 10 year [135] period. In addition, none of the studies investigating the association between thyroid status and body weight/BMI been conducted to date are randomised trials, and it is possible that other factors, such as lifestyle factors and illness etc, which were not controlled for in the analysis may be the reason that these associations were or were not identified. Therefore, our understanding of the role that thyroid hormones have in regulating body weight/weight gain in humans is still limited and further studies are needed.

**Children**

Most of the human studies which have investigated relationships between TSH and/or thyroid hormone concentrations and body weight/weight gain have been conducted in adults, with only 4 such studies in children. Three of these studies reported a positive relationship between TSH concentration in serum/plasma and body weight/BMI [136-138] while no association was found in the study of Prats-Puig and colleagues [139]. In addition, the 2 studies in children that measured fT3 levels both reported that these were also positively related to BMI/BMI z-score, independent of whether the children were normal weight, overweight or obese [136, 138]. However, there have been conflicting reports as to the relationship between
fT4 and BMI in the studies conducted to date. Two studies conducted in India and Spain [139] both reported a negative association between fT4 and BMI in euthyroid children. A study of 240 overweight and obese Italian children, however, did not find a significant association [138]. Similar to the adult studies, the child studies have only investigated the relationship between thyroid hormone status and body weight/BMI at one time point and no studies have examined the effects of thyroid status over a given time on the change in body weight/BMI over the same period. Almost all the studies conducted in children to date have also included a mixed population of males and females, which may have led to misleading results, given the suggestion that these relationships may be different between the sexes.
Table 1.6: Summary of population-based studies investigating associations between thyroid function and body composition

<table>
<thead>
<tr>
<th>Reference</th>
<th>Country</th>
<th>Study population</th>
<th>Thyroid status of population</th>
<th>Thyroid function assessments</th>
<th>Body composition assessments</th>
<th>Results</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alevizaki et al. [130]</td>
<td>Greece</td>
<td>303 males and females (33-53 years)</td>
<td>Euthyroid</td>
<td>TSH, T3 fT4, T3/fT4</td>
<td>1.BMI, weight ≥25kg/m², no ass. with fT4, no ass. with TSH, +ve with T3/fT4</td>
<td>1. After adjustment for BMI, For BMI ≥25kg/m²: -ve between fT4 and SF and SF/PF found in subject, For BMI &lt;25kg/m²: no ass between fT4 and SF and SF/PF</td>
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<td></td>
<td>2. Body weight: +ve with T3, no ass. with fT4, no ass. with TSH</td>
<td>2. After adjustment for sex, Male: SF and SF/PF -ve with fT4</td>
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<td></td>
<td>3. SF: -ve with fT4, +ve with TSH, +ve with T3, +ve with T3/fT4</td>
<td>Female: no ass. between SF and fT4, -ve ass between SF/PF and fT4</td>
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<td></td>
<td>4. PF: +ve with T3, no ass. with fT4, no ass. with TSH</td>
<td>3. After adjustment for age, sex, smoking, HOMA-IR</td>
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<td></td>
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<td></td>
<td>5. SF/PF: -ve with fT4</td>
<td>No ass between SF and TSH.</td>
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<tr>
<td>Ambrosi et al. [140]</td>
<td>Italy</td>
<td>581 overweight and obese males and</td>
<td>Euthyroid</td>
<td>TSH</td>
<td>BMI</td>
<td>BMI: +ve with TSH</td>
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<td></td>
<td></td>
<td>females (mean age 39.8±13.7 years)</td>
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<tr>
<td>Asvold, Bjoro &amp; Vatten [141]</td>
<td>Norway</td>
<td>27097 males and females (&gt;40 years)</td>
<td>Not stated</td>
<td>TSH</td>
<td>BMI</td>
<td>BMI +ve with TSH</td>
<td>Relationship present in both smokers and non-smokers and in both males and females</td>
</tr>
<tr>
<td>Diez &amp; Iglesias [142]</td>
<td>Spain</td>
<td>778 males and females (mean age 58.5±15.9 years)</td>
<td>Euthyroid</td>
<td>TSH</td>
<td>BMI</td>
<td>BMI +ve with TSH</td>
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<tr>
<td>Reference</td>
<td>Country</td>
<td>Study population</td>
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<tr>
<td>Adults</td>
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<tr>
<td>Dvorakova [132]</td>
<td>Czech</td>
<td>2637 males and females (18-65 years)</td>
<td>Not stated</td>
<td>fT3, fT4, TSH and T3/T4</td>
<td>1.BMI</td>
<td>1.BMI: +ve with fT3 in men, +ve with fT3/T4 in men; -ve with fT4 in women, +ve with TSH in women, +ve with fT3/T4 in women</td>
<td>Body fat distribution evaluated by CI CI: -ve with fT3 in female, +ve with TSH in male and female, -ve with fT3/T4 in female</td>
</tr>
<tr>
<td>Fox et al.</td>
<td>USA</td>
<td>2407 males and females (28-62 years)</td>
<td>TSH level between 0.5-5 mUI/l</td>
<td>TSH</td>
<td>BW at baseline and weight gain over 3.5 years</td>
<td>1. At baseline: BW +ve with TSH 2. At follow up: no ass. between either baseline TSH or TSH change and weight gain</td>
<td>Results adjusted for age, smoking status, menopausal status and/or baseline weight</td>
</tr>
<tr>
<td>Kumar et al.</td>
<td>India</td>
<td>45 females (mean age 32.6±9.6 years)</td>
<td>Not stated</td>
<td>Total T3, fT3, TSH and T4</td>
<td>BMI</td>
<td>BMI +ve with TSH, no ass. with fT4, no ass. with total T4, no ass. with fT3</td>
<td>-</td>
</tr>
<tr>
<td>Manji et al.</td>
<td>UK</td>
<td>401 males and females (mean age 48.2 years)</td>
<td>Euthyroid</td>
<td>TSH and fT4</td>
<td>BMI</td>
<td>BMI: no ass. with TSH and fT4</td>
<td>No difference in TSH and fT4 levels in obese vs lean subjects</td>
</tr>
<tr>
<td>Reference</td>
<td>Country</td>
<td>Study population</td>
<td>Thyroid status of population</td>
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</table>
| Nyrnes, Jorde & Sundsfjord [145] | Norway           | 6164 males and females (>24 years)      | Not stated                   | TSH                          | BMI                          | Analysis only including TSH values within normal range  
1. BMI: +ve with TSH in both non-smoking male and female, no ass. with TSH in smokers  
2. BMI change +ve with TSH change in non-smoker during 7 years follow-up, no ass. in smokers | Analysis conducted including all TSH values  
1. BMI: no ass with TSH in both smokers and non-smokers  
2. BMI change +ve TSH change in non-smoker during 7 years follow up, no ass. in smoker |
| Ren et al. [129]   | China            | 865 males and females (31 – 53 years)   | Euthyroid                    | fT3, fT4                     | 1.BMI, BW  
2.TFM and BFP by BIA          | 1. BMI: +ve with fT3, no ass. with fT4  
2. BW: +ve with fT3 and fT4  
3.TFM: no ass. with fT3 and fT4  
4. BFP: -ve with fT3 and fT4 | After adjustment for age and gender:  
fT3: +ve with BMI, BW, TFM, BFP  
fT4: no ass. with BMI, BW, TFM and BFP |
| Roef et al. [133]  | Belgium          | 941 male(25 – 45 years)                 | Euthyroid                    | TSH, fT3, fT4 and TBG        | 1.BMI, BW  
2. BFM assessed by DEXA      | 1. BMI: +ve with fT3, +ve with TBG, no ass. with fT4, no ass. with TSH  
2. BW: +ve with fT3, +ve with TBG, no ass. with fT4, no ass. with TSH  
3. BFM: +ve with fT3, +ve with fT4, +ve with TBG, no ass. with TSH | Data was adjusted for age for BMI model and both age and height for weight model. No unadjusted data reported. |
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<table>
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<tr>
<td>Ruhla et al. [146]</td>
<td>Germany</td>
<td>1333 males and females (&gt;18 years)</td>
<td>Euthyroid</td>
<td>TSH</td>
<td>BMI</td>
<td>BMI: +ve with TSH concentrations ranging from 0.3-2.5mU/l</td>
<td>The correlation persisted after adjustment for sex and age. Individuals with TSH in the upper normal range had significantly higher BMI</td>
</tr>
<tr>
<td>Shon et al. [131]</td>
<td>Korea</td>
<td>1572 females (mean age 46.2±11.2)</td>
<td>Euthyroid</td>
<td>TSH, fT4</td>
<td>BMI</td>
<td>BMI: -ve with fT4, no ass with TSH</td>
<td>Obese women had lower fT4 than lean women, but TSH levels did not differ between 2 groups</td>
</tr>
<tr>
<td>Svare et al.[135]</td>
<td>Norway</td>
<td>15020 males and females (&gt;19 years)</td>
<td>TSH of 0.5-3.5 mU/L at baseline and &lt;10mU/L at 10 year follow up</td>
<td>TSH</td>
<td>Baseline BMI, BMI change and weight gain during 10 years of follow up</td>
<td>1. BMI change: +ve with TSH change, no ass. with baseline TSH 2. Baseline BMI: weak -ve with TSH change 3. Weight gain: +ve with TSH change, +ve with TSH at follow up, no ass. with baseline TSH</td>
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<th>Reference</th>
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<th>Note</th>
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</thead>
<tbody>
<tr>
<td>Brufani et al. [138]</td>
<td>Italy</td>
<td>240 overweight and obese children (mean age 9.4±1.7 years)</td>
<td>Not stated</td>
<td>TSH, fT3 and fT4</td>
<td>BMI z-scores</td>
<td>BMI z-score: +ve with TSH; +ve with fT3, no ass with fT4</td>
<td>-</td>
</tr>
<tr>
<td>Ittermann et al. [137]</td>
<td>Germany</td>
<td>6435 children (3-10 years) 5918 adolescents (1117 years)</td>
<td>Not stated</td>
<td>TSH</td>
<td>BMI</td>
<td>BMI +ve with TSH</td>
<td>Association stronger in smokers than non-smokers.</td>
</tr>
<tr>
<td>Marwaha et al.[136]</td>
<td>Indian</td>
<td>1369 children (5-18 years)</td>
<td>Euthyroid</td>
<td>fT3, fT4, TSH</td>
<td>BMI</td>
<td>BMI: +ve with fT3, +ve with TSH, -ve with fT4</td>
<td>-</td>
</tr>
<tr>
<td>Prats-Puig et al.[139]</td>
<td>Spain</td>
<td>234 school-age boys and girls (mean age 6.9±0.1 years)</td>
<td>Euthyroid</td>
<td>fT4, TSH</td>
<td>1. BMI</td>
<td>1. BMI: -ve with fT4, no ass with TSH</td>
<td>After adjustment for sex,</td>
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<td>2. BFM by BIA and visceral fat by ultrasonography</td>
<td>2. BFM: no ass. with fT4, no ass with TSH</td>
<td>2. BFM: -ve with fT4only in females</td>
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<td></td>
<td>3. Visceral fat: -ve with fT4, ass. with TSH</td>
<td>3. Visceral fat: -ve with fT4 only in females</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviation: BMI, body mass index; BW, body weight; TFM, total fat mass; BIA, bioelectrical impedance analysis; BFM, body fat mass; BFP, body fat percentage, DEXA, dual-energy X-ray absorptiometry; SF, abdominal subcutaneous fat; PF, preperitoneal fat, HOMA-IR, homeostatic model assessment-insulin resistance; CI, confidence interval; ass., association.
1.7.1.2 Thyroid function and percent body fat

Almost all human studies which have investigated relationships between thyroid function and obesity have used BMI and body weight as markers of body size and composition, and only 5 studies to date have included measures of body composition/body fat mass. These studies include 4 in adults and 1 in children.

Adults

Despite the positive relationship between TSH and body weight reported in number of population studies, epidemiological studies provide little evidence of a relationship between TSH level and body fatness, with no studies reporting significant correlations between TSH levels and percentage fat mass [132, 133]. Interestingly, however, a study involving 303 euthyroid adults conducted in Greece and using B-mode US imaging reported that TSH levels was positively associated with subcutaneous fat thickness in these patients [130], suggesting that TSH levels may be more reflective of subcutaneous, rather than visceral or total fat mass. However further studies are needed to confirm this finding.

There is also conflicting data as to whether thyroid hormone levels are associated with body fat levels. Ren and colleagues [129] recently reported a negative association between thyroid hormone levels and percentage body fat, as assessed by bioelectrical impedance analysis (BIA). However, these results are not in agreement with a previous study by Dvoráková and colleagues [132], the results of which suggested that percentage body fat was negatively related to fT4 level, but not fT3 level. Whilst these studies included both male and female participants, Roef and colleagues [133] focused on 941 males to investigate the relationship between thyroid function and body fatness assessed by dual energy X-ray absorbance (DEXA). This study demonstrated that free thyroid hormone levels were positively associated with body fat mass after adjustment for age, height and weight. The use of different methods of measuring body fatness also makes it difficult to directly compare the results of these various studies because several studies have suggested that BIA and DEXA cannot be used interchangeably [147]. Interestingly almost all studies reported a change in the relationship between thyroid function and body fatness after adjustment for age sex and smoking status, compared to before adjustment. Therefore, further studies should ensure that these confounders are taken into account when assessing these relationships.
Children

Only one study to date has investigated the relationship between thyroid function and body fatness in children or adolescents. This study involved 234 euthyroid school-age children in Spain, and found that visceral fat thickness measured by ultrasonography was negatively associated with plasma fT4 concentrations, but not with plasma TSH concentrations [139]. In addition, this association was only present in girls. No other studies to date have investigated the relationships between thyroid function and body fat mass/percentage in children and this is an important area for further research.

1.7.2 Thyroid function and insulin sensitivity

A series of rodent studies have shown that thyroid hormones have the capacity to regulate insulin secretion by altering the expression of genes involved in insulin signalling and insulin synthesis [148-151]. However, there have been limited population-based studies in humans which have investigated the association between thyroid function and insulin sensitivity/insulin secretion. The majority of these studies have used the homeostasis model assessment-insulin resistance (HOMA-IR) in order to estimate the degree of insulin resistance. To date, only 9 such studies have been conducted. This includes 8 studies in adults and 1 in children, most of which have had sample sizes greater than 200. A summary of these studies is shown in Table 1.7.

Adults

Despite the evidence from animal studies that insulin secretion/sensitivity is altered in response to changes in thyroid hormone status, the evidence from human population-based studies is much less consistent.

A positive association between fT3 and both HOMA-IR and fasting insulin level was found in 2 adult studies, and these relationship were independent of body weight [129, 133]. This finding is not, however, in agreement with that of a smaller study involving 45 overweight and obese Indian females, in which neither HOMA-IR nor fasting insulin level were associated with fT3 [143]. However, this study also reported a positive correlation between T3, but not T4, level and both HOMA-IR and fasting insulin levels. In addition, the majority of studies measuring fT4 similarly reported no association between fT4 level and either HOMA-IR or fasting insulin level [133, 152-154] while the 2 remaining studies reported a negative correlation [155, 156]. Of the 6 studies measuring TSH level, 4 studies suggested
that TSH level was positively related to either HOMA-IR or fasting insulin level [143, 154-156] while 2 studies reported no association [133, 153]. One possible reason for the inconsistent findings between studies is differences in the thyroid status of populations and the range of TSH and thyroid hormone levels which were present. In addition, relatively small sample sizes and the use of different methods for measuring thyroid hormones and insulin sensitivity (HOMA-IR vs fasting insulin levels) also makes it difficult to directly compare the findings from these studies, and therefore to draw robust conclusions about the relationship between thyroid status and insulin sensitivity in humans.

Furthermore, almost all studies were cross-sectional studies measuring thyroid status at 1 timepoint, which may not be representative of past thyroid status. The exception was a study of 1173 euthyroid Korean participants, which reported that TSH levels measured at a given timepoint were positively associated with the degree of the change in fasting insulin level and HOMA-IR over the preceding 3 year period. This same study also reported that fT4 levels were positively correlated with the change in fasting insulin, but not HOMA-IR, over this same 3 year period. While these are interesting and potentially important results, they remain to be confirmed in other studies.

**Children**

In comparison with the number of studies of insulin resistance in relation to thyroid hormone status in adults, only 1 such study has been conducted in children/adolescents. This study, by Brufani and colleagues, included 240 overweight/obese pre-pubertal Italian children and reported a negative relationship between thyroid function, assessed by fT4 and TSH levels, and hepatic insulin resistance as well as the insulin sensitivity index (ISI) [138]. The findings of this study also showed that fT3 and TSH levels were negatively associated with insulin levels and the quantitative insulin sensitivity check index (QUICKI) whilst fT4 had no correlation with either of these indices. These complex and apparently conflicting findings make it difficult to draw clear conclusions about the relationship between insulin sensitivity and thyroid hormone status in children, and more studies are required.
Table 1.7: Summary of population-based studies of thyroid function and insulin sensitivity

<table>
<thead>
<tr>
<th>Reference</th>
<th>Country</th>
<th>Study population</th>
<th>Thyroid status of population</th>
<th>Thyroid function assessments</th>
<th>Assessment of insulin sensitivity</th>
<th>Results</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults</td>
<td>Turkey</td>
<td>226 obese/overweight females (mean age 43 years)</td>
<td>Euthyroid</td>
<td>TSH and fT4</td>
<td>HOMA-IR</td>
<td>HOMA-IR: +ve with TSH, no ass. with fT4.</td>
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<tr>
<td>Bastemir et al.[154]</td>
<td>Mexico</td>
<td>3033 males and females (18-70 years)</td>
<td>Euthyroid</td>
<td>TSH and fT4</td>
<td>FI and HOMA-IR</td>
<td>FI: +ve with TSH, -ve with fT4, HOMA-IR: +ve with TSH, -ve with fT4</td>
<td>Data reported after adjustment for age and sex</td>
</tr>
<tr>
<td>Garduno-Garcia Jde et al.[155]</td>
<td>India</td>
<td>45 obese/overweight females (18-56 years)</td>
<td>Not stated</td>
<td>TSH, T3, T4, fT3</td>
<td>FI and HOMA-IR</td>
<td>FI: +ve with TSH, +ve with T3, no ass. with T4, no ass. with fT3, HOMA-IR +ve with TSH, +ve with T3, no ass. with T4, no ass. with fT3</td>
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<tr>
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<tr>
<td><strong>Adults</strong></td>
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<tr>
<td>Park, Choi &amp;</td>
<td>Korea</td>
<td>1173 males and females &gt;18 years</td>
<td>Euthyroid</td>
<td>TSH and fT4 levels</td>
<td>1. FI and HOMA-IR at baseline</td>
<td>At baseline:</td>
<td>1. At baseline, data reported after adjustment for age, sex and BMI 2. At 3 years follow up, the relationship between TSH and FI and HOMA-IR reported after adjustment for age, sex, BMI, baseline TSH, smoking status, alcohol intake and exercise. The relationship fT4 and HOMA-IR reported after adjustment for age, gender, baseline fT4 and baseline HOMA-IR, but no association after adjustment for smoking, alcohol intake and exercise.</td>
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<td>Joo [153]</td>
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<td>2. Change of FI and HOMA-IR</td>
<td>2. HOMA-IR: no ass. with either TSH or fT4 AT 3 years follow up</td>
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<td></td>
<td>over 3 years follow up</td>
<td>1. Change of FI: +ve with TSH, no ass. with fT4</td>
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<td>2. Change of HOMA-IR: +ve with TSH, -ve with fT4</td>
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<tr>
<td>Ren et al. [129]</td>
<td>China</td>
<td>865 males and females (31-53 years)</td>
<td>Euthyroid</td>
<td>fT3 levels</td>
<td>FI and HOMA-IR</td>
<td>FI: +ve with fT3 HOMA-IR +ve with fT3</td>
<td>Relationships persisted after adjustment for age and sex</td>
</tr>
<tr>
<td>Roef et al. [133]</td>
<td>Belgium</td>
<td>941 males (25 -45 years)</td>
<td>Euthyroid</td>
<td>fT3, fT4, TBG and TSH levels</td>
<td>FI and HOMA-IR</td>
<td>FI: +ve with fT3, -ve with fT4, no ass. with TSH, no ass with TBG HOMA-IR: +ve with fT3, +ve with TBG, no ass. with TSH, no ass. with fT4</td>
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<tr>
<td>Reference</td>
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<tr>
<td>Roos, Bakker &amp; Thera P. Links [156]</td>
<td>Netherlands</td>
<td>2703 males and females (28-75 years)</td>
<td>Euthyroid</td>
<td>TSH and fT4 levels</td>
<td>HOMA-IR</td>
<td>HOMA-IR: -ve with fT4, +ve with TSH</td>
<td>The relationship between fT4 and HOMA-IR was independent of waist circumference, age and sex.</td>
</tr>
<tr>
<td>Tarcin et al.[152]</td>
<td>Turkey</td>
<td>211 obese males and females (mean age 39.7 ±11.7 years)</td>
<td>Not stated</td>
<td>fT4, TT3 and fT3/fT4</td>
<td>HOMA-IR</td>
<td>HOMA-IR: no ass. with fT4, no ass. with fT3/fT4, +ve with TT3</td>
<td></td>
</tr>
<tr>
<td>Brufani et al.[138]</td>
<td>Italy</td>
<td>240 overweight/obese pre-pubertal children</td>
<td>Not stated</td>
<td>TSH, fT4 and fT3 level</td>
<td>FI, hepatic insulin resistance, QUICKI and ISI</td>
<td>FI: -ve with TSH, -ve with fT3, -ve with fT4 Hepatic insulin resistance: -ve with TSH, -ve with fT3, -ve with fT4 QUICKI: -ve with TSH, -ve with fT3, no ass. with fT4 ISI: -ve with TSH, -ve with fT3, -ve with fT4</td>
<td>After adjustment for age, sex and measures of fatness, QUICK +ve with TSH Hepatic insulin resistance: +ve with fT3, +ve with fT4</td>
</tr>
</tbody>
</table>

Abbreviation: FI, fasting insulin, HOMA-IR, homeostastic model assessment-insulin resistance; QUICKI, quantitative insulin check index; ISI, insulin sensitivity index, +ve, positive correlation; -ve, negative correlation; ass., association
1.7.3 The relationship between thyroid status in early life and metabolic health outcomes later childhood

In the past decade, there has been an emerging field of research demonstrating that the nutritional environment an individual experiences before birth and in early infancy is a key determinant of their long-term health [157]. A series of studies in humans and animals have established that inappropriate maternal nutrition during pregnancy is associated with an increased risk of obesity and poor metabolic health in child and adult life [158]. While these studies initially focussed on global nutritional deficits, such as inadequate caloric intake or excess intake of fat, more recent studies have suggested that inadequate intakes of specific macronutrients (e.g. protein) or minor dietary components (e.g. omega-3 long chain polyunsaturated fatty acids (LCPUFA)) also have the capacity to program adipose tissue and/or insulin signalling pathways in the offspring and thereby predispose offspring to obesity and poor metabolic health in postnatal life [159, 160]. The role of iodine and thyroid hormones in regulating metabolism has led to the suggestion that exposure to inadequate iodine levels during early development may have long-term adverse consequences for the metabolic health of individuals.

The strongest evidence to date for a role of thyroid hormones in the early programming of metabolic health outcomes comes from an animal study conducted by Lagu and colleagues [161]. In this study, rats received intraperitoneal injections of either T4 (hyperthyroid group) or saline solution for the first 21 days after birth and body weight was measured in adulthood. The results of this study showed that the body weight gain of T4 treated rats was ~18% higher than that of saline treated rats at 90 days of age [161]. This suggested that exposure to excess thyroid hormones in the immediate postnatal period could promote weight gain through the life course. However, this finding is not in agreement with a similar study in which pregnant rats were injected daily with 50µg T-Levo-thyroxine (T4)/100g body weight during first 10 days of gestation [162].
In this latter study, the pups of T4-treated dams had a lower body weight in adulthood than control group. The conflicting data may result from the differences of dosage and timing of the thyroid hormone treatment, and imply that treatment at different stages of development has different long-term effects. Interestingly, the latter study also suggested that the pups of T4-treated dams had higher total fat mass than the control group. This finding highlighted that both body weight and total fat mass should be taken into account when the impact of neonatal thyroid hormone status/thyroid function on metabolic health outcomes in later life are investigated.

Only one human population-based study to date investigated the relationship between maternal thyroid hormone status and metabolic markers in the children [64]. This study included 5646 pregnant women in the Netherlands, and reported a negative correlation between maternal fT4 levels in early pregnancy and the BMI of their children at 6 years of age. The same study also demonstrated for the first time that lower maternal TSH levels in early gestation was associated with lower total fat mass and abdominal subcutaneous fat mass in their children. However, there have been no other similar studies conducted to date in order to confirm these findings.

There are also limited studies investigating the long-term impacts of neonatal thyroid function on later insulin sensitivity/insulin action in either humans or animal models. Faharani and colleagues directly explored the link between neonatal hypothyroidism and insulin resistance in adult male offspring in a rodent model [163]. In this study, treating lactating dams with 0.02% 6-propyl-2 thiouracil (PTU) to induce maternal and neonatal hypothyroidism, resulted in increased HOMA-IR (i.e. increased insulin resistance) in the adult offspring. Although not focus of their study, they also suggested that the impact of neonatal hypothyroidism on carbohydrate metabolism may act increase the risk of type 2 diabetes in later life [163]. However, there have been no
human studies to date which have investigated the association between thyroid function at birth and insulin resistance later in childhood.

**Summary**

The results of rodent studies have highlighted the potential existence of a relationship between thyroid function and metabolic health, including body composition and insulin sensitivity. However, whether thyroid function in early life can program later metabolic health outcomes in humans has yet to be established. To date, human studies which have attempted to link thyroid status with measures of metabolic health have produced conflicting results. It also remains unclear to what extent thyroid function at birth and in early postnatal life impacts on body composition and insulin sensitivity later in childhood. Therefore, further studies are required to establish whether neonatal as well as current thyroid function are related to markers of metabolic health, including body fat mass and insulin sensitivity, in children. Therefore, the aim of Chapter 6 of this thesis is to investigate the relationship between thyroid function at birth and in early childhood on measures of metabolic health (body weight, BMI z-score and HOMA-IR) in children at 5 years of age.

**1.8 AIMS OF THIS THESIS**

There are currently limited up to date and quality data on the iodine status of pregnant and lactating women in Australia from representative populations. In Australia, there have been few surveys of iodine status in pregnant and lactating women, particularly since the introduction of mandatory iodine fortification. In addition, most of these studies have been conducted in convenience samples and none of them in representative populations, and there have been limited attempts to compare the iodine status of pregnant and lactating women from the same region before and after the introduction of mandatory fortification. Thus, the effect the fortification on the iodine status of these
populations of women remains unknown. Furthermore, whilst the effects of severe iodine deficiency during pregnancy on neurodevelopment are well documented, there is virtually no information on how iodine status (mild or moderate iodine deficiency) at birth or in early childhood may affect metabolic health outcomes in children. Therefore, the aims of this project are:

1. To assess the iodine status of pregnant women, lactating women and their infants in Australia from a representative population after the introduction of mandatory iodine fortification
2. To compare the breast milk iodine levels of lactating women from the same region of Australia before and after mandatory iodine fortification
3. To examine the association between iodine/thyroid hormone level at birth and in early childhood and metabolic health outcomes (body mass index BMI, and insulin sensitivity) in children at 5 years of age.

This project will provide, for the first time, a clear picture of the iodine status of pregnant women, lactating women and their infants in a representative population in South Australia after the introduction of mandatory iodine fortification. The results presented in this thesis add to the extremely limited data on breast milk iodine levels of lactating women before the commencement of iodine fortification in 2009 and also provide information on the extent to which the introduction of iodine fortification has increased the average iodine content in breast milk of women in South Australia. The data presented in Chapter 5 of this thesis represents the first comparison of iodine concentrations in breast milk pre and post fortification in the same region in Australia.

In addition, the findings presented in Chapter 6 of this thesis provide novel data regarding to potential relationship between neonatal thyroid function and long-term
metabolic development of children as well as report additional data about the link between thyroid function and metabolic markers in childhood.
CHAPTER 2    ANALYTICAL METHODOLOGY AND VALIDATION PROCEDURES FOR MEASUREMENT OF UIC, TSH AND TG LEVELS

2.1 URINARY IODINE CONCENTRATION

Urinary iodine concentrations (UICs) in the pregnant women, lactating women and infants in this study were measured using the WHO method A. This method was originally described by Sandell-Kolthoff in 1936 [86]. In our laboratory, the method was modified to enable the use of a microplate reader to read absorbance in the place of a spectrophotometer. This method was validated as specified in the European Medicine Agency ICH Topic Q2 (R1) Validation of Analytical Procedures [164] prior to analysis of the experimental samples.

2.1.1 Principle of the method

This method based on the Sandell-Kolthoff reaction. First, urine is digested with ammonium persulphate to transfer all iodine to iodide, which is the catalyst in the reduction of ceric ammonium persulphate (yellow) to the cerous form (colourless). The assay is based on the fact that the concentration of iodine in the sample is proportional to the rate of colour disappearance (measured as absorbance) after the addition of the ceric ammonium persulphate solution.

![Figure 2.1: The Sandell-Kolthoff reaction](image)

Figure 2.1: The Sandell-Kolthoff reaction
2.1.2 Materials

Reagents for the digestion step, including high purity ammonium persulphate ($\text{H}_8\text{N}_2\text{O}_8\text{S}_2$) and arsenic acid ($\text{As}_2\text{O}_3$) were purchased from Sigma Aldrich (New South Wales, Australia). Ceric ammonium persulphate solution ($\text{Ce(NH}_4\text{)}_4(\text{SO}_4)\cdot 2\text{H}_2\text{O}$) was purchased from Ajax Fine Chemicals (New South Wales, Australia).

Stock iodine standard 1000mg/l for preparing working iodine standards was produced by Australia Chemical Reagents (Queensland, Australia).

Certified reference material (CRM), Seronorm Trace Elements urine, was purchased from Sero AS (Billingstad, Norway). This had a certified iodine level of $304 \pm 44\mu\text{g/l}$ and was used to assess the accuracy of iodine determination in all experiments.

High purity water generated by a Sartorius Stedim Biotech purification system (Lower Saxony, Germany) was used for the preparation of all reagents, standards and samples.

Borosilicate glass disposal culture tubes from Kimble Chase (New York, USA) were used to digest samples and parafilm (Adelab Scientific, Thebarton, South Australia) was used to cover the tubes during the digestion step. The incubation was carried out on a dry heating block (Adelab Scientific, Thebarton, South Australia). After digestion, the solution was transferred from tubes to microplates (Adelab Scientific, Thebarton, South Australia) and the results were read by a calibrated microplate reader (BioTek Instruments, USA).

2.1.3 Reagents and standards preparation

Ammonium persulphate solution (1.0mol/l) was prepared by dissolving 114.1g ammonium persulphate in 500ml high purity water. The solution was stored in the dark and kept refrigerated to minimise decomposition.
Sulfuric acid (5N and 3N) was prepared by slowly adding either 140ml or 97ml concentrated (36N) sulfuric acid to approximately 700ml high purity water. After cooling, the volume of the solution was made up to 1l by addition of high purity water.

Arsenic acid solution (0.025mol/l) was prepared by weighting 5g arsenic trioxide and 25g sodium chloride in a 1l Erlenmeyer flask, then slowly adding 200ml of 5N sulfuric acid. This solution was heated gently with constant mixing and then left to cool to room temperature. The solution was then diluted with 800ml high purity water and stored in the dark at room temperature.

Ceric ammonium sulphate solution (0.038mol/l) was prepared by dissolving 12g ceric ammonium sulphate in 500ml 3.5N H₂SO₄. This reagent was prepared at least 24 hours before use and stored in darkness at room temperature.

2.1.4 Procedure

Urine samples were allowed to reach room temperature, and were then mixed well. 250µl of each urine sample, working standards ranging from 0 to 600µg/l, 4 urine quality controls with iodine levels reflecting the low, middle and high ranges of the standard curve and Serenorm™ urine external standard were added into separate 10x75mm glass tubes. 1ml of ammonium persulphate was added into each tube and all tubes incubated at 91-95°C for 60 minutes. The tubes were then allowed to cool at room temperature before the addition of 2.5ml of arsenic acid solution and the mixing of the samples by inversion or brief vortex. The tubes were allowed to stand for a further 15 minutes at room temperature and 200µl of each sample was then transferred into a 96-well microplate. A multi-channel pipette was used to add 24µl of ceric ammonium persulphate solution to each well at 15-30 second intervals. The plate was incubated at room temperature for 30 minutes and then placed into the calibrated microplate reader.
The absorbance of all wells in the plate was read at 405nm exactly 30 minutes after the addition of ceric ammonium persulphate to the first tube.

2.1.5 Calculation of results

A standard curve was constructed by plotting the log of the absorbance at 405nm on the X-axis against the standard iodine concentration in µg/l on the Y-axis and the equation calculated in Microsoft Excel. Iodine concentrations in unknown samples were calculated by using the equation of the linear trend line of this plot. Any samples with absorbance values higher than 600µg/l (the top iodine standard) were diluted either 1:3 or 1:5 in high purity water and re-measured and the final concentrations were calculated by using the appropriate dilution factor.

2.1.6 Method validation

The method was validated in our laboratory in accordance with the European Medicine Agency ICH topic Q2 (R1) Validation of Analytical Procedures [164] based on 6 tests: linearity, recovery, parallelism, intra- and inter-assay variation, detection limit and reporting limit and biological validation on urine samples from pregnant women.

2.1.6.1 Linearity

6 standard iodine solutions, 0µg/l, 20µg/l, 40µg/l, 80µg/l, 120µg/l, 200µg/l, 300µg/l and 600µg/l, were prepared to construct a standard curve. These concentrations covered the range of UIC that were expected in the majority of the study population [43]. A linear relationship was expected between log of absorbance and iodine concentration. Slope, y-intercept, slope uncertainty and intercept uncertainty, residue and R² were estimated to evaluate linearity.
2.1.6.2 Recovery test

In order to test the accuracy of the assay, a recovery test was conducted in which samples were spiked by the addition of a known amount of iodine, and the relationship between the iodine concentration measured in the assay and calculated expected concentration was determined. The concentration of unspiked solution was determined from the assay results.

The expected iodine concentration in each tube was calculated (expected concentration) according to the equation:

\[
\text{Expected concentration in spiked solution} = \frac{\text{amount of iodine in unspiked solution} + \text{amount of iodine added to samples}}{\text{total volume}}
\]

All subsequent serial dilutions were at a ratio of 1:1 of the preceding dilution and the unspiked urine sample. Therefore, for the first dilution, the concentration can be calculated as the average of the expected concentration of spiked and concentration of unspiked solution. The same principle was applied for all subsequent dilutions.

\[
\text{Concentration of dilution} = \text{average } [C(\text{unspiked}) \text{and } C(\text{spiked})]
\]

Once the expected concentrations of spiked and unspiked solutions were calculated, the efficiency of the assay was calculated by determining the percentage recovery according to be equation:

\[
\text{Recovery} = \frac{\text{Observed concentration}}{\text{Expected concentration}}
\]

Where the observed value is the measure obtained in the assay and expected concentration is the calculated value based on the amount of iodine added.
2.1.6.3 Parallelism

Parallelism refers to a dilution linearity test of an authentic sample [165]. Two samples were serially diluted within the quantifiable range and each was measured as described above. The relationship between the dilution factor and the measured iodine concentration was then plotted to confirm that this was parallel to the standard curve.

2.1.6.4 Accuracy

The method was validated for accuracy by comparing the iodine concentration obtained for 184 replicates of the Seronorm™ Trace Elements Urine analysis. The relative standard deviation (RSD) of repeatability and 95% confidence interval (CI) were calculated from these results.

2.1.6.5 Precision

The precision test consisted of two parts: intra- and inter-assay. In order to determine the intra-assay coefficient of variation (CV), 20 samples with a wide range of iodine concentrations were run in triplicate in a single assay. Four samples (external standard, QC1, QC2, QC3 and QC4) were run in triplicate in separate assays all conducted on different days in order to determine the inter-assay CV.

2.1.6.6 Limit of detection (LOD), method quantitation Limit (MQL) and instrument quantitation limit

LOD refers to the lowest concentration where it is possible to distinguish a signal from the background. For this study, I used the definition of the limit of detection set by the International Union of Pure and Applied Chemistry (IUPAC) [166] and the National Association of Testing Authorities, Australia (NATA) [167]
According to this definition, LOD was calculated as the mean concentration plus 3 x the standard deviation (SD) of the concentration of a calibration blank measured in the same assay at least 7 times. In our study, 20 calibration blanks were measured to determine LOD.

The MQL is defined as the minimum concentration of an analyte that can be measured within specific limits of precision and accuracy and is calculated as 3 x LOD multiplied by the dilution factor. Instrument quantitation limit (IQL) is calculated as 3 x LOD.

2.1.6.7 Contamination check of components used in urine collection and analysis

The presence of iodine contamination in the tubes and consumables used for sample collection or in the assay was tested. This was done by first filling them with high purity water before leaving them to stabilise overnight. The high purity water were collected on the following day and tested for iodine contamination using the same method as described for the urine samples.

2.1.7 Validation results

2.1.7.1 Linearity

Three standard curves were constructed to examine the relationship between iodine concentration and log of absorbance. A strong linear relationship was found between iodine concentrations and log of absorbance. The evaluation of linearity is shown in Table 2.1

2.1.7.2 Recovery test

The percentage recovery of iodine in each of 2 samples ranged from 95% to 106% (Table 2.2). There was also strong linear relationship identified between expected and observed iodine concentration.
Table 2.1: The evaluation of linearity

<table>
<thead>
<tr>
<th></th>
<th>Standard curve 1</th>
<th>Standard curve 2</th>
<th>Standard curve 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope *</td>
<td>-525.6</td>
<td>-538.5</td>
<td>-521.5</td>
</tr>
<tr>
<td>Slope uncertainty (%)</td>
<td>-0.7</td>
<td>-0.6</td>
<td>-0.5</td>
</tr>
<tr>
<td>Correlation efficient</td>
<td>0.9997</td>
<td>0.9998</td>
<td>0.9998</td>
</tr>
<tr>
<td>Intercept</td>
<td>142.7</td>
<td>152.0</td>
<td>143.2</td>
</tr>
<tr>
<td>Intercept uncertainty (%)</td>
<td>0.9</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Residual sum of squares</td>
<td>74.7</td>
<td>61.6</td>
<td>47.7</td>
</tr>
</tbody>
</table>

*Negative slope showed the negative relationship between iodine concentration and log of absorbance.

Table 2.2: Recovery percentage of 2 tested urine samples

<table>
<thead>
<tr>
<th></th>
<th>Expected level (µg/l)</th>
<th>Observed level (µg/l)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>71.7</td>
<td>76</td>
<td>106</td>
</tr>
<tr>
<td>2</td>
<td>60.5</td>
<td>59.0</td>
<td>98</td>
</tr>
<tr>
<td>3</td>
<td>54.8</td>
<td>55.2</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>51.9</td>
<td>52.7</td>
<td>101</td>
</tr>
<tr>
<td>Sample B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>54</td>
<td>53.5</td>
<td>99</td>
</tr>
<tr>
<td>2</td>
<td>41.7</td>
<td>41.6</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>35.5</td>
<td>33.8</td>
<td>95</td>
</tr>
<tr>
<td>4</td>
<td>32.4</td>
<td>31.4</td>
<td>97</td>
</tr>
</tbody>
</table>

2.1.7.3 *Parallelism test*

A significant positive correlation was identified between urine volume and iodine concentration for both samples. This indicated that other substances in the urine matrix did not significantly interfere with the assay (Figure 2.2).
2.1.7.4 Accuracy

The results obtained (284.5 ± 12.2µg/l) was in agreement with the certified value (304 ± 44µg/l). The RSD was 4.3 with a 95%CI of 1.8.

2.1.7.5 Precision

The intra-assay CVs of iodine concentration of urine samples ranged from 0.4% to 3.5%. The inter-assay CVs of four urine samples were between 3.9% and 5.3%.

2.1.7.6 LOD, MQL and IQL

The LOD of our method was 11.9µg/l. Since the dilution factor of measured solution was 1, the IQL was equal to MQL, 35.9µg/l.
2.1.7.7 Contamination check of components used in urine collection and analysis

No contamination could be detected in any of the components used to collect and analyse urine samples and results were well below the assay reporting limit (30µg/l) for all tested materials.

2.2 ANALYSIS OF PLASMA TSH AND TG LEVELS

2.2.1 TSH ELISA method

2.2.1.1 Principle of the assay

In this assay, mouse monoclonal anti-TSH antibody is immobilised on the wells of a microtitre plate. After the addition of a sample containing TSH to the plate, TSH binds to the anti-TSH antibodies on the base of the wells. After this, an antibody-enzyme conjugate solution containing a goat anti-TSH anti-body conjugated with an enzyme (horseradish peroxidase) is added to the plate. The anti-TSH antibody in this sample will bind to any TSH molecules bound on the plate, such that TSH molecules are sandwiched between the two anti-TSH antibodies. After a 60-minute incubation at room temperature, to allow for maximum binding, the wells are washed with water which removes all unbound antibodies. In the final step, Tetramethybenzidine (TMB) substrate is added into the wells and the plate incubated for 20 minutes at room temperature. The TMB reacts with the horseradish peroxidase on the antibody enzyme conjugate, resulting in the appearance of a blue colour which is the oxidised product of TMB during the enzymatic degradation of H₂O₂ by horseradish peroxidase. The reaction is then terminated by the addition of a stop solution (1N of HCl), which changes the solution colour from blue to yellow. The absorbance is spectrophotometrically read at 450nm using a microplate reader (BioTek Instruments, USA). TSH concentrations in the sample are directly proportional to the amount of antibody enzyme conjugate bound.
to the plate, and therefore to the intensity of the yellow colouration of the samples after
the addition of the stop solution. The method is summarised in Figure 2.3.

Figure 2.3: The principle of the TSH sandwich ELISA (adapted from [168])

2.2.1.2 Assay procedure

The appropriate number of microplate wells for each standard (ranging from 0 to
25µIU/ml), 2 QCs and samples was determined and all samples and QCs were run in
duplicate. 100µl of standards, controls and samples were dispensed into the appropriate
wells and then 100µl of enzyme conjugate reagent was added into each well. The plate
was thoroughly mixed and incubated at room temperature (18-25°C) for 1 hour. The
incubation mixture was then removed by emptying the contents into a waste container.
The wells were rinsed and flicked 5 times with distilled water and absorbent paper or
paper towels used to remove all residual water droplets.100µl of TMB reagent was then
added into each well and the plate gently mixed for 5 seconds. The plate was incubated
at room temperature for 20 minutes. After incubation, 100µl of stop solution was
dispensed into wells and gently mixed. Absorbance at 450nm was read with a
microplate reader (BioTek Instruments, USA) within 15 minutes of addition of the stop solution.

2.2.1.3 Calculation of results

Mean absorbance value (OD$\textsubscript{450}$) for each set of standards, controls and samples was calculated. A standard curve was constructed by plotting the mean absorbance of each standard against its concentration in µIU/ml, with the absorbance on the y-axis and the concentration on x-axis. The mean absorbance value of controls and samples were used to determine the corresponding TSH concentration from the standard curve.

2.2.1.4 Validation

**Spiking recovery test**

2 plasma samples from volunteers were spiked with 25µl of the 10µIU/ml TSH standard provided in the TSH assay kit, and the TSH level in plasma were analysed as described above. The test was carried out in triplicate. The design is shown in Figure 2.4.

![Figure 2.4: Spiking recovery test](image)

Plasma sample 250µl plasma + 25µl of 10µIU/ml 150µl plasma + 150µl of from dilution 2 150µl plasma + 150µl of from dilution 3 150µl plasma + 150µl of from dilution 4
The percent recovery was calculated as described for the UIC validation.

**Parallelism**

Two plasma samples were serially diluted with high purity water and 5 concentrations were obtained. The TSH level was then analysed in each of the serial dilutions. The relationship between the dilution factor and the measured TSH concentration was then plotted to confirm that this was parallel to the standard curve.

**2.2.1.5 Result of validation**

**Spiking recovery test**

Two selected plasma samples (sample A and sample B) with different concentrations were used to conduct this test. The percentage recovery ranged from 90% to 124%. Strong correlations were identified between expected and observed concentration (Figure 2.5)

![Figure 2.5: The correlation between observed and expected concentration in recovery test](image-url)
**Parallelism test**

There was a strong correlation between plasma volume and TSH concentration in the parallelism test, and the $R^2$ of samples A and B were 0.97 and 0.99 respectively (Figure 2.6).

![Figure 2.6: The correlation between plasma volume (i.e. dilution factor) and TSH concentration in parallelism test.](image)

2.2.2 **Tg ELISA method**

2.2.2.1 *The principle of assay*

The assay utilised the sandwich technique with 2 selected polyclonal antibodies that bind to human thyroglobulin (Tg).

In this assay, high affinity polyclonal anti-human Tg antibodies are immobilised on the wells of microplates. After the addition of samples containing Tg to the plate, Tg is bound to the immobilised antibody during the first incubation. Then, a peroxidase-conjugated polyclonal rabbit anti human Tg antibody is added into each microplate wells. A sandwich of capture antibody-human Tg-peroxidase-conjugate is formed. After this, TMB substrate is added into the wells. The TMB reacts with the horseradish...
peroxidase on the antibody enzyme conjugate which changes the colour to blue. Finally, a stop solution is added to terminate the reaction and the colour of solution changes from blue to yellow. The absorbance of the samples is then spectrophotometrically read at 450nm using the microplate reader (BioTek Instruments, USA). The intensity of the yellow colour is directly proportional to the Tg concentration. The method is summarised in Figure 2.7.

![Figure 2.7: The procedure of Tg analysis (adapted from [168])](image)

2.2.2.2 Calculation of results

Sigma plot version 11.0 (Sigstat software Inc) was employed and the “four-parameter-algorithm” function was used to generate the standard curve and calculate the results for unknown samples.

2.2.2.3 Validation

The validation of the Tg kit was conducted based on the guidelines provided by the manufacturer.
**Spiking recovery test**

For the determination of recovery percentage, 3 sample series were assayed:

Series 1: 50µl standard/control + 50µl assay buffer

Series 2: 50µl sample + 50µl assay buffer

Series 3: 50µl sample + 50µl human thyroglobulin (hTg)-concentrate

These series were analysed in triplicate, and five plasma samples were used in each test. The percentage recovery was calculated according to the formula:

\[
%\text{recovery} = \frac{\mu g/l\text{ hTg (with recovery control)}}{\mu g/l\text{ hTg (without recovery control)} + 35\mu g/l} \times 100
\]

The recovery control contained 35µg/l hTg.

**Parallelism test**

2 plasma samples with different Tg concentrations were serially diluted with assay buffer to 5 different concentrations. The assay was performed in triplicate and used to determine the correlation between Tg concentrations and dilution factor.

**Accuracy**

The method was validated for accuracy by comparing Tg concentration of 2 QCs obtained in the assay and the value certified by company.

**2.2.2.4 Results of the validation**

**Recovery test**

The percentage recovery varied considerably between the 5 samples, from 77% to 105% (Table 2.3), indicative of an inconsistent recovery rate between samples.
Table 2.3: Recovery percentage of 5 samples in recovery test

<table>
<thead>
<tr>
<th>Sample</th>
<th>Neat (µg/l)</th>
<th>Spiked (µg/l)</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>17.6</td>
<td>42.7</td>
<td>81.2</td>
</tr>
<tr>
<td>Sample 2</td>
<td>14.5</td>
<td>52.4</td>
<td>106</td>
</tr>
<tr>
<td>Sample 3</td>
<td>6</td>
<td>31.7</td>
<td>77</td>
</tr>
<tr>
<td>Sample 4</td>
<td>7</td>
<td>36.2</td>
<td>86.3</td>
</tr>
<tr>
<td>Sample 5</td>
<td>6.7</td>
<td>37.6</td>
<td>90.3</td>
</tr>
</tbody>
</table>

**Parallelism test**

A strong correlation was found between absorbance and the assay buffer volume (dilution factor), and the $R^2$ of samples A and B were both in the order of 0.99 (Figure 2.8).

![Graph showing absorbance vs. ASYBUF volume](image)

**Accuracy**

The results of QC1 and QC2 obtained in the assay were 7.2µg/l and 19.6µg/l respectively with the RSD < 10%. These values were in a close agreement with the certified values (7.4µg/l for QC1 and 19.3µg/l for QC2).
2.2.3 Summary

The Tg ELISA assay which I intended to use for the determination of Tg in the clinical samples in this thesis could not be appropriately validated, due to poor and highly variable percentage of recovery between samples (77-105%). While the reason for this inconsistent and generally poor percentage recovery is uncertain, it appears likely to be due to the presence of Tg autoantibodies (TgAb) in some of the plasma samples, the interference of which can cause underestimation of the Tg content in samples [169]. It has been reported that TgAb are present in 5.5% of healthy adults in Australia [170], however the prevalence in the paediatric population is unclear. Since I were concerned that using an ELISA kit for Tg analysis which was unable to correct for the presence of TgAb would lead to inaccurate results, I made the decision not to use this assay for the Tg measurements in the clinical samples. Instead, these measurements were undertaken by an external provider, Institute of Medical and Veterinary Science (IMVS, South Australia), because they routinely determine the level of TgAb when assessing Tg levels in human plasma.
CHAPTER 3
Validation of an optimized method for the determination of iodine in human breast milk by inductively coupled plasma mass spectrometry (ICPMS) after tetramethylammonium hydroxide extraction

Dao Huynh, Shao Jia Zhou, Robert Gibson, Lyndon Palmer, Beverly Muhlhausler

Published in Journal of Trace Elements in Biology and Medicine (2014)
# Statement of Authorship

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## Author Contributions

By signing the Statement of Authorship, each author certified that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate’s thesis.

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<tr>
<th>Name of Principal Author (Candidate)</th>
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<td>Contribution to the Paper</td>
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<td>Name of Co-Author</td>
<td>Shao Jia Zhou</td>
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<td>Contribution to the</td>
<td>Established the protocol of breast milk iodine analysis, performed validation test, contributed to manuscript construction and evaluation</td>
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CHAPTER 3   VALIDATION OF AN OPTIMISED METHOD FOR THE DETERMINATION OF IODINE IN HUMAN BREAST MILK BY INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY (ICPMS) AFTER TETRAMETHYLAMMONIUM HYDROXIDE EXTRACTION

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Short title: Human milk iodine analysis by ICPMS

3.1 SUMMARY

In this study a novel method to determine iodine concentrations in human breast milk was developed and validated. The iodine was analysed by inductively coupled plasma mass spectrometry (ICPMS) following tetramethylammonium hydroxide (TMAH) extraction at 90°C in disposable polypropylene tubes. While similar approaches have been used previously, this method adopted a shorter extraction time (1 hour vs. 3 hours)
and used Antimony (Sb) as the internal standard, which exhibited greater stability in breast milk and milk powder matrices compared to Tellurium (Te). Method validation included: defining iodine linearity up to 200µg/l; confirming recovery of iodine from NIST 1549 milk powder. A recovery of 94-98% was also achieved for the NIST 1549 milk powder and human breast milk samples spiked with sodium iodide and thyroxine (T4) solutions. The method quantitation limit (MQL) for human breast milk was 1.6µg/l. The intra-assay and inter-assay coefficient of variation for the breast milk samples and NIST powder were <1% and <3.5% respectively. NIST 1549 milk powder, human breast milk samples and calibration standards spiked with the internal standard were all stable for at least 2.5 months after extraction. The results of the validation process confirmed that this newly developed method provides greater accuracy and precision in the assessment of iodine concentrations in human breast milk than previous methods and therefore offers a more reliable approach for assessing iodine concentrations in human breast milk.

3.2 INTRODUCTION

Iodine is a major constituent of thyroid hormones, and an adequate supply of iodine before birth and in early infancy is essential for achieving optimal physical growth and mental development [8]. Breast milk is the sole source of iodine for exclusively breastfed infants, and it is therefore critical to ensure that the iodine content of the breast milk is sufficient to meet the nutritional needs of this infant population. However, relatively few studies have reported breast milk iodine concentrations, and this is largely due to the lack of a robustly validated method for assessing iodine concentrations in human breast milk.

Since breast milk is a complex matrix, consisting of a range of bioactive components and nutrients, existing studies which have measured iodine concentrations in breast milk
have applied comparable methods to those used for assessing iodine content of food. A number of analytical methods have been used for the determination of iodine in foodstuffs including the classic Sandell and Kolthoff kinetic-catalytic method [97, 171], ion chromatography [172, 173], inductively coupled plasma mass spectrometry (ICPMS) [174-177], flame atomic absorbance spectrometry [178], high performance liquid chromatography [179] and ion-specific electrodes [177, 179]. Of these methods, ICPMS is considered to be the gold standard, due to its high level of accuracy, precision and low detection limit, and is the most widely used approach for iodine quantification in foods. Moreover, ICPMS analysis following extraction by TMAH has been adopted by the European Committee for Standardization as the official method for the quantification of iodine concentrations in foodstuffs (EN 15111:2007) [180].

However, while the ICPMS method is routinely used for the assessment of iodine in foods [99, 177, 181-184], its suitability for the assessment of iodine in human breast milk has not been systematically assessed, and various aspects of the method have not been optimized. It is not clear, for example, whether TMAH is a strong enough digesting agent to liberate iodine from T4 in breast milk or whether the internal standard used in the assay is suitable for the human milk matrix. In addition, the limit of determination of the previously reported method is relatively high (30µg/kg) [99], raising concerns about its sensitivity, and the extent of carryover between samples has not been thoroughly tested. The process of preparing the milk samples for analysis, in particular the procedure for homogenization of milk samples after thawing, has also not been clearly described.

Therefore, the aim of this study was to modify existing approaches for assessing iodine concentrations in breast milk to address these issues and thereby develop an ICPMS method which was suitable for the accurate and reproducible determination of iodine in human breast milk.
3.3 MATERIALS AND METHODS

3.3.1 Reagents and equipment

Two human milk samples were collected in 50 mL polypropylene (PP) tubes with screw caps (Cat no. 227261, Greiner Bio-One GmbH, Frickenhausen, Germany) and frozen at -20°C until analysis for concentration, long term stability and spiked recovery.

Certified reference material (CRM) produced by the National Institute of Standard and Technology (NIST), NIST 1549 non-fat milk powder (Maryland, USA) with a certified iodine level of 3.38 ± 0.02mg/kg was used to assess the accuracy of iodine determination.

Reagents for milk digestion included: high purity TMAH powder (Cat. No. T7505-100G) from Sigma-Aldrich (New South Wales, Australia), a commercial stock iodine standard (1000mg/l) from Australia Chemical Reagents (Queensland, Australia), two internal standard stock solutions, 1000±3mg/l Tellurium (Te) in 2% HNO₃ + 0.2% HF and 1000±3mg/l Antimony (Sb) in 5% HNO₃ + 0.1% HF from High-Purity Standards (South Carolina, USA). L-Thyroxine (T4) powder (Cat. No. T2376-1G) for recover tests was purchased from Sigma-Aldrich (New South Wales, Australia).

High purity water generated by a Sartorius Water Purification System (Sartorius Stedim Australia Pty. Ltd., Dandenong South Victoria, Australia) was used for the preparation of all reagents, standards and samples.

Graduated 50 mL PP digestion tubes with screw caps (Cat. No. SC475, Environmental Express South Carolina, USA) were used for digestion/extraction with TMAH.
Graduated 15 mL PP tubes with screw caps (Cat. No. 188261 Greiner Bio-One GmbH, Frickenhausen, Germany) purchased from Interpath Services Pty Ltd (South Australia, Australia) were used as the analysis tube for the ICPMS.

Millex HV disposable syringe filters (33 mm diam. 0.45 μm pore size; Millipore Corp, MA, USA) and Terumo 10ml syringes (Binan Laguna, Philippines) were used to filter the digested breast milk samples.

An IKA T25 digital Ultra Turrax homogenizer (IKA Ltd, Germany) was used to macerate breast milk samples before analysis.

The digestion step was performed in a 54 well HotBlock™ heating block with digital temperature control (Environmental Express Cat. No. SC154) purchased from DKSH Pty. Ltd. Australia.

All reagent additions and dilutions were carried out using a semi-automated Gilson 402 diluter (John Morris Scientific, Keswick, South Australia). The dilutor tubing (FEP-Fluorinated ethylene propylene) was soaked in 8% TMAH overnight and thoroughly rinsed with high purity water before use.

Pure water was dispensed with a Brand® bottle top dispenser (5-50 mL Dispensette® Organic, Digital Cat. No. 4730 360)

The TMAH powder and all containers and equipment used to prepare the samples or store/collection the breast milk were checked for iodine contamination before use.

### 3.3.2 Reagents and standard solution preparation

To prepare the 25% TMAH and 8% TMAH solutions, 125g or 40g respectively of TMAH was dissolved in high purity water and made up to 500 ml. These solutions were stored at room temperature.
3.3.2.1 Internal standard stock solutions.

1.6ml of Te stock standard, 2ml of Sb stock standard and 40ml of 25% TMAH were diluted in high purity water to a final volume of 1000ml to make solutions of 1.6mg/l Te and 2mg/l Sb which were stored at room temperature. These solutions were further diluted during the calibration standard preparation and digestion process to yield 40µg/l Te and 50µg/l Sb in the final analysed solutions.

3.3.2.2 Iodide spiked solutions.

To prepare iodide spiked solutions, the 1000mg/l iodide stock solution was diluted with high purity water to yield 50mg/l iodide solution. This solution was further diluted in 1% TMAH to produce final concentrations of 0.2, 0.4 and 0.8mg/l. These solutions in turn were used as spiked solutions in recovery tests, producing concentrations of 2.5, 5.0 and 10µg/l in the final analysed samples.

3.3.2.3 Thyroxine spiked solutions.

To prepare thyroxine (T4) spiked solutions, 0.1531g of T4 was dissolved in 20ml of 25% TMAH and diluted to 2l with high purity water to yield a 50mg/l T4 iodine solution in 1% TMAH. This solution was further diluted in 1% TMAH to produce final concentrations of 0.2, 0.4 and 0.8 mg/l. These solutions in turn were used as spiked solutions for recovery tests producing concentrations of 2.5, 5.0 and 10µg/l T4 in the final analysed samples.

3.3.2.4 Iodine calibration solutions (0, 1, 2.5, 5, 10, 25, 50, 100 and 200µg/l)

The iodine stock solution (1000mg/l) was diluted in high purity water to a concentration of 5mg/l (intermediate stock standard). This solution was further diluted to prepare intermediate standard 1 and 2 with concentrations of 1.0 and 0.05mg/l respectively and these 2 standards in turn were used to make the calibration solutions in 1% TMAH. 1ml
of internal standard solution was added to 40ml of each these iodine calibration solutions. The calibration solutions were stored at room temperature and fresh solutions prepared every 4 months.

3.3.2.5 Iodine drift correction standard solutions (0 and 5µg/l)

The iodine intermediate standard 2 solution (0.05mg/l) was used to make the drift correction standard. Both these standards were prepared in 1% TMAH. 1ml of internal standard solution was added into 40ml of each these iodine drift correction standard solutions.

3.3.2.6 Blanks

Four types of blanks were used; a calibration blank, a method digestion blank processed in exactly the same way as the samples and containing all the reagents used in the assay, a drift correction blank and a system blank. All blanks were prepared in 1% TMAH and also contained the internal standard mix. The calibration blank was used to establish the analytical calibration curve, the method digestion blank was used to account for batch to batch variation and to overcome system memory effects from the instrument by subtraction during result calculation, the drift correction blank was used to normalize the drift standard solution every 25 samples during each analytical run and the system blank was used to monitor the overall system memory from the instrument during each analytical run.

3.3.2.7 Wash solutions

Three types of wash solutions were used. Two of the solutions, the auto-sampler wash station rinse solution and an extra clean wash solution, consisted of 1% TMAH and high purity water. Another pre-wash solution was prepared with 1% ammonia (NH₄) in high purity water.
3.3.3 Instrumentation

Iodine determination was carried out using an Agilent 7500ce ICPMS system consisting of an Integrated Sample Introduction System (ISIS) unit plus a CETAC ASX-510 autosampler (Agilent Technologies Australia) equipped with a Ceramic VeeSpray nebulizer (Glass Expansion Pty. Ltd, Melbourne, Australia). The ISIS peristaltic pump program was used to reduce the washout time and speed up sample uptake to the instrument between samples to reduce analysis time per sample. Instrument performance optimization, including nebulizer gas flow rate, ion lens voltage and torch alignment, was set up following the manufacturer’s instructions and optimized before each run. Operating conditions for the systems are shown in Table 3.1.
Table 3.1 Agilent ICPMS operating conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>RF power (W)</td>
<td>1500 W</td>
</tr>
<tr>
<td>RF matching (W)</td>
<td>1.66 W</td>
</tr>
<tr>
<td>Frequency (MHz, free running)</td>
<td>27 MHz</td>
</tr>
<tr>
<td>Sampling depth (mm)</td>
<td>0.8 mm</td>
</tr>
<tr>
<td>Carrier gas (L/min)</td>
<td>1.00 L/min</td>
</tr>
<tr>
<td>Makeup gas (L/min)</td>
<td>0.20 L/min</td>
</tr>
<tr>
<td>Nebulizer</td>
<td>Ceramic VeeSpray</td>
</tr>
<tr>
<td>Spray chamber</td>
<td>Double Pass</td>
</tr>
<tr>
<td>Nebulizer pump (rps)</td>
<td>0.20 rps</td>
</tr>
<tr>
<td>Lens Settings</td>
<td>Optimized with each run</td>
</tr>
<tr>
<td>Iodine (I)-Mass</td>
<td>127 Mass</td>
</tr>
<tr>
<td>Antimony (Sb)-Mass</td>
<td>121 Mass</td>
</tr>
<tr>
<td>Tellurium (Te)-Mass</td>
<td>128 Mass</td>
</tr>
<tr>
<td>Scanning mode</td>
<td>Peak hoping</td>
</tr>
<tr>
<td>Points/peak</td>
<td>3</td>
</tr>
<tr>
<td>Number of replicates</td>
<td>3</td>
</tr>
</tbody>
</table>

All raw concentration data from the ICPMS was exported to Microsoft Excel. Blank subtraction, drift correction and other data processing (mass and volume adjustments) were performed off-line, using custom-written macro programs operated within Excel.

### 3.3.4 Sample Digestion

1 mL of homogenized breast milk measured using the Gilson 402 diluter or 0.1g of milk powder was placed into labelled 50ml PP tubes, 5ml of 8% TMAH plus 0.75ml of pure water was then added to each of the tubes using the diluter and the tubes recapped. Samples were mixed by shaking/vortexing at low speed and allowed to stand overnight in a fumehood at room temperature. On the following day, samples were mixed again by shaking/vortexing and digested at 90°C for 1 hour using the heating block system. Samples were mixed by shaking/vortexing at least twice during the incubation period to
ensure complete digestion. The tubes were then removed from the heating block and cooled at room temperature. 1 mL of internal standard solution plus 2.25 ml of pure water was added to all tubes using the diluter and the volume made up to 40 ml by the addition of 30 ml high purity water using a Brand® bottle top dispenser. The tubes were tightly recapped, shaken/vortexed until thoroughly mixed and then 5-10 ml of each digested solution was filtered and transferred into graduated 15 ml PP tubes prior to ICPMS analysis.

3.3.5 Method Optimisation

3.3.5.1 Optimal digestion time and temperature for iodine (iodide and T4) extraction from human breast milk

The efficiency of iodine extraction from breast milk and NIST 1549 milk powder by TMAH was tested under 3 different conditions: (1) 80°C for 1 hour, (2) 90°C for 1 hour and (3) 90°C for 2.5 hours. The recovery of iodine from the NIST 1549 milk powder and 2 human milk samples were tested under each of these conditions.

3.3.5.2 Instrument/system memory effect

Depending on the prior use of the ICPMS instrument (nitric acid digests or TMAH digestions) the system showed various levels of iodine contamination. The most effective pre-wash solution was determined by comparing the time taken for the background count to decrease to an acceptable and stable level (600-1000 counts/second for iodine) following a continuous pre-wash with 1% TMAH or 1% ammonia.

The efficiency of cleaning up the ICPMS system with the 1% TMAH pre-wash solution was tested for 3 levels of iodine contamination, namely highly contaminated (0.46 µg/l), slightly contaminated (0.3 µg/l) and a clean system (0.06 µg/l). The cleaning efficiency of the 1% NH₄ pre-wash solution was also tested on a highly contaminated system.
Furthermore, the clean-up efficiency of the pre-wash solution was monitored in each analytical run by collecting iodine concentrations of all the blank solutions defined above to assess the instrument memory effect.

3.3.5.3 Sample-to-sample carryover

Previous studies in our group had indicated that 4% perchloric acid digests and high salt matrices had the potential to cause precipitation within a range of nebulizers leading to significant drift throughout long analytical runs. These studies also suggested the use of wet argon in the nebulization gas could prevent significant drift and reduce sample-to-sample carry over. In the present study, sample carryover from a previous analytical sample was determined after washing the nebulizer with either dry or wet argon.

The sample carryover in these tests was determined by assessing iodine concentration in 15 successive replicate blanks following the measurement of iodine in a highly concentrated standard solution (200µg/l).

3.3.5.4 Stability of the iodine calibration standards

Three of the iodine standard solutions (2.5, 5 and 10µg/l) were analysed on repeated occasions over a 2.5 month period to assess the stability of iodine standards during storage. The iodine concentrations measured at each of the time points across the 2.5 month period were compared to those in the equivalent standard immediately after preparation.

3.3.5.5 Stability and reliability of internal standards

The stability and reliability of two internal standards, Sb and Te, were tested in the human breast milk matrix in order to assess their suitability for routine analysis. Human breast milk samples, NIST non-fat milk powder reference material and standards were prepared as described earlier with the addition of a mixture of 2 internal standards (Sb
and Te). The raw intensity (raw counts) of iodine (I), Sb and Te were monitored to check for stability. The raw I counts were also normalized to both the raw Sb and Te counts to evaluate the stability and reliability of each internal standard across the analytical run.

3.3.5.6 *Human breast milk homogeneity*

After collection, the breast milk sample was mixed by shaking vigorously, split into 2 aliquots and then frozen at -20°C until analysis. After thawing, one aliquot was homogenized at 20,000 min⁻¹ for 30 seconds while the other milk was allowed to separate into two phases, the aqueous and fatty fractions. The iodine level of each fraction was measured and compared to the concentration measured in the homogenized sample.

3.3.5.7 *Stability of extracted samples*

To assess the stability of iodine in samples post digestion, NIST 1549 milk powder and 2 human milk samples were digested by using the digestion procedure described above and iodine levels measured on the day of digestion and following 2.5 months storage at room temperature.

3.3.5.8 *Contamination check of components used in the milk collection and analysis*

A small amount of high purity water was placed into the various containers and devices used in the milk collection process. This included 2 types of collection containers, 2 types of breast pump systems and 2 types of storage tubes. The water was left to stabilize for periods varying from 3 hours to 2.5 weeks. Samples of this high purity water were then collected and tested for iodine contamination using the same method as for human milk samples. The contamination check was also conducted for all the disposable PP tubes used in the digestion and analysis steps.
3.3.6 Method Validation

3.3.6.1 Linearity

The iodine standards (0, 1, 2.5, 5, 10, 25, 50, 100, 200µg/l) were used to establish the calibration curve. In order to ensure accuracy, the iodine concentrations of all samples analysed are required to fall within the range of the calibration curve, otherwise the samples need to be diluted.

3.3.6.2 Recovery

A breast milk sample, NIST 1549 milk powder and a method digestion blank were used to determine the percent recovery of various levels of added iodine in the samples. The breast milk and milk powder samples were spiked with 5 and 10µg/l of iodide solution and also with 5µg/l and 10µg/l of T4 iodide solution. The method digestion blank was spiked with 2.5 and 5.0µg/l of iodide solution and also with 2.5µg/l and 5.0µg/l of T4 iodide solution. The unspiked samples and all spiked samples were measured in quadruplicate. The measured iodine concentration was divided by the expected value in order to determine the percent recovery.

3.3.6.3 Precision

The intra-assay and inter-assay variation were determined by analysing 2 breast milk samples and NIST 1549 milk powder either 4 times in a single run (intra-assay coefficient of variation (CV)) or on 4 different days in 4 separate assays (inter-assay CV).
3.3.6.4 Accuracy

The method was validated for accuracy by comparing the iodine concentration obtained for 78 replicates of NIST CRM 1549 milk powder analysis carried out over a period of ~3 years. The CV of repeatability and 95 % confidence interval (CI) were calculated.

3.3.6.5 Limit of Detection (LOD) and Method Quantitation Limit (MQL)

LOD refers to the lowest concentration where we can just distinguish a signal from the background. For this publication we used the definition of the limit of detection set by the International Union of Pure and Applied Chemistry (IUPAC) [166] and the National Association of Testing Authorities, Australia (NATA) [167]

According to this definition, LOD was calculated as the mean concentration plus 3 X the standard deviation of the concentration of a calibration blank measured in the same assay at least 7 times.

The MQL is defined as the minimum concentration of an analyte that can be measured within specified limits of precision and accuracy and is calculated as 3 X LOD multiplied by the dilution factor [167]. This takes into account any matrix related effects. Instrument Detection Limit (IDL) is equivalent to the LOD in our case. The Instrument Quantitation Limit (IQL) is calculated as 3 X LOD.
3.4 RESULTS

3.4.1 Method optimisation

3.4.1.1 Optimal digestion time and temperature for iodine extraction from human breast milk

It was critical to achieve a complete sample digestion to ensure the accuracy of iodine measurements conducted in downstream applications, including ICPMS. As illustrated in Table 3.2 the efficiency of extraction of iodine from human breast milk was similar under all tested conditions. The extraction efficiency for the NIST CRM 1549 milk powder also showed good agreement with the certified value (3.38±0.02mg/kg) for all 3 processes tested. We chose to use a digestion temperature of 90°C for 1 hour for all subsequent experiments to reduce the overall time required for sample preparation.

Table 3.2: Comparison of iodine concentration determined in the sample NIST 1549 milk powder and human breast milk samples for each of the 3 digestion conditions

<table>
<thead>
<tr>
<th>Materials</th>
<th>80°C / 2.5h</th>
<th>90°C / 1h</th>
<th>90°C / 2.5h</th>
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<tr>
<td>NIST 1549 milk powder (mg/kg)</td>
<td>3.39 ± 0.03</td>
<td>3.37 ± 0.01</td>
<td>3.38 ± 0.01</td>
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<td>(N = 3)</td>
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<tr>
<td>Human milk 1 (µg/l)</td>
<td>88.7 ± 0.5</td>
<td>89.7 ± 2.8</td>
<td>88.0 ± 0.02</td>
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<tr>
<td>(N = 2)</td>
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<tr>
<td>Human milk 2 (µg/l)</td>
<td>74.3 ± 0.2</td>
<td>74.2 ± 0.1</td>
<td>74.4 ± 0.06</td>
</tr>
<tr>
<td>(N = 2)</td>
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</tr>
</tbody>
</table>

3.4.1.2 Instrument/system memory effect

We found 2 significant areas within the ICPMS that showed memory effect; the autosampler wash station and the uptake tubing/nebuliser/spray chamber area. There was a marked difference in the time taken for the background counts to drop to acceptable levels between the 1% TMAH and 1% NH₄ pre-wash solutions. When using 1% TMAH
to wash out the the 2 areas identified above, the background count levels decreased gradually and often did not reach an acceptable level even after ~2 - 3 hours from the start of the wash procedure or well into the actual analysis run. In contrast, using a 1% NH₄ solution resulted in a sharp drop in the background counts to an acceptable level after only 10 minutes in the uptake area. The auto-sampler wash station also required extra soaking with 1% NH₄ to remove long term buildup (data not shown).

The data presented in Figure 3.1 supports these findings. In the clean system, achieved after 1 day of running routine breast milk iodine analyses, the iodine concentration in the blank decreased only slightly at the start of the run before stabilizing, indicating that there was minimal iodine contamination prior to the start of the run. In contrast, in both the highly contaminated and slightly contaminated systems, the iodine concentrations in the blank solutions analyzed after the pre-wash with 1% TMAH dropped sharply at the beginning of the analysis run before declining to acceptable stable iodine levels for the blank sample, indicating that 1% TMAH wash-out was not effective at reducing the iodine concentrations to acceptable levels prior to the analytical run. In the highly contaminated system cleaned using the 1% NH₄ pre-wash solution, however, we achieved a similar washout profile to the clean system washed with 1% TMAH. This suggested that a 1% NH₄ pre-wash solution could be used once before each run to achieve an acceptable background count at the beginning of the analytical run.

We also observed a substantial and variable drop in the baseline counts depending upon the extent of the system contamination, indicating that the way the method digestion blank is handled is critical with respect to the calculation of the final results. We therefore subtracted our method digestion blank value from each batch of samples as a part of calculation.
Iodine concentration of the drift correction blank (0µg L⁻¹) over the course of an ICPMS run at 3 levels of system contamination; Contaminated system after 1% TMAH pre-wash solution (■), slightly contaminated system after 1% TMAH pre-wash solution (▲) and clean system after 1% TMAH pre-wash solution (●), contaminated system after 1% NH₄ pre-wash solution (○). The iodine concentration at t=0 represents the iodine zero calibration concentration. The negative iodine concentrations indicate the extent of washout during each analytical run.

3.4.1.3 Sample-to-sample carryover

Figure 3.2 shows the result of the sample carryover experiment comparing the extent of carryover after using either wet or dry argon to rinse the nebulizer between samples. There was no significant difference in the reduction of carry over effects between the wet and dry argon in this system.
3.4.1.4 Stability of the iodine calibration standards

Over the 2.5 month period, the percentage error for the measurement of the 3 iodine calibration solutions ranged from -1.3 to +0.5% with respect to their actual concentration. Iodine standard solutions appeared to be very stable after preparation for a period of at least 6 months (data not shown).

3.4.1.5 Stability and reliability of internal standards

As expected with ICPMS the raw count stability over the various runs varied significantly for both internal standards as well as iodine (data not shown). When the raw iodine counts were normalized to the raw counts for the 2 internal standards in the drift check standard, the stability for iodine concentration measured across the run improved significantly for both Sb and Te. When Sb was used the internal standard, the values for the NIST milk powder certified reference material were consistent with the

Figure 3.2 Sample-to-sample carryover:

Iodine washout with dry argon (—) and wet (---) argon after analysing 200 µg L\(^{-1}\) iodide. IQL, instrument quantitative limit; IDL, instrument detection limit.
expected value. However, when the iodine counts were normalized to the Te standard counts, the calculated result for the NIST milk powder certified reference material exceeded the expected value by 10% of the expected value. Sb was therefore selected as the internal standard used to correct for the variability caused by matrix effects and instrument drift in this assay.

3.4.1.6 Human breast milk homogeneity

The iodine level in the fatty fraction (145±48μg/l) was considerably higher and more variable than in the aqueous fraction (88.5±2.1μg/l). The iodine concentration in the homogenized samples (105.7±0.6μg/l) was notably less variable, and was intermediate to that of the two separate fractions. These iodine concentrations were within the expected range of breast milk iodine levels in breast-feeding women from iodine sufficient populations [93, 185-188].

3.4.1.7 Stability of extracted samples

There was no difference in the iodine concentration measured in digested samples immediately after digestion or after 2.5 months of storage post digestion (Table 3.3).

Table 3.3: The stability of extracted samples

<table>
<thead>
<tr>
<th>Materials</th>
<th>0 month</th>
<th>2.5 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIST 1549 milk powder (mg/kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N = 2)</td>
<td>3.39 ± 0.01</td>
<td>3.39 ± 0.01</td>
</tr>
<tr>
<td>Human milk 1 (μg/l)</td>
<td>106 ± 1.0</td>
<td>105 ± 1.0</td>
</tr>
<tr>
<td>(N = 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human milk 2 (μg/l)</td>
<td>79.8 ± 1.7</td>
<td>81.0 ± 1.4</td>
</tr>
<tr>
<td>(N = 4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values were expressed as mean ± SD.
3.4.1.8 Contamination check of components used in milk collection and analysis.

No iodine contamination was detected in any of the equipment used to collect and analyze human milk. All results were below the MQL (1.6µg/l) for all tested components.

3.4.2 Method Validation

3.4.2.1 Linearity

The standard curve was linear up to 200µg/l iodine and the slope and coefficient of correlation were 0.0162 and 0.9999 respectively.

3.4.2.2 Recovery

For the NIST SRM (n=4), recoveries between 95.5 % and 96.5 % were achieved for solution spiked with both low (5µg/l) and high (10µg/l) amounts of iodine (Table 3.4). For human breast milk samples, the percentage recovery of iodine from all the spiked samples was between 96.5 % and 97.2 % (n=4 for each spiked concentration level). A recovery of ~ 96 % also found in breast milk samples and NIST milk powder spiked with the 2 different concentrations of T4. The percentage recoveries for all T4-spiked blanks were between 96.2 % and 98.2 % with the variation of 0.5 %.
Table 3.4: Iodine recovery percentage for samples spiked with iodide and T4.

<table>
<thead>
<tr>
<th></th>
<th>Iodine concentration</th>
<th>T4 concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5µg/l</td>
<td>5µg/l</td>
</tr>
<tr>
<td>Blank</td>
<td>93.8±0.5</td>
<td>97.2±0.5</td>
</tr>
<tr>
<td>NIST milk powder</td>
<td>-</td>
<td>96.0±1.6</td>
</tr>
<tr>
<td>Breast milk</td>
<td>-</td>
<td>97.2±0.5</td>
</tr>
</tbody>
</table>

Values expressed as mean % ± SD. (N = 4)

3.4.2.3 Precision

The intra-assay CVs for iodine concentration of 2 breast milk samples were 0.1% and 1.8%, respectively whilst NIST 1549 milk powder were 0.97 %. The inter-assay CVs were 2.2%, 3.06% for 2 breast milk samples and 0.25 % for NIST 1549 milk powder.

3.4.2.4 Accuracy

The results obtained for the NIST milk standard using this method was 3.38 ± 0.02 mg/kg (N = 78) was in a close agreement with the certified value of 3.38 ± 0.02mg/kg. The CV was 0.5 % with a 95 % CI of 0.005.

3.4.2.5 Detection limits

The IDL for iodine in human milk was 0.013µg/l if carryover and contamination were eliminated as described in this paper. Assuming a dilution factor of 40, the MQL was 1.6µg/l.
3.5 DISCUSSION

This paper describes the development and validation of a method for the assessment of iodine concentration in human breast milk which offers significant improvements over existing methods in relation to detection/quantitation limit, choice of internal standard and minimization of instrumental memory effect during analysis.

The performance of the method was evaluated with respect to linearity, recovery, precision, accuracy and quantitation limit. The method exhibited strong linearity ($R^2 > 0.999$ and slope of 0.0162) and achieved high recovery (>94%) of iodine from milk samples spiked with either iodine or T4. The intra- and inter-assay of the breast milk and NIST milk powder samples were both <3.5% and the iodine level measured in NIST 1549 milk powder using this method were in close agreement with the certified value, indicating a high degree of reliability and precision. The quantitation limit for this method of 1.6µg/l, was much lower than the quantitation limits which have been reported in previous methods, which have ranged from 14-30µg/l [99, 171, 181, 189]. Importantly, the quantitation limit we achieved in our method is also well below the expected range of iodine concentrations in human breast milk, even in regions classified as iodine deficient (32-78µg/l) [190-192], suggesting that the method is appropriate for the assessment of iodine concentrations in breast milk across a broad range of populations.

Sample digestion is a critical step in ensuring accurate determination of the iodine content of samples by ICPMS, and establishing a method for the digestion of human milk samples which did not affect iodine concentrations in the sample was a critical component of this study. Previous studies had shown that extraction by acidic and ammonia media had the potential to affect the accuracy of the analysis, by inducing instrument memory effects [101] and variations in the efficiency of iodine extraction.
between samples [193, 194]. As a result, we chose to use another widely applied digestion medium, TMAH, in this method. The use of TMAH in iodine extraction for ICPMS analysis has previously been applied to infant formula [177], milk powder [99, 177, 183, 195] and herb milk [196], but this is the first report of its successful application in the assessment of iodine in human breast milk samples. In addition, we showed that we were able to obtain complete and reproducible digestion with considerably shorter heating times compared to previous studies (1 hour vs 2-3 hours) [99, 175, 183] thus reducing the total time required for the experimental procedure.

Although previous studies have suggested that iodine species in biological samples can be extracted by TMAH, there had been no reports of successful extraction of iodine from iodine-containing compounds, such as T4. The failure to liberate iodine from such compounds during the digestion process would result in underestimation of the iodine content of the sample, thus impacting on accuracy and reliability of the measurements. By assessing the recovery of iodine from human breast milk samples spiked with T4 in the present study, we confirmed that TMAH digestion was able to release iodine from this complex, and therefore provide an accurate measure of the total iodine content of the sample.

We did not find any difference between wet and dry argon in the efficacy of reducing carryover between samples in this study. Although not the focus of our study, flushing the system with wet argon is likely to be preferable in practice, since it is able to prevent the build of iodine contamination within the argon jet in the nebulizer, which has the potential to introduce random errors during large analytical runs (unpublished data). We identified the auto-sampler wash station as a major source of instrument carryover when the ICPMS machine had previously been utilized for the assessment of other elements and using other wash out solutions (e.g. nitric acid solutions). Thus, thoroughly flushing
the system with a 1% NH₄ solution prior to commencing any new assay is necessary to avoid carryover effects due to previous analyses conducted on the same instrument.

Drift is an analytical error leading to the poor accuracy, and arises when instrument responses change through the run. The drift can be corrected by analysing drift standards after every four to five samples [197], however no previous studies involving assessment of iodine content in breast milk have included an approach for drift correction. Instrument performance in this method was monitored by including a high drift standard (5µg/l) and drift correction blank (0µg/l) after every 25 samples, and we were able to use this to appropriately correct for the drift in the experimental procedure. Measurement of drift standards after 25 samples, instead of 4-5 samples reduces the cost and reading time in routine analysis. It was also noted that the largest difference between successive drift iodine levels occurred at the beginning of the run and this also needed to be taken into account during the drift correction to achieve the most accurate results.

Te has commonly been used as an internal standard in ICPMS methods and was selected due to the fact that its ionization status is closer to iodine than other elements [99]. However, the Te signal in the milk powder experiment was lower and more variable than expected, resulting in reduced accuracy in the assessment of iodine concentrations. This may be because Te precipitates with some components within the human milk matrix, and indicates that it is not the most appropriate internal standard for this method. However, Sb exhibited much greater stability in the human milk matrix in the present study and appears to be a more suitable internal standard for ICPMS assessment of human breast milk.

In a previous study reporting the analysis of iodine in food, high-cost tubes were used during sample digestion, which were washed and re-used for subsequent assays. Even
with thorough washing, there is the potential for trace amounts of iodine to remain in the tubes, which could produce carryover effects and introduce inaccuracies in the results [99]. In this method, we eliminated this source of contamination by using disposal screw cap polypropylene tubes [198]. Disposable auto-sampler tubes and pipette tips were also used to minimize the potential for contamination. Importantly, we confirmed that all the containers and equipment that were in contact with the sample at any stage in the process of collection, processing and analysis were free from iodine contamination, and can thus be confident that any iodine detected in this assay originated from the breast milk sample. Confirming the absence of contamination is critical to ensuring that the results of the assay are a true reflection of concentrations in the sample and should be standard practice in any application of this method.

Appropriate sample preparation is essential to achieve reliable results. Despite the fact that human milk is known to separate into 2 distinct layers after thawing or during extended periods of standing, no previous method has provided detailed information regarding sample preparation. This study confirmed that iodine concentration differs markedly between the fatty and aqueous fractions of human milk and that complete homogenization of the samples is required prior to digestion in order to obtain reliable results.

The stability of extracted human milk samples had not been tested in previous methods. In this modified method we found that iodine level in extracted human milk also containing the internal standard was stable for at least 2.5 months when stored at room temperature. The finding increases the potential for this method to be utilized in large-scale clinical trials and population screening programs, since it makes it possible for samples collected at different times to be analysed in the same ICPMS run.
In conclusion, we have successfully validated a method for the assessment of iodine in human breast milk which overcomes the limitations of previous approaches and highly accurate, reproducible and precise. The modified method is able to recover over 95% of iodine from spiked solutions, has a lower quantitation limit than previous method and has inter-and intra-assay coefficients of variation well below 5%. This method represents a significant advance in the assessment of iodine concentrations in human breast milk. This assay is currently being applied for routine assessment of iodine concentration in breast milk samples in our laboratory.

3.6 ACKNOWLEDGMENTS

We are grateful to Waite Analytical Service, School of Agriculture, Food and Wine, University of Adelaide for assistance of method development and validation. Beverly Muhlhausler is supported by a Career Development Award from the National Health and Medical Research Council of Australia (NHMRC). Robert Gibson is supported by a NHMRC Senior Research Fellowship. Dao Huynh is sponsored by Vietnamese Ministry of Education and Training (project 322) and the University of Adelaide. This method was developed for the assessment of iodine concentrations in breast milk in a cohort study supported by a NHMRC project grant (ID 626800).
CHAPTER 4
CHAPTER 4    CURRENT IODINE STATUS OF PREGNANT
WOMEN, LACTATING WOMEN AND THEIR INFANTS IN
SOUTH AUSTRALIA

4.1 INTRODUCTION

Iodine deficiency was not considered to be a significant issue in Australia in the early
1990s, however more recent surveys of iodine status of school children, pregnant
women and healthy adults beginning in 2001 have provided evidence of a re-emergence
of iodine deficiency in Australia [39]. This has raised concerns about the impact this
may have on the health of the population.

In an attempt to correct this deficiency, mandatory iodine fortification of iodized salt in
bread making was introduced in Australia in 2009 [85]. In 2010, the National Health
and Medical Research Council (NHMRC) also introduced recommendations that
pregnant women, breast feeding women and women planning pregnancy consume an
iodine supplement providing 150μg of iodine/day [25]. This is because of the need for
pregnant and lactating women to supply iodine to their developing fetus/infants as well
as meet their own requirements, which places them at increased risk of iodine
deficiency.

As highlighted in literature review, there are limited data on the iodine status of
pregnant and lactating women and their infants in Australia after the introduction of
mandatory iodine fortification. It is therefore currently unclear whether the fortification
program has been successful in preventing iodine deficiency in these vulnerable
populations. To date, only three relatively small cohort studies have assessed iodine
status of pregnant women after the commencement of iodine deficiency. One study was
undertaken in Gippsland, Victoria, and showed no significant improvement in the iodine
status of pregnant women in this region after the introduction of mandatory fortification (median IC=96µg/l before iodine fortification vs 95.5µg/l after iodine fortification) [111]. Another study conducted in the Illawarra region of Australia reported that iodine status post fortification was only improved in those pregnant women who were taking iodine-containing supplements (median UIC from 87.5µg/l (2008, n=110) to 145.5µg/l (2011, n=106) and 166 µg/l (2012, n=95), and that only pregnant women taking iodine supplement had an iodine-sufficient status [116]. A study of 196 pregnant women in South Australia also reported that women who were not taking iodine supplements were classified as iodine deficient after the introduction of iodine fortification, although it is important to note that this study was conducted in a relatively socially disadvantaged region of Adelaide [117].

There have been even fewer studies investigating the iodine status of breast feeding women following the introduction of mandatory iodine fortification. One cross-sectional study which included breast feeding women within the first 6 months postpartum from South East Sydney suggested an improved iodine status in breast feeding women following mandatory fortification; with a median UIC of 123µg/l (IQR 71-236µg/l) [119]. Although this study was the first to report iodine status of lactating women post fortification, the small sample size (n=60) and use of convenience samples (seven early childhood centres operated by South East Sydney Illawarra Area Health Service), means that it is not possible to apply these results to the general population.

Overall, therefore, the studies to date which have assessed the iodine status of pregnant and lactating women in Australia after the introduction of mandatory iodine fortification of bread flour have had relatively small sample sizes and have generally been conducted in convenience samples that are unlikely to be representative of the population as a whole. In addition, there have been no studies to date which have determined the iodine status of infants in pre and post fortification. Therefore, the aim of this Chapter was to
assess the current iodine status of pregnant women, lactating women and their infants in a representative sample in South Australia after the introduction of mandatory iodine fortification.

4.2 METHODS

4.2.1 Study design

This study was undertaken as part of a larger NHMRC-funded cohort study which aimed to examine the relationship between iodine status in pregnancy and neurodevelopmental outcomes in the child at 18 months of age (Pregnancy Iodine and Neurodevelopment in Kids, PINK). Approval for the study was granted by Women’s & Children’s Health Network Human Research Ethics Committee and Southern Adelaide Clinical Human Research Ethics Committee. All women provided written informed consent prior to enrolment.

4.2.2 Participants and recruitment

The study was carried out at Women’s and Children’s Hospital and Flinders Medical Centre in Adelaide, South Australia. The recruitment period for the study was from September 2011 to November 2012. Pregnant women were approached by study staff at their first antenatal appointment and screened for their eligibility for the PINK study. Eligibility criteria were women who were less than 20 weeks of gestation and who were able to give informed consent. Pregnant women with a history of thyroid disease, drug/alcohol abuse, and known fetal abnormality were excluded. Because the PINK study involved the administration of a developmental assessment in English, pregnant women were not eligible if English was not the main language spoken at home. Pregnant women who met the inclusion criteria were invited to participate. Those
women who provided written informed consent were then enrolled in the PINK cohort study and the women and their infants followed up during pregnancy and lactation.

4.2.3 Assessments of UIC

4.2.3.1 Sample collection

A spot urine sample (10-20mls) was collected from each woman at the time of enrolment, 28 weeks of gestation and at 3 months postpartum. A spot urine sample was also collected from their infants at 3 months of age. After collection, urine samples were stored at 4°C and then transferred to the laboratory on ice. Once the sample arrived at the laboratory, they were each divided into 3 aliquots of ~2ml and stored at -20°C until the assessment of UIC.

4.2.3.2 UIC assessment

UIC was employed as indicator of iodine status of pregnant women, lactating women and infants in this study. In the present study, the UIC was measured using the modified WHO method A as described in detail in Chapter 2. The detection limit and reporting limit of the assay were 5.5µg/l and 18.3µg/l respectively. Intra-assay coefficient of variation (CV) and inter-assay CV were both less than 5%.

4.2.4 Other assessments

A number of factors have been found in previous studies to potentially impact on the iodine status of pregnant and lactating women, including maternal factors, such as marital status, maternal education and income and as well as maternal BMI and smoking during pregnancy [199, 200]. The intake of nutritional supplements containing iodine also clearly has an impact on maternal iodine status. A secondary aim of this Chapter was to examine the differences in UIC of pregnant and lactating woman in
relation to socioeconomic, lifestyle and maternal factors, including maternal age and
employment status, gestational age at the time of sample collection, and maternal BMI,
smoking status, alcohol intake, parity and intake of iodine supplements during
pregnancy and lactation. The BMI of pregnant women at enrolment was calculated as
following formula:

\[ \text{BMI} = \frac{\text{Weight (kg)}}{(\text{height (m)})^2} \]

Participants with BMI\( \geq 25 \)kg/m\(^2\) were considered overweight/obese [201]. The present
study also looked at the differences in UIC levels in mothers in relation to the mode of
infant feeding at 3 months postpartum (breast feeding or non-breast feeding). The
difference in UIC levels in breastfed and non-breastfed infants was also investigated.

4.3 DATA ANALYSIS

Data analysis was performed using version 17 (SPSS Inc, Chicago, IL, USA). Median
and interquartile range of UIC level were calculated at each time point. The percentage
of participants with UIC less than 150µg/l for pregnant women and 100µg/l for lactating
women were then determined. Iodine nutrition status of these populations was classified
according to the WHO criteria on median UIC categories for pregnancy and lactation
(Table 1.4). Descriptive statistics were determined for baseline information, pregnancy
information and maternal and infant data. Normally distributed data were expressed as
mean ±SD and non-normally distributed data were expressed as median (interquartile
range). Due to the non-normal distribution of UIC data, the Wilcoxin Mann-Whitney
test was used to assess differences in the UIC levels where there were 2 independent
categories for a given variable (employment status (yes/no), smoking status (yes/no)
and parity (0 and \( \geq 1 \)) and the Kruskal-Wallis test was used to compare the 3 groups of
iodine supplements (non-iodine supplements, iodine supplements <100µg/day and
iodine supplements ≥100µg/day). Spearman’s rho test was used to investigate the relationship between UICs and continuous variables (maternal age, gestational age and maternal BMI at baseline)

4.4 RESULTS

Of the 783 pregnant women less than 20 weeks of gestation who were enrolled in the PINK study, 781 provided urine samples at enrolment, 730 at 28 weeks of gestation and 686 at 3 months postpartum. 628 urine samples were collected from their infants at 3 months of age, including 11 sets of twins.

The median age of pregnant women was 33 (29.5-36) years, and ranged from 18-51 years. The majority of women were Caucasian (84.2%), well-educated (82.8% completed secondary education and 86.1% completed further education) and were currently in paid employment (75.7%). Approximately half of the women (53.9%) were nulliparous (in their first pregnancy) and almost all were enrolled into the study during the second trimester (≥13 weeks of gestation). Only a small proportion of the women (13%) smoked either at study entry or in the 3 months leading to pregnancy. Similarly, only 5.1% of women were drinking at the time of enrolment. Nearly half of women were classified as overweight or obese at the time of enrolment. Baseline demographic, lifestyle and pregnancy-related clinical information for the women enrolled in the trial is presented in Table 4.1.
Table 4.1: Demographic, lifestyle and pregnancy-related characteristics of women at enrolment

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n^a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enrolment site</strong></td>
<td></td>
</tr>
<tr>
<td>Women’s and Children’s Hospital</td>
<td>386 (49.3)</td>
</tr>
<tr>
<td>Flinders Medical Centre</td>
<td>397 (50.7)</td>
</tr>
<tr>
<td><strong>Mother age at baseline, median (IQR), years</strong></td>
<td>33 (29.5-36)</td>
</tr>
<tr>
<td><strong>Gestational age at baseline, median (IQR), weeks</strong></td>
<td>16.4 (14.9-18.1)</td>
</tr>
<tr>
<td><strong>Caucasian race</strong></td>
<td>659 (84.2)</td>
</tr>
<tr>
<td><strong>Highest qualification</strong></td>
<td></td>
</tr>
<tr>
<td>Higher Degree</td>
<td>78 (10)</td>
</tr>
<tr>
<td>Degree</td>
<td>326 (41.6)</td>
</tr>
<tr>
<td>Certificate or Diploma</td>
<td>270 (34.5)</td>
</tr>
<tr>
<td><strong>Years of education</strong></td>
<td>15 (13-17)</td>
</tr>
<tr>
<td><strong>Completed secondary school</strong></td>
<td>648 (82.8)</td>
</tr>
<tr>
<td><strong>Currently in the work force</strong></td>
<td>650 (83)</td>
</tr>
<tr>
<td><strong>Currently in paid employment</strong></td>
<td>593 (75.7)</td>
</tr>
<tr>
<td><strong>Parity = 0</strong> have children</td>
<td>422 (53.9)</td>
</tr>
<tr>
<td><strong>Weight at enrolment, median (IQR), kg</strong></td>
<td>67.3 (60-78)</td>
</tr>
<tr>
<td><strong>Height at enrolment, mean±SD, m</strong></td>
<td>1.66±0.1</td>
</tr>
<tr>
<td><strong>BMI at enrolment, median (IQR), kg/m^2</strong></td>
<td>24.9 (22.6-28.3)</td>
</tr>
<tr>
<td><strong>Smoked at baseline</strong></td>
<td>45 (5.7)</td>
</tr>
<tr>
<td><strong>Drank alcohol at baseline</strong></td>
<td>40 (5.1)</td>
</tr>
</tbody>
</table>

Abbreviation: BMI, body mass index; IQR, interquartile range.

^a Data expressed as No (%) unless otherwise indicated,

### 4.4.1 Iodine status during pregnancy

The median UIC of pregnant women during pregnancy was 189µg/l (IQR 112-308µg/l) at baseline (<20 weeks of gestation) and 172µg/l (IQR 103-281µg/l) at 28 weeks of gestation. Although the median UICs indicated an iodine sufficient status of pregnant women 37.4% at enrolment and 42.5% at 28 weeks gestation had UICs <150µg/l which is the accepted cut-off for iodine deficiency in this population group [43] (Table 4.2).
Table 4.2: UICs at enrolment and 28 weeks of gestation

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>28 weeks of gestation</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>781</td>
<td>730</td>
</tr>
<tr>
<td>Median UIC (IQR)</td>
<td>189 (112-380)</td>
<td>172 (103-281)</td>
</tr>
<tr>
<td>UIC &lt;150µg/l</td>
<td>292 (37.4)</td>
<td>310 (42.5)</td>
</tr>
<tr>
<td>UIC 150-249µg/l</td>
<td>210 (26.9)</td>
<td>197 (27)</td>
</tr>
<tr>
<td>UIC 250-499 µg/l</td>
<td>213 (27.3)</td>
<td>179 (24.5)</td>
</tr>
<tr>
<td>UIC &gt;500 µg/l</td>
<td>66 (8.5)</td>
<td>44 (6)</td>
</tr>
</tbody>
</table>

* a: n (%)

I also explored the differences in UICs at these 2 timepoints in the relation to baseline characteristics of the women collected at the time of enrolment (Table 4.3). There were no significant differences in median UICs either at enrolment or at 28 weeks gestation in relation to parity, highest qualification levels, employment status or smoking status (P>0.05). Gestational age at baseline was significantly correlated with baseline UIC, but not with 28-week UIC.

Baseline median UICs of non-supplemented women were at the borderline of the cut-off for iodine deficiency (153µg/l) whereas women who were not taking iodine supplements at 28 weeks had median UICs below the cut-off (136µg/l) (Figure 4.1). The median UIC was significantly higher in the women who consumed iodine supplements at the time of enrolment, compared to women who did not take iodine supplements (P<0.05) (Figure 4.1). No difference in median UIC at enrolment was however found between women who consumed iodine supplements providing less than 150µg/day and women taking supplements with an iodine dose of at least 150µg of iodine/day (183µg/l vs 217µg/l). Those women who currently took supplements which contained equal to or greater than 150µg iodine/day had significantly higher UICs at 28
weeks of gestation than women who either did not take iodine supplements or who took supplements which provided less than 150µg iodine/day (Figure 4.1).

Figure 4.1: UIC during pregnancy according to the use of iodine supplements at baseline, and 28 weeks of gestation. Different superscripts indicate median values that were significantly different.
Table 4.3: Differences in median UIC at baseline and 28 weeks of gestation in relation to demographic, lifestyle and clinical characteristics of the women

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Baseline</th>
<th>28 weeks of gestation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (%) Median (IQR) P value</td>
<td>N (%) Median (IQR) P value</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>420 (46.2) 193 (104.5-317.5) ns</td>
<td>396 (54.8) 167.5 (101.5-268) ns</td>
</tr>
<tr>
<td>≥ 1</td>
<td>361 (53.8) 184 (123-294) ns</td>
<td>334 (45.2) 176 (112-298) ns</td>
</tr>
<tr>
<td>Gestational age at enrolment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;14 weeks</td>
<td>107 (13.7) 207 (126.5-346) ns</td>
<td>99 (13.6) 194 (99.75-297) ns</td>
</tr>
<tr>
<td>≥14 weeks</td>
<td>674 (86.3) 187.5 (109-300)</td>
<td>631 (86.4) 169 (104-277)</td>
</tr>
<tr>
<td>Highest education level</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degree or higher degree</td>
<td>403 (51.6) 184 (101.5-308) ns</td>
<td>380 (52.1) 179 (102-302.5) ns</td>
</tr>
<tr>
<td>Certificate or Diploma</td>
<td>269 (34.4) 196(131-313)</td>
<td>247 (33.8) 171 (112.5-260)</td>
</tr>
<tr>
<td>Secondary school or no further education</td>
<td>109 (14) 184 (113-288)</td>
<td>103 (14.1) 163 (89.8-272)</td>
</tr>
<tr>
<td>Currently in paid employment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>190 (24.3) 207.5 (127-307) ns</td>
<td>178 (24.4) 175.5 (105-303) ns</td>
</tr>
<tr>
<td>Yes</td>
<td>593 (75.7) 182 (106.5-308)</td>
<td>552 (75.6) 182 (102.5-274)</td>
</tr>
</tbody>
</table>
Continued

| Smoking status at baseline |  | Baseline |  | 28 weeks of gestation |  |
|---------------------------|------------------|-----------|-----------------------|-----------|
|                           | N (%)            | Median (IQR) | P value | N (%)            | Median (IQR) | P value |
| No                        | 736 (94.2)       | 188.5 (108-308) | ns      | 690 (94.5)       | 172 (105-283) | ns      |
| Yes                       | 45 (5.8)         | 212 (128-294)  | ns      | 40 (5.5)         | 139 (89.5-236) | ns      |
| Intake of iodine supplements | No supplements | 178 (23)     | 153 (88.4-214) | 0.01 | 142 (21) | 136 (86.8-213) | 0.01 |
|                            | <150µg/day       | 70 (9)       | 183 (105.5-295.5) | 0.01 | 85 (12.5) | 148 (90.95-234.5) | 0.01 |
|                            | ≥150µg/day       | 533 (68)     | 217 (126.5-344.5) | 0.01 | 453 (66.5) | 192 (116.5-339) | 0.01 |

Abbreviations: IQR, interquartile range; ns means “not significant”

Mann-Whitney test or Kruskal-Wallis test used to compare the differences of UIC level between 2 or groups of categorised variables of background information

iodine supplement intake was assessed at the same time as the UIC assessments (i.e. at enrolment and at 28 weeks of gestation)
4.4.2 Iodine status at 3 month postpartum

The median UICs of women at 3 months postpartum was 125µg/l (IQR 76.4-200µg/l), indicative of iodine sufficient status (Table 4.4). However, of the 686 participants whose UICs were assessed at this time-point, 38.2% had UICs below the cut-off, indicative of iodine deficiency (<100µg/l).

The differences between median UICs of the women in relation to baseline demographic, socioeconomic and lifestyle factors are presented in Table 4.5. Women who reported smoking at the time of enrolment had a significantly higher UIC at 3-months postpartum compared to non-smokers (194µg/l vs 122µg/l). There was no difference in UIC between women who were and were not breast feeding at the time of sample collection. There were also no differences in UICs between women of different parity, gestational age at enrolment or employment status at baseline. There was a positive correlation between maternal age at baseline and their UIC at 3 months postpartum ($R^2=0.108$, $P<0.05$), but no association was found between 3-month UICs and either gestational age or maternal BMI at the time of enrolment ($P>0.05$).

The median UIC of women who did not take iodine supplements during pregnancy was not significantly different from that of women who consumed iodine supplements, irrespective of the iodine dose ($P>0.05$) (Table 4.5). Median UIC was however significantly higher in women who had supplements providing at least 150µg of iodine/day, compared to women who were taking supplements with an iodine dose of less than 150µg/day (129µg/l vs 117µg/l) (Figure 4.2)
Table 4.4: UICs in women and their infants at 3 month postpartum

<table>
<thead>
<tr>
<th></th>
<th>Mothers</th>
<th>Infants</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>686</td>
<td>628</td>
</tr>
<tr>
<td><strong>Median UIC (IQR) (µg/l)</strong></td>
<td>125 (76.4-200)</td>
<td>197.5 (121-295)</td>
</tr>
<tr>
<td><strong>UIC&lt;100µg/l</strong></td>
<td>262 (38.2)</td>
<td>109 (17.4)</td>
</tr>
<tr>
<td><strong>UIC≥100µg/l</strong></td>
<td>424 (61.8)</td>
<td>519 (82.6)</td>
</tr>
</tbody>
</table>
Table 4.5: Differences in median UIC at 3 months postpartum in relation to demographic, lifestyle and clinical characteristics of the women

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>Median (IQR)</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parity</td>
<td>0</td>
<td>376 (54.8)</td>
<td>130.5 (74.5-213)</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>≥ 1</td>
<td>310 (45.2)</td>
<td>120.5 (77.8-189)</td>
<td></td>
</tr>
<tr>
<td>Gestational age at enrolment</td>
<td>&lt;14 weeks</td>
<td>90 (13.1)</td>
<td>143.5 (80-198)</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>≥14 weeks</td>
<td>596 (86.9)</td>
<td>122 (75.5-201.5)</td>
<td></td>
</tr>
<tr>
<td>Highest level of education</td>
<td>Degree or higher degree</td>
<td>360 (52.5)</td>
<td>119 (68.2-218.50)</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Certificate or Diploma</td>
<td>238 (34.7)</td>
<td>130 (83.9-211)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Secondary school or no further education</td>
<td>88 (12.8)</td>
<td>135.50 (86.2-218.5)</td>
<td></td>
</tr>
<tr>
<td>Currently in paid employment</td>
<td>No</td>
<td>162 (23.6)</td>
<td>136.5 (85.8-205)</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>524 (76.4)</td>
<td>123.5 (73.2-199)</td>
<td></td>
</tr>
<tr>
<td>Smoking at enrolment</td>
<td>No</td>
<td>653 (95.2)</td>
<td>122 (73.8-197)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>33 (4.8)</td>
<td>194 (132-235)</td>
<td></td>
</tr>
</tbody>
</table>
Continued

<table>
<thead>
<tr>
<th>Baseline</th>
<th>N (%)</th>
<th>Median (IQR)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intake of iodine supplements</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>During pregnancy</td>
<td>No supplements</td>
<td>127 (18.5)</td>
<td>122 (79-192)</td>
</tr>
<tr>
<td></td>
<td>&lt;150µg/day</td>
<td>128 (18.6)</td>
<td>117 (66.2-178)</td>
</tr>
<tr>
<td></td>
<td>≥150µg/day</td>
<td>431 (62.9)</td>
<td>129 (78.2-214)</td>
</tr>
<tr>
<td>Breast feeding at 3 months postpartum</td>
<td>No</td>
<td>35 (5.1)</td>
<td>142 (101.5-207.5)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>650 (94.89)</td>
<td>123.5 (74.7-200)</td>
</tr>
</tbody>
</table>

Abbreviations: IQR, interquartile range; ns means “not significant”

Mann-Whitney test or Kruskal-Wallis test used to compare the differences of UIC level between 2 or groups of categorised variables of background information; Spearman’s rho test used to test the relationship between UIC level and continuous variables of background information.
Figure 4.2: UIC at 3-months postpartum in mothers and infants in relation to the average dose of iodine supplements consumed during pregnancy. Different superscripts indicate median values that were significantly different.
4.4.3 Iodine status of infants at 3 months of age

The median UIC of infants at 3 month postpartum was 197.5µg/l (IQR 121-295.25µg/l) which is well above the cut-off, indicative of iodine sufficient status of infants (Table 4.4). There were no significant differences in the UIC of infants who were or were not being breastfed at the time of sample collection (195µg/l vs 198µg/l, P>0.05). There was also no difference between median UICs of infants whose mothers took iodine supplements during pregnancy and whose mothers did not (P>0.05). Similarly, the median UIC of infants who mothers took supplements which supplied equal to or greater than 150µg of iodine/day was not different from that of infants whose mothers took iodine supplements providing less than 150µg/day (200µg/l vs 197µg/l). The median UICs of infants whose mothers did not take iodine supplements at any time during pregnancy was 173.5µg/l, indicative of iodine sufficient status of this population (Figure 4.2).

4.5 DISCUSSION

This study aimed to assess the iodine status of vulnerable populations, including pregnant women, lactating women and their infants in South Australia. The median UIC for pregnant women at the time of enrolment (<20 weeks gestation) and at 28 weeks of gestation were 189µg/l (IQR 112-308) and 172µg/l (IQR 103-281) respectively in this study. The median UIC of lactating women at 3 month postpartum was 126µg/l (IQR 76.4-200) and that of infants was 197.5µg/l (IQR 121-295). These findings suggest that iodine deficiency is unlikely to be a major health concern in the majority of pregnant and lactating women and their infants in South Australia at this time.
4.5.1 Iodine status of pregnant women in Australia after the introduction of mandatory iodine fortification

The present study has added to the limited data regarding iodine status of pregnant women in South Australia after the commencement of mandatory iodine fortification in salt used in bread industry in Australia since 2009 and is the first study to be conducted in a representative sample of women in South Australia. The UIC results in pregnant women presented in this Chapter were in contrast to a previous study in South Australian women, the results of which suggested that the median UIC of pregnant women in South Australia post fortification was mildly deficient (median UIC 82µg/l) [117]. The previous study included UIC data of both pre and post fortification as the recruitment was between January 2009 and July 2010. This may contribute to the decrease in median UIC of the population. Furthermore, the study was conducted in only one region of South Australia (Elizabeth Playford region), which is considered to be relatively disadvantaged and has a low socioeconomic status, and had a relatively small sample size (n=96). Consequently, the participants included in this previous study may not be representative of the pregnant population in South Australia as a whole.

Recently, the 2011-2012 National Health Measures Survey (2013), which began 18 months after mandatory iodine fortification was introduced, reported adequate iodine nutrition status in the Australian population. This conclusion was based on the finding that Australian adults had a population median UIC of 124µg/l and only 12.8% had an UIC below 50µg/l, i.e. the cut off for iodine deficiency [202]. However, this survey did not present any data indicating whether the iodine status of pregnant and lactating women had also improved since the introduction of iodine fortification to the levels where these populations were also classified as iodine sufficient. This is an important distinction, since pregnant and breast feeding women have higher iodine requirements than the general adult population, as detailed in the literature review. To date, there are relatively few studies evaluating the effectiveness of mandatory iodine fortification in improving the iodine status of pregnant and lactating women. Rahman
and colleagues [34] conducted a cross-sectional study of 86 pregnant women in the Gippland region of Victoria and found no improvement in UIC in pregnant women after iodine fortification was introduced (96µg/l pre fortification vs 95.5µg/l post fortification). More recently, another study conducted in Illawara region of Australia suggested that mandatory iodine fortification program contributed to improve iodine status of pregnant women [116], with an increase in the median UIC level from 87.5µg/l prior to fortification (2008, n=110) to 145.5µg/l in 2011 (n=106) and 166µg/l in 2012 (n=95). The difference in UIC measured in different regions of Australia highlights the geographical variation in iodine status in similar population groups from different parts of the country. Notably, all of these previous studies are limited by having relatively small sample sizes [203], which makes it more difficult to extrapolate the findings to the whole pregnant population.

Cross-sectional studies in Australia about iodine status of pregnant women post fortification have also examined how the UIC of this population has been affected by the increased prevalence of women taking iodine supplements. Almost all studies, including the present study, reported an increase of UIC in women taking iodine supplements during pregnancy compared to those not taking supplements. Interestingly, 2 other studies suggested that pregnant women were still at risk of iodine deficiency after iodine fortification even if they were taking iodine supplements during pregnancy [111, 117]. Although the median UIC of the women in the current study who did not take iodine supplements was at the borderline of iodine sufficiency (153µg/l) at the time of enrolment (<20 weeks of gestation), the median UICs at 28 weeks of gestation was indicative of iodine deficient status in the population. This finding highlights that although the median UIC of pregnant women has significantly improved since iodine fortification, pregnant women are still at potential risk of iodine deficiency if they do not continuously consume iodine supplements during pregnancy.

Of a hundred harmful substances for human’s health contained in cigarette smoke, thiocyanate is a goitrogenic factor due to the competitive inhibition of iodine uptake into
thyroid gland [204]. Several studies have recently addressed an impact of smoking on iodine status of pregnant women. Interestingly, almost all these studies, including the present study, showed no difference in UIC between smoking and non-smoking women [205, 206]. It is worth noting that, in all of these studies, the number of women who smoked was much lower (n<30), compared to the numbers of non-smoking women (110-200 participants), because almost all women stopped smoking before pregnancy. Furthermore, a study recently conducted by Brucker-Davis and colleagues to investigate the impacts of smoking in early pregnancy on maternal thyroid function found that smoking women had higher thyroid volume and Tg level than non-smoking women [205]. Taken together, these studies showed that maternal smoking may contribute to abnormal thyroid function in pregnant women, but also highlighted the need for further investigation since the impact of maternal smoking status on UICs in pregnancy remains unclear.

4.5.2 Iodine status of lactating women at 3 months postpartum after the introduction of mandatory iodine fortification

There is currently only one other study which has reported the iodine status of lactating women in Australia after the introduction of mandatory iodine fortification. Our finding demonstrated that the UIC of lactating women at 3 months postpartum after iodine fortification was 126µg/l, indicating an iodine sufficient status. This result is in agreement with another smaller study conducted in Illawara region of Australia, the results of which suggested the UIC level of breast feeding women was 123µg/l [119]. However, it is worth noting that the Illawara study only included breast feeding mothers and excluded non-breast feeding mothers. It would be expected that in the face of equivalent iodine intakes from diet and supplements the UIC of breast feeding women would be lower than those who are not breast feeding, since breast feeding mothers transfer a significant proportion of the iodine they consume to their infants via breast milk, and therefore have higher iodine requirements. However, the difference in median UIC between breast feeding and non-breast feeding
mothers in the present study was not statistically significant. These findings are in line with another study conducted in Switzerland, where a universal iodized salt program was commenced in 2004 [207], which also found no significant differences between the UIC of breast feeding and non-breast feeding women. However, it is worth noting that neither information on dietary iodine intake nor intake of iodine supplements during lactation was assessed in these studies, which makes it difficult to draw clear conclusions.

In the present study, I also explored the differences in UIC postpartum between women who took iodine supplements during pregnancy and those who did not. I found no significant difference in UIC between iodine-supplemented mothers and unsupplemented mothers during pregnancy, and the median UIC of both supplemented and unsupplemented women were indicative of iodine sufficient status of lactating mothers at 3 months postpartum. This finding suggested that iodine fortification alone has improved the iodine nutrition status of lactating women to a level indicative of an iodine sufficient status. The present study also found significantly higher UICs in mothers consuming iodine supplements which provided a dose of equal to or greater than 150µg/day during pregnancy, compared to mothers who consumed supplements containing less than 150µg of iodine/day. This finding suggests that taking high-dose iodine supplements during pregnancy was associated with increased in the UIC of the women postpartum. It is worth noting, however, that women may stop taking iodine supplements after they give birth, which would clearly affect their level of iodine intake, and therefore UIC, and this was not assessed in this study. Therefore, it will be important to obtain information on the intake of iodine supplements postpartum, and to take this into account when the relationship between iodine supplementation during pregnancy and UIC during lactation is investigated.

Interestingly, while there was no difference in the UIC of smokers and non-smokers during pregnancy, I found a significant difference in UICs at 3 month postpartum between mothers who did not smoke and mothers who were smokers at the time of enrolment. The result of my
present study is not in line with that of previous study which reported no differences in median UICs of smoking and non-smoking lactating women at 5 days postpartum [208]. It is possible that this discrepancy is because many women who stop smoking during pregnancy may start again after they give birth [209]. Current smoking status of the women at 3 months postpartum however was not assessed in this study. It is also important to point out that the method to collect information regarding smoking status in the present study was self-report, which is known to lead to an underestimation of smoking status of Australian adults [210], and this is reflected in the low number of smoking mothers in this study. Exposure to passive smoking, information on which was not collected in this study, has also been associated with a higher risk of thyroid dysfunction [211]. In attempt to limit the confound of self-report, almost all studies investigating the impacts of smoking during pregnancy and lactation on UICs thoroughly explored the relationship between thiocyanate levels measured in urine and serum and UICs level [208], rather than the difference between smoking status classified by self-report, and it would be interesting to do this in the future studies in order to determine the accuracy of the self-reported data.

4.5.3 Iodine status of infants at 3 months of age after the introduction of mandatory iodine fortification

The present study has provided novel data regarding to the iodine status of infants in Australia. The infants at 3 months of age had UIC considerably above the threshold for sufficiency of 100µg/l (197.5µg/l), indicative of iodine sufficient status. Our finding is consistent with those from several other studies conducted in iodine sufficient countries. Zhang and colleagues [212] conducted a study in 97 breastfed infants at 1 month of age in Beijing, and reported iodine sufficiency of infants (median UIC 183µg/l). More recently, a cross-sectional study in Boston (USA) found median UIC of 197.5µg/l for infants less than 3 months of age [213]. The iodized salt program interestingly provided adequate iodine intake for school age children and pregnant women, but was not able to protect Swiss infants against
iodine deficiency, as indicated by a median of UIC of 91µg/l in this population, found in a study involving 228 infants at 6 months of age [207]. Interestingly, almost all studies which have reported UICs of both mothers and their infants at the same time have shown the UIC to be significantly higher in the infants, compared to their mother [187, 214]. A similar finding was reported in the present study; the median UIC of breast feeding mothers was 123.5µg/l whereas that of infants was 198µg/l. It is worth noting that almost all studies conducted in lactating women in Australia to date have classified this population group as being iodine deficient based on their UICs, and recommended that these women would benefit from iodine supplementation without assessing the iodine status of their infants. Taken together, the recommended dose of iodine supplementation for breast feeding mothers should be thoroughly considered based on both UIC and breast milk iodine levels, to avoid the excessive iodine transfer from mothers to infants, which could potentially have negative effects on infant development.

The studies conducted to date which have examined the iodine status of infants have also looked closely at the impact of feeding pattern on their UIC. The most recent study, conducted in 95 infants less than 3 months of age from Boston (USA), demonstrated no significant difference in median UICs between infants classified as exclusively breastfed, exclusively formula-fed or a combination of both feeding modes [213]. The present study confirmed this finding in a larger population of 628 3-month infants and showed that the median UIC of non-breastfed infants was not different to that of breastfed infants. Interestingly, both studies found UICs in infants of ~200µ/l i.e. well above the cut-off for iodine sufficiency. Although the adverse impacts of excessive iodine status on thyroid function and hence on the child development have been documented in the literature [46], the upper cut-off of infant UICs, indicative of excessive iodine status, has not yet been established. Hence further studies are required to determine whether there is an upper threshold of UICs that may have adversely influence development of infants.
The current study also found no differences in UICs between infants whose mothers took iodine supplements and those who took no iodine supplements. The median UIC of infants whose mothers did not consume iodine supplements during pregnancy was well above the cut-off of 100µg/l. These finding suggested that iodine status of infants after the commencement of iodine fortification is sufficient, irrespective of iodine supplementation during pregnancy. It is also worth noting that UICs only reflect iodine intake over the preceding few days. Thus, to investigate the impact of maternal iodine supplementation during pregnancy on markers of long-term iodine nutrition, such as biomarkers of thyroid function in the women and their infants, e.g. TSH level and thyroid hormone levels, are likely to be more informative.

4.5.4 The limitations of the present study

The UIC measurements are not likely to be representative of dietary intake during the whole of pregnancy and lactation, and cannot be used as a predictor of maternal thyroid function over the longer term. Furthermore, the present study excluded pregnant women who did not use English as main language at home. This exclusion may lead to an overestimation of median UIC in the population, since previous studies have identified non-English speaking women in Australia as being higher risk of iodine deficiency [113]. The analysis of more than one urine sample from women/infants at each time point would also have allowed for adjustment for intra-individual variation in the UIC, thus resulting in a narrower population distribution and increasing the statistical power to detect differences between groups [215]. Similarly, collection of 24 hour urine samples would have provided a more reliable measure of iodine status and dietary iodine intake of Australian pregnant women, compared to that of casual urine sample [84]. However, due to the numbers of women (n=781) included in the clinical trial, it was not feasible logistically to collect 24 hour urine samples or repeated samples from all participants. Another limitation of the present study is that the information on smoking status and iodine supplements of lactating women which may impact the iodine status of the women at 3 months postpartum was not collected.
4.5.5 Summary

Iodine fortification has alone successfully improved UIC of lactating women and their infants to level compatible with iodine sufficient status. However, median UIC of pregnant women who did not taking iodine supplements during pregnancy may be suboptimal, as indicated by the borderline of UIC level. The majority of pregnant women are likely to still require iodine supplementation to supply adequate iodine level for both mothers and their developing fetus in order to maintain normal developments.
CHAPTER 5
CHAPTER 5  BREAST MILK IODINE CONCENTRATION OF LACTATING WOMEN IN SOUTH AUSTRALIA PRE AND POSTFORTIFICATION

5.1 INTRODUCTION

Iodine is a trace element essential for the synthesis of the thyroid hormones, triiodothyronine (T3) and thyroxine (T4) and plays an important role in physical growth and mental development before birth and in early life [8]. For exclusively breastfed infants, their mother’s breast milk provides their only source of iodine. The recommended adequate intake of iodine is 90µg/day for infants 0-6 months and 110µg/day for infants 7-12 months [26], and it has been suggested that a breast milk iodine concentration (BMIC) of at least 100 µg/l is required to ensure that a full-term breastfed infants received a sufficient supply of iodine to meet these requirements [21].

Previous studies internationally have provided evidence that iodine breast milk levels differ significantly between regions classified as iodine sufficient and iodine deficient. Thus, BMICs have been reported to range from 135.9µg/l to 259µg/l in populations considered to be iodine sufficient [93, 185-188] while they are typically lower than <100µg/l in women living in iodine deficient regions [190-192, 216]. However, information on the iodine content of breast milk in Australian women is scarce. Only one study to date has reported on BMIC in Australian women prior to the introduction of mandatory iodine fortification and although the iodine content of breast milk in this study varied considerably between individual women in the study population (25-234µg/l), the median level (84µg/l) indicated that the breast milk of Australian women would not, on average, provide adequate iodine to meet infant requirements [217]. However, as this study had only a small sample size (n=49) and women were recruited from one tertiary referral hospital in Sydney, Australia, these data are unlikely to be representative of the general Australian population. Furthermore, there are no studies to
date which have assessed iodine level in breast milk in Australian women after the introduction of mandatory iodine fortification in 2009. There is thus limited data on the iodine content of breast milk in Australia women, and whether mandatory iodine fortification has been effective in increasing iodine levels in breast milk remains unknown. Therefore, the aim of this Chapter was to determine breast milk iodine level in lactating women post fortification in South Australia and compare these to iodine levels in the breast milk of women from the same region of South Australia before mandatory iodine fortification was introduced.

5.2 METHODS

5.2.1 Study design

The current study used a sub-set of two cohorts from whom breast milk samples had been collected. The first cohort was a sub-set of women who participated in the DOMInO (DHA to Optimise Mother Infant Outcome) randomised controlled trial, in which breast milk samples were collected between 2006 and 2007 (pre fortification) and the second was the PINK cohort study, in which breast milk samples were collected between 2011-2013 (post fortification). The breast milk samples for both studies were obtained from women who were enrolled at the Women’s and Children’s Hospital and or Flinders Medical Centre in Adelaide, South Australia. Both studies were approved by Women’s & Children’s Health Network Human Research Ethics Committee and Southern Adelaide Clinical Human Research Ethics Committee (South Australia).

5.2.1.1 DOMInO study population (pre iodine fortification)

The DOMInO trial is double blind randomised controlled trial which aimed to determine the effect of maternal n-3 long chain poly unsaturated fatty acid (LCPUFA), chiefly as Docosahexaenoic acid (DHA), during the second half of pregnancy on symptoms of postnatal depression in mothers and on neurodevelopmental outcomes in infants at 18 months of age.
The inclusion criteria were women with singleton pregnancy and able to give informed consent. Women with multiple births, a known major fetal abnormality, history of drug and alcohol abuse, bleeding disorders that would contraindicate taking fish oil or who were already taking prenatal supplements containing DHA were excluded. Women were also ineligible if no English was spoken at home because the child needed to understand and follow English instructions to undertake the neurodevelopmental assessments that were part of this study.

The DOMInO trial was a multi-centre study conducted in 5 maternity hospitals around Australia, including the Women’s & Children’s Hospital and Flinders Medical Centre (South Australia), Sunshine Hospital (Victoria), Royal Brisbane & Women's Hospital (Brisbane), Campbelltown Hospital (New South Wales) and Private Hospitals. The current study only included participants who were recruited in South Australia (Women’s & Children’s hospital and Flinders Medical Centre). At the time of providing informed consent for the DOMInO study, participants were asked to indicate if they agreed for a sample of their breast milk to be stored for future non-genetic analyses. The original DOMInO consent form for all the participants were checked in order to confirm that they had agreed to storage and future analysis of their breast milk samples prior to the current analysis. Ethical approval was obtained from the Women’s & Children’s Health Network Human Research Ethics Committee to undertake iodine analyses on these stored breast milk samples prior to commencing this study.

Detailed information on sociodemographic, clinical and lifestyle factors were collected from all DOMInO participants at the time of enrolment into the study. Data collected included maternal education, maternal employment/occupation, parity, maternal alcohol, smoking, drug use, and intake of non-DHA dietary supplements at baseline.
5.2.1.2 *PINK cohort (post iodine fortification)*

These breast milk samples were collected from participants in the PINK cohort study, which is described in detail in Chapter 4.

5.2.2 Assessments

5.2.2.1 *Breast milk iodine concentration (BMIC)*

**Sampling and storage of pre fortification samples**

In the DOMInO study, a 70ml sterilized pot (Southern Cross Scientific Ltd) was sent to DOMInO participants to collect one breast milk sample of approximately 5mls of breast milk samples in the first 7 days following delivery. No specific instruction was provided regarding the time of sample collection, or whether this is done before or after a feed. The DOMInO breast milk samples were stored in the 70ml pots at -20°C until analysis. In total, 485 breast milk samples were collected from women in DOMInO study enrolled in South Australian Centres between 2006 and 2007. After samples of participants who did not indicate further analysis in consent forms and samples with insufficient volume (<1ml) were excluded, 291 breast milk samples were available for assessment of iodine content.

**Sampling and storage of post fortification samples**

The same type of pot as for the DOMInO samples (70ml yellow-topped collection container) together with a sheet of clear instructions and diagram to assist women with breast milk collection was issued to PINK participants within the first 7 days of birth and at 3 months postpartum. The breast milk was collected in the morning before the first feed between 5am-9am and only foremilk (the first milk of the feed) was collected. Breast milk was collected in either the clinic or in the women’s home.
The breast milk samples were frozen until the batches of samples were ready for delivery to the coordinating centre for storage. If the sample was collected at home, participants were instructed to freeze the samples in their home freezer until collection by study staff. During transportation from participants home or the clinic centre to the laboratory, the samples were kept cold using an insulated bag or container with an ice block or freeze brick. The PINK samples were stored at -80°C until analysis.

Iodine breast milk analysis

Breast milk iodine concentration (BMIC) was determined using a modified method of the determination of iodine in food samples by ICPMS after TMAH extraction as described in detail in Chapter 3. The method quantitative limit for human breast milk was 1.6µg/l.

Pilot study to examine the iodine stability in breast milk samples

In order to verify that the different storage conditions (-20°C in 70ml pots vs -80°C in the same type of containers) of the DOMInO and PINK breast milk samples would not affect the iodine content, a pilot study was conducted to compare the stability of iodine stored for up to 18 months under these two storage conditions. Seven breast milk samples were divided into aliquots immediately after collection and separate aliquots stored either at -20°C or -80°C in 70ml yellow pots for periods up to 18 months. The results demonstrated that iodine levels in breast milk samples were stable for at least 18 months when stored at -20°C (RSD <2%) (Table 5.1). The results also showed no significant difference in iodine concentrations when the same breast milk sample was stored under these different conditions (RSD <10%) (Table 5.2). Taken together, this pilot study provided evidence that the different storage conditions for breast milk samples collected pre and post fortification would not impact on the results, and confirmed the validity of using these two study populations to compare breast milk iodine levels before and after the introduction of mandatory iodine fortification.
Table 5.1: The relative standard deviation of 7 samples during 18 month storage

<table>
<thead>
<tr>
<th>Sample</th>
<th>MEAN (µg/l)</th>
<th>SD</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>274.50</td>
<td>2.69</td>
<td>0.98</td>
</tr>
<tr>
<td>Sample 2</td>
<td>146.00</td>
<td>1.87</td>
<td>1.28</td>
</tr>
<tr>
<td>Sample 3</td>
<td>152.25</td>
<td>1.92</td>
<td>1.26</td>
</tr>
<tr>
<td>Sample 4</td>
<td>201.50</td>
<td>1.12</td>
<td>0.55</td>
</tr>
<tr>
<td>Sample 5</td>
<td>201.50</td>
<td>1.66</td>
<td>0.82</td>
</tr>
<tr>
<td>Sample 6</td>
<td>60.08</td>
<td>0.58</td>
<td>0.97</td>
</tr>
<tr>
<td>Sample 7</td>
<td>116.25</td>
<td>0.83</td>
<td>0.71</td>
</tr>
</tbody>
</table>

Abbreviation: RSD, relative standard deviation; SD, standard deviation

*: Average of breast milk iodine level measured at 3-month, 6-month, 9-month and 18-month storage.

Table 5.2 Average relative standard deviation of breast milk samples stored at -20°C vs -80°C

<table>
<thead>
<tr>
<th>Timepoints of analysis</th>
<th>Number of samples</th>
<th>Average RSD of samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 months</td>
<td>4</td>
<td>0.83</td>
</tr>
<tr>
<td>6 months</td>
<td>4</td>
<td>0.40</td>
</tr>
<tr>
<td>9 months</td>
<td>9</td>
<td>3.30</td>
</tr>
<tr>
<td>12 months</td>
<td>5</td>
<td>1.37</td>
</tr>
<tr>
<td>18 months</td>
<td>5</td>
<td>5.50</td>
</tr>
</tbody>
</table>

Abbreviation: RSD, relative standard deviation

5.2.2.2 Other assessments

In addition to the comparison of median BMIC of non-iodine supplemented mothers before and after the introduction of mandatory iodine fortification, this Chapter also investigated the differences in BMIC between women who reported taking iodine supplements compared to those that took no supplements both before and after the introduction of mandatory iodine fortification. The effects of mothers’ anthropometric and demographic characteristics at enrolment on BMIC both pre and post fortification were also examined.

5.3 DATA ANALYSIS

Full term infants require a daily iodine intake of 15µg/kg to achieve positive iodine balance, which would equate to a BMIC of 100µg/l. In the present study, this normative value was used as a marker of sufficient iodine intake for full-term breastfed infants.
SPSS version 17 was used for data processing and analysis. The Shapiro-Wilk test was used to determine if the data followed a normal distribution. Normally distributed data were expressed as mean ±SD and non-normally distributed data were expressed as median (interquartile range). The percentage of women with a breast milk iodine concentration less than 100µg/l was also determined both pre and post fortification.

In the post fortification cohort, where breast milk samples were collected both at birth and at 3 months postpartum, the Wilcoxin paired signed-rank test was used to compare iodine levels in breast milk samples collected at these 2 timepoints. I also compared BMICs pre and post fortification in either all participants or non-dietary iodine supplement participants using Mann-Whitney tests. Due to no differences in BMICs between placebo and DHA-treated groups in the DOMInO, all participants who did not take dietary iodine supplement at trial entry in both groups were included in the comparison. Differences in BMICs between subgroups based on anthropometric and demographic variables were determined by Mann-Whitney tests (where they were 2 sub-categories) or Kruskal-Wallis test (where there were >2 sub-categories), respectively. A non-parametric Spearman rho’s test was used to investigate the relationship between BMICs and demographic continuous variables.

5.4 RESULTS

5.4.1 Breast milk iodine concentration of breast feeding women before and after the introduction of mandatory iodine fortification

5.4.1.1 Before mandatory iodine fortification

Demographic information of the DOMInO women from whom breast milk samples were available for analysis at trial entry is presented in Table 5.3. The majority of the women were Caucasian, had completed a high school and were employed at the time of recruitment into the trial. The proportion of women smoked or drank at trial entry, 11.7% and 12.4%
respectively. Only 22% of the pregnant women were consuming vitamin supplements at study entry. The majority of infants were born at term and were still being breastfed at 6 weeks of age. The average BMI of the women in this population was 26.4kg/m², and 43% had BMI >25kg/m², placing them in the overweight or obese category (43%).

Prior to iodine fortification, the iodine concentration in breast milk of lactating women at birth was 103µg/l (72.8-156µg/l), which was just over the cut-off indicating adequate iodine supply for breastfed infants (100µg/l). However, almost half of mothers pre fortification had a BMIC below the 100µg/l cut-off, suggesting that they would have insufficient iodine to meet the requirements of full-term infants (Table 5.5)

Mothers who completed secondary school had significantly higher breast milk iodine level than mothers who did not; 108µg/l and 89µg/l, respectively. The breast milk of mothers who smoked contained less iodine than that of non-smoking mothers, 62µg/l vs 108µg/l. Other demographic characteristics, maternal age, maternal BMI maternal completion of a higher degree, employment and alcohol intake at enrolment did not appear to impact on the iodine level in breast milk. There was also no significant difference in BMICs in the early postpartum period between women who were randomised to the DHA and placebo treatment groups or who reported taking other non-DHA containing nutritional supplements (Table 5.4).
### Table 5.3: Demographic information of DOMInO women at trial entry

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at baseline, median (IQR), years</td>
<td>30 (26-34)</td>
</tr>
<tr>
<td>Caucasian ethnicity</td>
<td>273 (93.8)</td>
</tr>
<tr>
<td>Mother completed secondary school</td>
<td>196 (67.4)</td>
</tr>
<tr>
<td>Mother completed further education</td>
<td>211 (72.5)</td>
</tr>
<tr>
<td>Gestational age at baseline, median (IQR), weeks</td>
<td>19 (19-20)</td>
</tr>
<tr>
<td>Mother’s BMI at baseline, median (IQR), kg/m²</td>
<td>26.4 (23-30.1)</td>
</tr>
<tr>
<td>Parity = 0</td>
<td>114 (39.2)</td>
</tr>
<tr>
<td>Mother smoked at baseline</td>
<td>34 (11.7)</td>
</tr>
<tr>
<td>Mother drank alcohol at baseline</td>
<td>36 (12.4)</td>
</tr>
<tr>
<td>Mother employed at baseline</td>
<td>223 (76.6)</td>
</tr>
<tr>
<td>Clinical diagnosis of gestational diabetes in DOMInO pregnancy</td>
<td>13 (4.5)</td>
</tr>
<tr>
<td>Clinical diagnosis of pre-eclampsia in DOMInO pregnancy</td>
<td>10 (3.4)</td>
</tr>
<tr>
<td>Gestational age at randomisation, weeks</td>
<td>19 (19-20)</td>
</tr>
<tr>
<td>Feeding mode at 6 weeks</td>
<td></td>
</tr>
<tr>
<td>Breast milk only</td>
<td>193 (66.6)</td>
</tr>
<tr>
<td>Formula only</td>
<td>52 (17.9)</td>
</tr>
<tr>
<td>Both breast milk and formula</td>
<td>45 (15.5)</td>
</tr>
<tr>
<td>Maternal intake of vitamin supplements</td>
<td>65 (22.3)</td>
</tr>
</tbody>
</table>

Abbreviation: IQR, interquartile range, DOMInO, DHA to Optimise Mother Infant Outcome; MSSI, maternal social support index.
Data were expressed as n (%), unless otherwise indicated.
Table 5.4: Differences in breast milk iodine levels (µg/l) in relation to demographic, lifestyle and clinical parameters of women in both pre and post fortification

<table>
<thead>
<tr>
<th></th>
<th>Pre fortification</th>
<th>Post fortification</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (IQR)</td>
<td>P value</td>
<td>Median (IQR)</td>
<td>P value</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>At birth</td>
<td>At 3 months</td>
</tr>
<tr>
<td>Parity</td>
<td>0</td>
<td>ns</td>
<td>189 (272-126)</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>&gt;=1</td>
<td></td>
<td>186 (279-137)</td>
<td>127 (84-178)</td>
</tr>
<tr>
<td>Mother completed secondary school</td>
<td>No</td>
<td>89 (63.5-134.5)</td>
<td>0.01</td>
<td>174.5 (97.6-255)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>108 (77-161)</td>
<td>189 (133.5-279)</td>
<td>126.5 (87.8-187)</td>
</tr>
<tr>
<td>Mother completed further education</td>
<td>No</td>
<td>96.5 (68-156.5)</td>
<td>ns</td>
<td>192 (114.5-284)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>104 (74.5-155)</td>
<td>186 (131-270)</td>
<td>127 (84-189.5)</td>
</tr>
<tr>
<td>Mother employed</td>
<td>No</td>
<td>108.5 (60.5-165.5)</td>
<td>ns</td>
<td>202 (129-294)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>99 (74.5-152.5)</td>
<td>185 (130-265)</td>
<td>124.5 (84.5-187)</td>
</tr>
<tr>
<td>Treatment group at trial entry</td>
<td>1</td>
<td>97 (68-163)</td>
<td>ns</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>106 (77-144)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother smoked at trial entry</td>
<td>No</td>
<td>108 (76-162)</td>
<td>0.01</td>
<td>190 (134.5-279)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>62 (36-89)</td>
<td>65.7 (51.8-154)</td>
<td>71.7 (47.8-148)</td>
</tr>
</tbody>
</table>
Continued

<table>
<thead>
<tr>
<th>Dietary supplements at trial entry</th>
<th>Pre fortification</th>
<th>Post fortification</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>Median (IQR)</td>
<td>P value</td>
</tr>
<tr>
<td></td>
<td>105 (72.5-155)</td>
<td>ns</td>
</tr>
<tr>
<td>Yes</td>
<td>100 (73-156.5)</td>
<td></td>
</tr>
<tr>
<td>Average iodine supplements during pregnancy</td>
<td>No supplements</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>158 (106-231)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>175 (137-231)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>203 (133.5-297.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 (124-303)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: IQR, interquartile range; ns, not significant

Mann-Whitney test or Kruskal-Wallis test used to compare the differences of UIC level between 2 or groups of categorised variables of background information
5.4.1.2 After mandatory iodine fortification

Demographic information of the PINK women from whom breast milk samples were available for analysis at trial entry was previously presented in Chapter 4. The BMICs were 187µg/l (130-276µg/l) at birth and 126µg/l (84-184µg/l) at 3-months postpartum. Although the BMICs at 3 months postpartum were significantly less than that at birth, the median 3-month iodine level was still above the cut-off of 100µg/l. However, the percentage of women in the sample with a breast milk iodine concentration of <100µg/l was significantly higher at 3 months postpartum (~35%) than in the period immediately after birth (~13%) birth (Table 5.5).

The iodine content of the breast milk at birth was higher in women who had completed secondary school compared with women who had not whereas no difference was found in BMICs between these groups at 3-months postpartum. Women who reported smoking at baseline also had lower iodine concentrations in their breast milk compared to non-smokers, both at birth (65.7µg/l vs 190µg/l) and at 3-months postpartum (71.7µg/l vs 127µg/l) (Table 5.4). There were no differences in breast milk iodine levels either at birth or at 3 months postpartum between women with different parity, further education, employment status at baseline. Maternal age at study entry was positively correlated with breast milk iodine level at both birth and 3 month postpartum. However, there was no association between breast milk iodine level and BMI at baseline.

Iodine concentration in breast milk at birth in women who consumed any iodine supplements during pregnancy, independent of the dose, was significantly higher than that of women who did not consume iodine supplements (Table 5.4). In addition, women who took higher-dose iodine supplements, providing at least 150µg iodine/day had significantly higher BMICs than mother who took supplements containing less than 150µg/day, both at birth and 3 month postpartum. However, there was no difference between iodine content in breast milk at 3-
months postpartum of mothers who had not consumed iodine supplements at any time during pregnancy compared to those who had consumed supplements which provided less than 150µg of iodine/day (Figure 5.1). Importantly, however, the breast milk iodine concentration of unsupplemented mothers was still higher than the cut-off of 100µg/l, indicating that mothers post fortification were able to supply adequate iodine amount for full-term infant development, irrespective of iodine supplementation in pregnancy.

Table 5.5: Breast milk iodine levels of breast feeding mothers post fortification

<table>
<thead>
<tr>
<th></th>
<th>Pre fortification</th>
<th>Post fortification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>At birth</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td>291</td>
<td>653</td>
</tr>
<tr>
<td>Median (IQR) (µg/l)</td>
<td>103 (72.8-156)</td>
<td>187 (130-276)</td>
</tr>
<tr>
<td>Breast milk iodine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>concentration b ≥100µg/l</td>
<td>149 (51.2)</td>
<td>566 (86.7)</td>
</tr>
<tr>
<td></td>
<td>142 (48.8)</td>
<td>87 (13.3)</td>
</tr>
</tbody>
</table>

a: the number of samples collected
b: data express as n (%)
Figure 5.1: The difference in breast milk iodine levels between iodine supplement groups at birth and 3-month postpartum. Different superscripts indicate median values which are significantly different.
5.4.2 Comparison of breast milk iodine concentrations pre and post fortification

A comparison of breast milk iodine concentrations in the period immediately postpartum before and after the introduction of mandatory iodine fortification is presented in Table 5.5, Figures 5.2 and Figure 5.3. The median BMIC was significantly higher in the post fortification sample in comparison to the pre fortification sample (P<0.05) (103 vs. 187µg/l). The percentage of mothers who had BMICs below 100µg/l also decreased from ~50% before fortification to ~13% after fortification.

The majority of the women in the post fortification samples were taking iodine-containing supplements at enrolment (77%). Women in the pre fortification sample were not specifically asked whether the supplements they were taking contained iodine. However, although the proportion of DOMInO women who were on iodine supplements is not known, it was uncommon for prenatal supplements to contain iodine in the period during which the DOMInO women were recruited [108]. To determine whether mandatory iodine fortification, in the absence of iodine supplementation, resulted in an increase in BMIC, I therefore compared the breast milk iodine concentrations of those who did not take iodine supplements at enrolment in the post fortification sample with those of women in the pre fortification sample who did not take any dietary supplements. This would eliminate any impacts of iodine supplementation on iodine level in breast milk. In this comparison, the BMIC before the introduction of mandatory iodine fortification were still significantly lower than those after iodine fortification (105 vs. 165µg/l) (Figure 5.3), and the percentage of women with BMICs ≤100µg/l was significantly higher (~48% vs. ~20%) (Figure 5.3).
Figure 5.2: The difference in breast milk iodine level between pre and post fortification in all women and in the sub-group of mother who were not taking iodine supplements. Different superscripts indicate median values which are significantly different.
Figure 5.3: The proportion of women with BMIC above and below 100µg/l before and after fortification in the whole sample and in women not consuming dietary iodine supplements during pregnancy.
5.5 DISCUSSION

My findings have added to the extremely limited data regarding the breast milk iodine level of lactating women in Australia before the commencement of mandatory iodine fortification of salt used in bread making. The present study has also provided important information as to the breast milk iodine level in lactating women in South Australia post fortification. The median BMIC at birth prior to the iodine fortification was 103µg/l, and the BMICs post fortification were 187µg/l at birth and 126µg/l at 3-month postpartum. The median iodine contents in both pre and post fortification were indicative of adequate iodine supply for infant requirement; however the values prior to fortification were at the borderline of the cut-off suggested as a marker of sufficiency. Furthermore, this is the first study to directly compare the iodine concentrations in breast milk in the same region before and after iodine fortification, and has provided evidence that iodine fortification has resulted in a significant increase in the iodine content of breast milk in this population.

5.5.1 The breast milk iodine concentrations of mothers pre and post-fortification

Data on breast milk iodine level of lactating women in Australia are extremely limited. Only one study (n=49) conducted in 2003 in Sydney suggested that the breast milk of women prior to iodine fortification contained an inadequate amount of iodine to meet the requirements of full-term infants; 84µg/l compared to 100µg/l [217]. The present study, which included a larger sample size, produced similar findings, suggesting that the median BMIC in South Australian women prior to the introduction of iodine fortification was at the borderline of the level considered to suggest adequate supply for breastfed infant requirement (103µg/l). It is also important to note that almost half of women in the present study had breast milk iodine levels below 100µg/l, indicating that over half of exclusively breastfed infants may not have been receiving an adequate supply of iodine to support normal growth and development before iodine fortification was introduced [219].
The present study furthermore provided some of the first data regarding the breast milk iodine content of lactating women after the introduction of iodine fortification in South Australia. Median iodine concentrations in breast milk of lactating both at birth and 3 month postpartum were well above the suggested cut-off for iodine deficiency, which suggests that their breast milk provides an adequate iodine intake for full term breastfed infants in South Australia. It is worth highlighting that the percentage of women who had breast milk iodine level under 100µg/l was only ~13% at birth and ~35% at 3 months. I also found significantly higher iodine level in breast milk at birth, compared to 3 month postpartum. This result is consistent with the results of previous studies, which have also reported that iodine level in breast milk decreased as the duration of breast feeding (time postpartum) increased [216, 220, 221]. Furthermore, the evidence provided from a double-blind, randomised, placebo-controlled trial indicate that iodine supplementation during lactation increases iodine content in breast milk [216]. The relative impacts of the iodine fortification on BMIC compared with that of iodine supplementation during breast feeding is unclear as data on the intake of iodine supplements at 3 months postpartum in the post fortification samples was being collected in a separate study and was not available for this thesis.

As mentioned in the literature review, the NHMRC recommended in 2010 that breast feeding women should consider taking an iodine supplement providing 150µg/day of iodine in addition to their normal diet [25]. Several recent studies have highlighted the importance of iodine supplements for increasing the iodine content of the breast milk above the levels required to meet infant requirements, particularly for infants who are exclusively breastfed, and these studies all suggested that maternal use of iodine supplements increases the iodine level in their breast milk [222, 223]. It is worth noting that these previous studies had relatively small sample sizes (less than 100 women). The present study has replicated these findings in a larger sample size, and supports the suggestion that maternal iodine supplementation is an effective strategy for increasing the iodine content of the breast milk.
However, while the iodine levels in breast milk of unsupplemented mothers was lower than supplemented mothers; they were still well above the cut-off of 100µg/l, indicative of adequate iodine supply for their infants. In the present study, I also found that mothers who were taking supplements which provided equal to or greater than 150µg iodine/day had significantly higher BMICs than women taking lower dose supplements. However, it is important to note that no upper threshold of breast milk iodine level, indicative of the excessive iodine supply for infants as well as no cut-off of UICs, indicative of excessive iodine status in infants has been established. Information about the dose of iodine in the nutritional supplements taken by women pre fortification was not been collected in DOMInO, so similar sub-group analyses are not possible. However, I found no difference in breast milk iodine concentrations between DOMInO women who reported taking non-DHA containing dietary supplements during pregnancy and those who did not take supplements.

As described in literature review, iodine is transported from maternal circulation to breast milk via the mammary gland by membrane protein called the sodium iodide symporter (NIS). Several similarly charged anions may competitively inhibit this transporter, and therefore interfere with iodine transport. One such anion is thyocianate which accumulates in blood and tissue of smokers [224, 225]. There have been very few studies investigating the impacts of smoking on BMICs. Laurberg and colleagues found that serum of smoking mother contained higher thiocyanate level, compared to non-smoking mothers, which may responsible for the decrease of iodine level in breast milk [208]. The present study confirmed that iodine levels in breast milk were significantly lower in smokers than in non-smokers in both the pre fortification and post fortification samples. Taken together, these findings provide evidence that smoking during the postpartum period can impair the transport of iodine from mothers to breastfed infants via breast milk, hence increasing the risk of iodine deficiency in infants. Lauberg and colleagues [208] also suggested that increasing the dose of iodine in nutritional supplements consumed by women during lactation, could potentially restore breast milk
iodine levels in smoking mothers to those in non-smokers, but this was not specifically assessed in the present study.

5.5.2 The comparison of breast milk iodine concentrations pre and post fortification

Our study has provided the first direct comparison of breast milk iodine content in lactating women in the same region of South Australia before and after the introduction of mandatory iodine fortification. The results have provided evidence in support of the efficacy of mandatory iodine fortification of salt used in bread making in increasing both median breast milk concentrations and reducing the percentage of women whose breast milk iodine concentrations were below the recommended cut-off.

It is worth noting that the percentage of lactating women pre fortification in Australia consuming iodine supplements was only ~20% [44]. In some populations, such as that in a study by Chan and colleagues assessing the postpartum maternal iodine status, none of the women in their study population were taking iodine supplements [217]. This is in contrast to the current situation, following the introduction of both mandatory iodine fortification and NHMRC recommendations regarding iodine supplementation in pregnant and breast feeding women, in which the majority of women are taking some form of iodine supplement [25]. In the present study, almost 80% of the pregnant women in the post fortification sample were taking iodine supplements at the time of enrolment (early-mid pregnancy). Thus, I compared not only the breast milk iodine concentrations pre and post fortification across the whole study population, but also between the sub-group of women who did not consume dietary supplements at trial entry, in order to assess the effects of mandatory iodine fortification, as distinct from increased use of supplements, on breast milk iodine content.

As highlighted in Chapter 4, UIC of lactating mothers significantly improved and reached iodine sufficient status after the commencement of iodine fortification. The findings of this
Chapter provide evidence of the success of iodine fortification in enhancing iodine nutrition status not only for lactating women but also for their infants. The current study suggested that breast milk concentrations in ~90% of lactating women post fortification was above the recommended cut-off for achieving adequate iodine intake for breastfed infants in Australia, irrespective of whether or not the women were taking iodine supplements at trial entry. The iodine fortification reduced the prevalence of mothers who had breast milk iodine levels below the 100µg/l cut-off by ~28%. The findings of our study are not, however, in line with another study conducted by Brough and colleagues in New Zealand, [226] who also implemented iodine fortification in bread making since 2009, and which found no significant increase in breast milk iodine content pre and post fortification; notwithstanding that majority of women post fortification were consuming iodine supplements. These discrepancies may be due to the small sample size in the New Zealand study (n=31 pre fortification vs n=33 post fortification). More importantly, New Zealand historically had lower iodine status than Australia. The inclusion of women across a broad range of times postpartum (at least 3 weeks postpartum, actual weeks postpartum were not reported) in the New Zealand study may also have confounded the assessment because the breast milk iodine levels are known to decline across the lactation period [216].

5.5.3 The limitations of the present study

Numerous studies demonstrated that the nutrient composition is different between foremilk and hindmilk, such that the latter contains more fat and energy but less zinc and copper than former [227-229]. To date, no study has addressed the differences in iodine content between foremilk and hindmilk. However, for practical and logistical reasons, only foremilk samples were collected from the PINK participants. If there is a difference in iodine level between foremilk and hindmilk, the results of present study may therefore not accurately reflect the actual supply of iodine to the breastfed infant. It is hence important to acknowledge that
further studies assessing iodine level in breast milk samples collected at different phases of breast feeding and from different breast milk fractions are needed.

In addition, although I demonstrated in a pilot experiment that the iodine concentrations in breast milk samples stored under the same conditions as the DOMInO samples were stable across an 18 month period, the pre fortification were stored for almost 5 years before analysis, and I cannot exclude the possibility of losses occurring at later time points. One possibility is that the evaporation of water from the samples could have occurred during the storage period. If this was the case, however, then the actual iodine levels in breast milk pre fortification would be even lower than that measured in the current study (103µg/l), and definitely less than the iodine level post fortification, and this would therefore not impact on the main conclusion of this Chapter.

Furthermore, Bouhouch and colleagues recently reported in a double-blind, randomised, placebo-controlled trials that iodine supplementation during lactation resulted in the elevated iodine level in breast milk [230]. However, the information of iodine supplementation during lactation was not collected in this study. Therefore, whether the intake of iodine supplements postpartum confounds the comparison of breast milk iodine levels between pre and post fortification has not been determined.

5.5.4 Summary

In summary, the current study has provided novel data regarding breast milk iodine level after the commencement of iodine fortification in salt for bread making, 187µg/l at birth and 126µg/l at 3 months postpartum, indicative of a sufficient iodine supply for full-term infants. This is also the first study to test the impact of mandatory iodine fortification on the iodine concentrations in breast milk, and therefore the iodine supply to breastfed infants in Australia. This study demonstrated an improvement of breast milk iodine level after the mandatory iodine fortification compared to pre fortification, and that the introduction of iodine
fortification and recommendations regarding iodine supplementation in pregnant and breast feeding women reduced the incidence of mothers who had iodine level in breast milk less than 100µg/l by 28%, suggesting that these measures have been effective in increasing the iodine supplied to the average South Australian infants, and is consistent with the results of Chapter 4, indicating that the iodine status of the infants in the post fortification sample were well above the cut-off for iodine sufficiency.
CHAPTER 6
6.1 INTRODUCTION

The prevalence of overweight and obesity has significantly increased in both developing and developed countries over the past three decades [231]. Both adults and children have been affected, and in a survey conducted in 2011/2012 over 25% of Australian children aged from 5 to 17 years were classified as overweight or obese [232]. It is well-established that overweight and obesity are associated with an increased risk of several chronic diseases, in particular insulin resistance and type 2 diabetes, hypertension and stroke [233, 234].

While a number of different factors have been implicated in the obesity epidemic, diet and lifestyle are thought to be one of the most important causes. In addition to increases in overall energy intake, alterations in intakes of specific micronutrients have also been implicated as contributing factors [235]. Iodine is a key component of thyroid hormones, which are known to play a critical role in the regulation of basal metabolic rate and endogenous energy expenditure in humans [236], and this has led to suggestions that poor iodine nutrition may be a contributing factor to the current epidemic of childhood obesity.

The relationship between thyroid function and body weight/body fat mass has been investigated in a range of population-based studies, and studies in adults have reported significant relationships between body composition including body weight, body fat mass and BMI and thyroid function as determined by circulating fT3, fT4 and TSH level [129, 237]. While there have been fewer such studies in children, these have provided some evidence to suggest that thyroid function may also influence body composition of children. In one study, Chen and colleagues reported that the incidence of obesity was higher in 6-7 year-old children who suffered from congenital hypothyroidism than in the general population [238]. Studies in
overweight and obese paediatric populations have also reported significant positive associations between fT3 and/or TSH concentrations and BMI-z score [138, 239, 240]. However, studies conducted in the general paediatric population have provided less consistent results. In one study, BMI was positively associated with fT3, but negatively associated with fT4 [136]. A positive correlation between BMI and TSH level was reported in a study involving 13691 Indian healthy children [136], but another study with a smaller sample size (234 school-age children) showed no association [139]. This latter study also found no correlation between either TSH level or fT4 levels in the circulation and body fat mass in the general child population [139]. Taken together, while these studies suggest the presence of a relationship between thyroid function and body weight/composition in obese/overweight children, whether these relationships exist in the general paediatric population is unclear and further studies are required.

In addition to their role in regulating energy metabolism and body weight, thyroid hormones have also been shown to have a role in regulating insulin secretion and insulin action, and therefore insulin sensitivity [241]. An experimental study in dogs demonstrated that hypothyrodism was associated with the development of insulin resistance [242]. However, few studies have investigated the relationship between thyroid function and markers of insulin sensitivity, including fasting insulin levels or HOMA-IR, in humans, and those that have produced conflicting results. In one study of 240 overweight or obese pre-pubertal children, Brufani and colleagues reported that both fasting insulin concentrations and hepatic insulin resistance were negatively correlated with thyroid function [138]. However, other studies in overweight/obese children have failed to replicate these findings [239, 243, 244]. The studies conducted to date which have attempted to determine the presence of relationships between thyroid function and either body weight, body fat mass or insulin sensitivity in children have produced inconsistent results, and further studies are required. All studies to date have also
included children across a broad range of ages (from around 5 to 18 years-old), and therefore failed to take into account the possibility that these relationships may vary with age.

In addition to current nutritional intake, the importance of nutritional exposures experienced before birth and in early infancy for the metabolic health of individuals later in life is increasingly recognised [158]. The role of iodine and thyroid hormones in adult metabolism has led to the suggestion that iodine nutrition status, and hence thyroid function, in fetal and early postnatal life may influence the metabolic health of an individual later in childhood. In support of this, Godoy and colleagues recently reported a significant relationship between maternal TSH/fT4 level in early pregnancy and BMI and body fatness of their children at 6 years of age in a prospective cohort study of 5646 mother-child pairs [64]. However, this study only assessed thyroid status of pregnant women at one time point and at less than 18 weeks of gestation. Therefore, how maternal and fetal iodine nutrition and thyroid status in late pregnancy, the major period of development of adipose tissue and other key metabolic regulatory systems, impacts on body composition later in childhood is unclear. Only one animal study to date has investigated the relationship between iodine nutrition in the perinatal period and insulin sensitivity in the offspring later in life, and showed that pups exposed to maternal hypothyroidism during the suckling period had higher plasma glucose concentrations and were more insulin resistant than controls at 90 days of age [163]. However, no study has been conducted to confirm whether similar relationships are present in humans.

In summary, while there is some evidence in support of a role for thyroid hormone status in the regulation of body weight and insulin sensitivity in children, the evidence from existing studies has been inconsistent. In addition, the recognised importance of the nutritional environment experienced before birth in determining long term metabolic health outcomes in individuals has also led to the suggestion that iodine nutrition in early development, as reflected by neonatal TSH levels, may also be related to metabolic health later in childhood.
Therefore, the primary aim of this Chapter was to assess relationships between both current Tg/TSH status and TSH status in the early neonatal period on markers of metabolic health, including body mass index (BMI), body fat mass and insulin sensitivity at 5 years of age.

6.2 METHODS

6.2.1 Study design

The study utilised samples and clinical data collected from children who were participating in the 3 and 5 year growth and insulin resistance follow-up of the DOMInO randomised controlled trial. The primary aim of this follow up study was to assess the effect of maternal dietary supplementation with omega-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA), chiefly as docosahexaenoic acid (DHA), in the second half of pregnancy on BMI-z score and body fat mass at 3 and 5 years of age, and a secondary aim was to determine the effect of this intervention on insulin sensitivity, as measured by HOMA-IR, in the children at 5 years of age. This follow-up study was conducted at the Women’s and Children’s Hospital and Flinders Medical Centre. Ethical approval was obtained from Children’s Health Network Human Research Ethics Committee to extract TSH data from the routine neonatal screening database and to undertake the analysis of Tg and TSH concentrations in the blood samples collected at the 5 year for which the participants have provided consent for these samples to be stored and used for additional non-genetic analyses.

6.2.2 Participants and recruitment

The participants included in this study represented a sub-set of children who were participating in the 3 and 5 year growth and insulin follow up of the DOMInO study. Of the 1407 DOMInO children who attended clinic appointments at 5 years of age, blood samples were collected from 715, and 678 of these provided consent for non-genetic tests to be carried out on the stored samples once the primary analytes (glucose and insulin) had been measured.
Of these samples, 199 had sufficient volume of plasma remaining to undertake both TSH and Tg analyses. From this sub-population, 100 children (stratified for sex and treatment group) were randomly selected for the current study by an independent statistician.

TSH level at birth were extracted from the results of routine neonatal screening conducted on neonatal screening cards collected within few days after birth for the same 100 DOMInO children.

6.3 ASSESSMENTS

6.3.1 Thyroid status

6.3.1.1 Thyroid function of DOMInO children at birth

TSH levels at birth were extracted from the neonatal screening results database (TSH is measured in all infants at birth as a part of routine neonatal screening).

6.3.1.2 Thyroid function of children at 5 years of age

TSH and Tg concentrations were measured in plasma samples collected at the 5 year appointment for participants in the DOMInO 3 and 5 year follow up study.

TSH level were measured using a TSH ELISA kit for human serum (ALPCO Diagnostics, US); the sensitivity was determined to be 0.054µIU/ml; the intra-assay coefficient of variation (CV) and inter-assay CV were 6.4-92% and 7.6-12.9% respectively. I undertook all of the TSH analyses for this study.

Tg level was measured by an external pathology laboratory (the Institute of Medical and Veterinary Sciences, IMVS, Adelaide) using the Immulite 2000 system and a solid-phase chemiluminescent immunometric assay. The analytical sensitivity of this method was 0.2ng/ml. The level of Tg autoantibodies were also measured in each sample and taken into
consideration when calculating the plasma Tg concentrations in each specimen. Tg level of one plasma sample could not be measured because of the presence of an unknown contaminant which interfered with the Tg assay.

### 6.3.2 Anthropometric parameters and body composition

#### 6.3.2.1 Anthropometrics and body mass index (BMI)

At 5 years of age, standard anthropometric measures, including weight, height, waist circumference and hip circumference was undertaken by research staff.

Weight in kg was measured to the nearest 10g using an electronic scale calibrated every 6 months. Children were weighed alone without shoes and outer clothing.

Height was measured to the nearest 0.1cm using a portable or fixed stadiometer. Children stood on the baseboard or floor with feet slightly apart. Feet were together, body was aligned and the head was placed in the Frankfort plane.

The weight and height measurements for each child were used to calculate BMI (weight in kg/height in m$^2$). Height, weight and BMI z-score was determined using the WHO Child Growth Standards [245] which provided clear BMI cut off points in childhood based on international data of healthy breastfed infants and young children raised in environments that do not constrain growth.

The parameters of waist and hip circumference were measured using non-stretch tape. Hip circumference was measured at the greatest posterior protuberance of the buttocks. Waist circumference was measured at the narrowest point between the lower costal border and the iliac crest.
6.3.2.2 Body fat mass

The impedance data measured from the Bioelectrical Impedance Spectroscopy (BIS) was used to calculate total body water based on the equation developed by Kushner and colleagues [246]. The value for total body water was then used to estimate fat free mass using the age and sex-specific equations developed and validated by Goran and colleagues [247]. Fat free mass was subtracted from total body weight to determine fat mass, and this figure was divided by body weight to obtain the percentage of body fat.

6.3.3 Insulin sensitivity

The children were instructed to fast for at least 4 hours prior to their 5 year clinic appointment. Blood samples (5ml) were collected by venepuncture into tubes containing heparin as an anti-coagulant and kept on ice until they were transferred to the laboratory for processing.

Glucose concentrations were measured in the samples using the Infinity Glucose Hexokinase kit (Thermo Electron, Pittsburgh, PA, USA) on the Konelab 20XTi system (Thermo Scientific, Vantaa, Finland). This system is a clinical chemistry analyser for colorimetric, immuno-turbidimetric and ion-selective electrode measurement. This test was performed by a trained researcher from the FOODplus Research Centre. The method detection limit and reporting limits were 0.026mmol/l and 0.053mmol/l, respectively.

Insulin concentration was measured using an insulin ELISA kit for human serum and plasma. (ALPCO Diagnostics, US); the method detection limit and reporting limit were 0.0000502µIU/mL and 0.00167µIU/mL; intra-assay variation was 1.9% and inter-assay variation was 6.8%. The test was performed in the laboratory of FOODplus Research Centre (University of Adelaide) by a trained research assistant.
These measures were used to provide an estimate of insulin sensitivity of the child using the insulin resistance index (HOMA-IR index), according to the equation:

\[
HOMA-IR\ index = \frac{\text{Glucose (mmol/l)} \times \text{insulin (mU/l)}}{22.5}
\]

6.3.4 Other assessments

6.3.4.1 Maternal socioeconomic and demographic background at DOMInO trial entry

Detailed information related to maternal age, BMI, parity and smoking status were collected at the time of enrolment into the DOMInO trial. Mothers were also asked to provide information about their education and employment status at trial entry.

6.3.4.2 Pregnancy-related outcomes at birth

Information relating to the DOMInO pregnancy, including a diagnosis of gestational diabetes or pre-eclampsia and gestational age at delivery was collected from all women in the DOMInO study. Infant outcomes, including birth weight, birth length and head circumference, were also collected. Information on the feeding mode (breast feeding/ formula feeding/mixed feeding) at 6 weeks postpartum was also collected in DOMInO.

6.3.4.3 Maternal measures at 5 years follow up

BMI, education and employment of biological mother was also collected at the time of the 3 and 5 year follow up of the DOMInO trial.

6.3.5 Sample collection and processing

Blood samples were collected into heparinised tubes from the DOMInO children as a part of the clinic appointment at 5 years of age. After collection, the samples were immediately refrigerated and sent to the analytical laboratory within 24 hours. When the samples arrived at the laboratory, the whole blood was centrifuged at 3200rpm for 15mins at room temperature.
After centrifugation, three different fractions were distinguishable. The top plasma layer was carefully removed without disturbing the intermediate layer by glass transfer pipette and put in appropriately labelled barcoded cryo-tubes (Thermo Fisher Scientific, USA). All samples were processed within 24 hours of collection. Plasma was frozen at -80°C until analysis.

6.4 DATA ANALYSIS

SPSS version 17 (SPSS Inc, US) was employed for all data analysis and P<0.05 was considered statistically significant. The Shapiro-Wilk test was applied to all variables in order to determine if they were normally distributed prior to analysis. Continuous variables which were normally distributed are presented as mean ±SD while non-normally distributed data are presented as median (IQR). Categorical variables were reported as number (%). Differences in the anthropometric and biochemical variables between male and female groups were determined using an independent T-test for normally distributed variables or Mann Whitney test for non-normally distributed variables. Correlations between TSH or Tg concentrations at birth or at 5 years of age and measures of metabolic health (body fat mass, BMI z-score, weight z-score, hip and waist circumference, fasting glucose and insulin and HOMA-IR) at 5 years of age were assessed using simple Pearson or Spearman correlation tests for normally and non-normally distributed data as appropriate, both in the whole group and in male and female infants separately. The adjustments of TSH/Tg levels at 5 years of age were performed by partial correlation.

6.5 RESULTS

The maternal characteristics at trial entry and at the 5 year follow up are shown in Table 6.1. Half of women were over 30 years old at the time of recruitment, and the majority had a BMI that placed them in the overweight and obese category both at baseline and at the time of the 5 year follow-up. The majority of women had completed further education and were employed both at baseline and at the time of 5 year follow up. The majority of women did not smoke or
drink at baseline. There were few diagnoses of gestational diabetes or preeclampsia in the women in their DOMInO pregnancy.

All children selected for this sub-study were born at term (39 weeks, 38-41) with a mean birth weight of 3.4kg, mean birth length of 49.2cm and mean birth head circumference of 34.2cm. Approximately half of the children were exclusively breastfed up to 6 weeks postpartum.

6.5.1 Anthropometric and hormonal measures in different sex

The anthropometric and biochemical characteristics of children at birth and at 5 years of age are presented in Table 6.2 and 6.3, separated by sex. Male neonates were longer and had larger head circumferences, compared to female neonates (P<0.05). However, there was no difference in birth weight between male and female infants. At 5 years of age, male children were significantly taller than female children (P<0.05), but there was no significant difference in BMI z-score between the sexes. There was a significant difference in the percentage fat mass at 5 years of age between the sexes, with girls having higher percentage fat mass than boys. Fasting glucose and insulin concentrations and HOMA-IR was not different between males and females in this study. There were also no differences in neonatal TSH level and TSH or Tg levels at 5 years of age between DHA group and placebo group (P>0.05)
Table 6.1: Maternal anthropometric and demographic characteristics at trial entry and follow-up of 5 years

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>At trial entry</th>
<th>At 5-year follow up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Mean±SD), years</td>
<td>29.8±6</td>
<td>-</td>
</tr>
<tr>
<td>Gestational age (Median, IQR), weeks</td>
<td>20 (19-20)</td>
<td>-</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26 (23.4-30.2)</td>
<td>26.6 (23.2-30.1)</td>
</tr>
<tr>
<td>Parity =0 (%)</td>
<td>35</td>
<td>-</td>
</tr>
<tr>
<td>Current maternal smoking (%)</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>Current maternal drinking (%)</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>Mother completed secondary school (%)</td>
<td>72</td>
<td>72.6</td>
</tr>
<tr>
<td>Mother completed further education (%)</td>
<td>60</td>
<td>68.4</td>
</tr>
<tr>
<td>Mother currently employed (%)</td>
<td>80</td>
<td>68.4</td>
</tr>
<tr>
<td>Clinical diagnosis of gestational diabetes in the DOMInO pregnancy (%)</td>
<td>7.5</td>
<td>-</td>
</tr>
<tr>
<td>Clinical diagnosis of preeclampsia in the DOMInO pregnancy (%)</td>
<td>2.1</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviations: SD, standard deviation; BMI, body mass index; IQR, interquartile range.

Table 6.2: Neonatal anthropometric and biochemical parameters of participants according to sex

<table>
<thead>
<tr>
<th></th>
<th>All participants</th>
<th>Males</th>
<th>Females</th>
<th>P value ( ^{a} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSH (µUI/ml)</td>
<td>2 (1-2.9)</td>
<td>1.6 (1.1-3.3)</td>
<td>2 (1-2.7)</td>
<td>ns</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>3.4±0.6</td>
<td>3.4±0.5</td>
<td>3.43 (2.9-3.8)</td>
<td>ns</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>49.2±3</td>
<td>49.8±2.3</td>
<td>49 (47-50)</td>
<td>0.01</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>34.2±2.1</td>
<td>34.8±1.7</td>
<td>34 (33-35)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Data are expressed as Median (interquartile range) or mean±SD

\( ^{a} \): statistical test used to compare between male group and female group at 95% confidence. Independent T-test used for normal distribution variables and Mann Whitney test used for non-normal distribution variables. ns: not significant
Figure 6.1: The distribution and differences in BMI z-score, body fat mass and HOMA-IR between male and female children. Different superscripts indicate median values which are significantly different.
Table 6.3: Anthropometric and biochemical parameters of participants at 5 years of age according to sex

| Parameter                  | All participants | Males   | Females  | P value  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg (µg/l)</td>
<td>2 (1-4)</td>
<td>2 (1-4)</td>
<td>2 (1-4)</td>
<td>ns</td>
</tr>
<tr>
<td>TSH (µIU/ml)</td>
<td>2.5 (1.9-3.8)</td>
<td>2.6 (2.1-3.6)</td>
<td>2.4 (1.6-3.9)</td>
<td>ns</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.1±1</td>
<td>4.2±1.1</td>
<td>4±0.9</td>
<td>ns</td>
</tr>
<tr>
<td>Insulin (µIU/ml)</td>
<td>4.9 (3.1-10.7)</td>
<td>4.3 (2.9-8.3)</td>
<td>5.6 (3.6-11.6)</td>
<td>ns</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.9 (0.6-1.7)</td>
<td>0.7 (0.5-1.4)</td>
<td>1 (0.7-1.8)</td>
<td>ns</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>110.7±4.5</td>
<td>111.8±4.1</td>
<td>109.6±4.6</td>
<td>0.02</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>20.3 (18.8-21.8)</td>
<td>20.8 (19.4-21.9)</td>
<td>19.6 (17.7-21.4)</td>
<td>ns</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>53.4 (50.9-55.6)</td>
<td>54.1 (52-55.7)</td>
<td>52.6 (50.2-55.4)</td>
<td>ns</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>59.2 (57.1-61.5)</td>
<td>59.1±3.4</td>
<td>59.7±4.4</td>
<td>ns</td>
</tr>
<tr>
<td>Weight-z-score</td>
<td>0.6±0.9</td>
<td>0.8±0.8</td>
<td>0.5±0.9</td>
<td>ns</td>
</tr>
<tr>
<td>BMI-z-score</td>
<td>0.8±0.7</td>
<td>0.8 (0.3-1.4)</td>
<td>0.6 (-0.1-1.5)</td>
<td>ns</td>
</tr>
<tr>
<td>Height-z-score</td>
<td>0.2±0.9</td>
<td>0.3±0.9</td>
<td>-0.01±1</td>
<td>ns</td>
</tr>
<tr>
<td>Body fat mass (%)</td>
<td>23.9±6.6</td>
<td>21.8 (16.3-23.8)</td>
<td>26.8 (24.3-30.9)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Abbreviation: HOMA-IR, homeostatic model assessment-insulin resistance; BMI, body mass index; IQR, interquartile range; ns, not significant

Data are expressed as Median (interquartile range) or mean±SD. a: statistical test used to compare between male group and female group at 95% confidence. Independent T-test used for normally distributed variables and Mann Whitney test used for non-normally distributed variables.

6.5.2 Correlation between neonatal thyroid function anthropometric parameters at birth

Neonatal TSH levels were not correlated with any anthropometric parameters at birth, including gestational age, weight, length and head circumference when data from both male and female infants were combined (Table 6.4). There was also no correlations between these variables when the correlations were determined separately in male and female infants (P>0.05).
Table 6.4 The correlation between neonatal TSH level and anthropometric parameters at birth

<table>
<thead>
<tr>
<th></th>
<th>Correlation coefficient</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age at birth</td>
<td>0.19</td>
<td>ns</td>
</tr>
<tr>
<td>Birth weight</td>
<td>0.13</td>
<td>ns</td>
</tr>
<tr>
<td>Birth length</td>
<td>0.16</td>
<td>ns</td>
</tr>
<tr>
<td>Birth circumference</td>
<td>0.11</td>
<td>ns</td>
</tr>
</tbody>
</table>

Abbreviation: ns, not significant
Simple Pearson or Spearman correlation tests were applied.

6.5.3 Correlations between neonatal TSH levels and metabolic parameters of children at 5 years of age

Neonatal TSH levels were unrelated to both TSH and Tg levels at 5 years of age when data from all children were combined and in females when children were separated by sex (P>0.05). In males, however, there was a direct relationship between TSH concentrations at birth and both TSH and Tg concentrations at 5 years of age (P<0.01).

There were no significant correlations between neonatal TSH levels and fasting insulin level, HOMA-IR, BMI and body fat mass in early childhood in the combined data set (Table 6.5). However, TSH level at birth was positively correlated with fasting plasma glucose level measured at 5 years of age (P<0.05). This positive correlation was only present in males when males and females were separated in the analysis (Figure 6.2). These positive correlations between neonatal TSH concentrations and fasting glucose levels did not, however, persist after the effects of TSH/Tg levels at 5 years of age were controlled for in the analysis. TSH levels at birth were also positively correlated to HOMA-IR at 5 years of age in males whereas there was no relationship between these measures in females (Figure 6.4). Higher neonatal TSH level was associated with a lower height/height z-score in males (Figure 6.5 and 6.6), but not in females. The correlations between neonatal TSH levels and either HOMA-IR or height/height z-score in males persisted after adjustment for TSH/Tg levels at 5 years of age. There were no correlations between TSH levels and other metabolic markers including insulin
level, weight/weight z-score, waist circumference, hip circumference, BMI z-score and body fat mass (P>0.05) in either the combined dataset, or when the data from male and female infants were analysed separately, irrespective of whether or not TSH/Tg levels at 5 years of age were controlled for.
Figure 6.2: The relationship between neonatal TSH levels and glucose level at 5 years of age

(a) all children; (b) male children and (c) female children
Figure 6.3: The relationship between neonatal TSH levels and BMI z-score at 5 years of age,
(a) all children, (b) male children, (c) female children
Figure 6.4: The relationship between neonatal TSH levels and HOMA-IR at 5 years of age,
(a) all children, (b) male children, (c) female children
Figure 6.5 The relationship between neonatal TSH levels and height at 5 years of age (a) all children, (b) male children and (c) female children.
Figure 6.6: The relationship between neonatal TSH levels and height z-score at 5 years of age.

(a) all children, (b) male children and (c) female children
Figure 6.7: The relationship between neonatal TSH levels and body fat mass at 5 years of age, (a) all children, (b) male children (c) female children.
Table 6.5: Correlations between neonatal TSH level and TSH/Tg level and metabolic parameters of children at 5 years of age

<table>
<thead>
<tr>
<th></th>
<th>All children</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Correlation coefficient</td>
<td>P</td>
<td>Correlation coefficient</td>
</tr>
<tr>
<td>TSH level</td>
<td>0.19</td>
<td>ns</td>
<td>0.39</td>
</tr>
<tr>
<td>Tg level</td>
<td>0.09</td>
<td>ns</td>
<td>0.32</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>0.21</td>
<td>0.043</td>
<td>0.33</td>
</tr>
<tr>
<td>Insulin (µIU/ml)</td>
<td>0.04</td>
<td>ns</td>
<td>0.27</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.09</td>
<td>ns</td>
<td>0.29</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>-0.04</td>
<td>ns</td>
<td>-0.33</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>-0.02</td>
<td>ns</td>
<td>-0.12</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>-0.08</td>
<td>ns</td>
<td>-0.07</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>-0.09</td>
<td>ns</td>
<td>-0.21</td>
</tr>
<tr>
<td>Weight-z-score</td>
<td>-0.02</td>
<td>ns</td>
<td>-0.15</td>
</tr>
<tr>
<td>BMI-z-score</td>
<td>0.01</td>
<td>ns</td>
<td>0.11</td>
</tr>
<tr>
<td>Height-z-score</td>
<td>-0.06</td>
<td>ns</td>
<td>-0.36</td>
</tr>
<tr>
<td>Body fat mass (%)</td>
<td>-0.08</td>
<td>ns</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Note: The significant correlation between TSH at birth and height or height z-score at 5 years of age still persisted after the adjustment for TSH/Tg at 5 years of age. Other significant correlations became non-significant after the adjustment.

Abbreviations: HOMA-IR, homeostatic model assessment-insulin resistance; BMI: body mass index. Simple Pearson or Spearman correlation tests were applied. P<0.05 presents a significant correlation. ns means “not significant”
6.5.4 Correlation between thyroid function and metabolic parameters of children at 5 years of age

There was a significant positive correlation between TSH and Tg concentrations at 5 years of age in males (P<0.05), but not in females. Furthermore, there were no significant correlations between either TSH or Tg concentrations at 5 years of age and any of the markers of metabolic health assessed in the children at this time point, including height, weight, waist circumference, hip circumference, weight z-score, height z-score, BMI z-score, and body fat mass. Similarly, no correlation was found between thyroid function and fasting glucose and insulin concentrations or the HOMA-IR index in this study population (P>0.05). The correlations between TSH/Tg level and metabolic markers are shown from Figure 6.8 to Figure 6.13. Pearson’s correlation coefficient for the association between thyroid function and either body composition parameters or insulin resistance are shown in Table 6.6.

The same correlations were also assessed separately male and female groups. There was no association between TSH and Tg level and body composition or insulin resistance at 5 years of age in either males or females.
Figure 6.8: Correlation of TSH level among all children with metabolic parameters; (a) BMI z-score, (b) Body fat mass, (c) HOMA-IR
Figure 6.9: Correlation of Tg level among all children with metabolic parameters; (a) BMI z-score, (b) Body fat mass, (c) HOMA-IR
Figure 6.10: The correlation of TSH level among male children with metabolic parameters:

(a) BMI z-score, (b) body fat mass, (c) HOMA-IR
Figure 6.11: The correlation of Tg level among male children with metabolic parameters: (a) BMI z-score, (b) body fat mass, (c) HOMA-IR
Figure 6.12: The correlation of TSH level among female children with metabolic parameters:

(a) BMI z-score, (b) body fat mass, (c) HOMA-IR
Figure 6.13: The correlation of Tg level among female children with metabolic parameters:

(a) BMI z-score, (b) body fat mass, (c) HOMA-IR
Table 6.6: Correlation analysis between either TSH or Tg concentrations and metabolic parameters in children at 5 years of age

<table>
<thead>
<tr>
<th></th>
<th>Tg level</th>
<th>TSH level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Correlation coefficient</td>
<td>P</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>0.15</td>
<td>ns</td>
</tr>
<tr>
<td>Insulin (µIU/ml)</td>
<td>-0.01</td>
<td>ns</td>
</tr>
<tr>
<td>HOMA-IR</td>
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<td>ns</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>-0.14</td>
<td>ns</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>-0.09</td>
<td>ns</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>-0.12</td>
<td>ns</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
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<td>ns</td>
</tr>
<tr>
<td>Weight-z-score</td>
<td>-0.11</td>
<td>ns</td>
</tr>
<tr>
<td>BMI-z-score</td>
<td>0.03</td>
<td>ns</td>
</tr>
<tr>
<td>Height-z-score</td>
<td>-0.18</td>
<td>ns</td>
</tr>
<tr>
<td>Body fat mass (%)</td>
<td>-0.11</td>
<td>ns</td>
</tr>
</tbody>
</table>

Abbreviations: HOMA-IR, homeostatic model assessment-insulin resistance; BMI: body mass index. Simple Pearson or Spearman correlation tests were applied.
6.6 DISCUSSION

The present study is the first to investigate the relationship between thyroid function in the early neonatal period (as reflected by TSH levels at birth) and metabolic health in early childhood. It is also the first study to evaluate the relationship between current Tg levels and markers of metabolic health in young children.

6.6.1 The association between TSH status in the neonatal period and metabolic health at 5 years of age

The present study has provided the first data regarding the relationship between neonatal TSH levels and markers of metabolic health measured at 5 years of age. I identified a weak positive correlation between TSH levels at birth and fasting glucose levels at 5 years of age in the full dataset. This relationship appeared to be driven mainly by males, as it was only present in males when the sexes were analysed separately. However, while this relationship was statistically significant, it is important to note that the correlation co-efficient was only 0.21, indicating that TSH levels at birth could explain only ~4% of the variation in glucose levels at 5 years of age. Accordingly, the clinical significance of this relationship is likely to be limited. I also found that higher neonatal TSH levels were associated with a higher HOMA-IR at 5 years of age in males, but not in females. Similarly, a negative association between TSH levels at birth and height/height z-score at 5 years of age was found only in male children.

A large number of clinical, epidemiological and experimental studies have provided evidence that the nutritional environment experienced by an individual before birth and in early infancy can have long-term effects on an individual’s metabolic health. A review by Gonzalez-Barranco & Rios-Torres [248] suggested that young men who suffered from malnutrition in early postnatal life were at increased risk of insulin resistance and glucose intolerance in adulthood. However, this review did not mention whether the same impact was found in female participants. The impact of impaired thyroid function during the perinatal period on
metabolic health in later life has previously been investigated in a recent animal study. Farahani and colleagues used a rat model to explore the relationship between neonatal hypothyroidism, induced by administering a 0.02% solution of 6-propyl-2-thiouracil to rat dams during lactation, on carbohydrate metabolism and insulin levels in their offspring in adulthood [163]. They reported that both plasma glucose levels and HOMA-IR of offspring exposed to neonatal hypothyroidism was significantly higher than in offspring of control dams, while no difference in plasma insulin levels was found between 2 groups. Although not the focus of their study, the authors also reported that offspring of hypothyroid dams were lighter than the offspring of control dams from 15 days to 90 days of age. However, there have been no comparable human studies conducted to date. The present study is the first to assess whether thyroid function at birth is associated with the measures of metabolic health later in childhood. I found male children with higher TSH levels at birth had higher fasting glucose levels and higher insulin resistance index (as assessed by HOMA-IR) in early childhood. This may suggest that thyroid function at birth can influence glucose metabolism later in childhood although it is important to stress that the current study only indicates the presence of associations between these variables rather than causal relationship. Our data also highlighted the lack of any relationships between neonatal TSH levels and fasting glucose metabolism or insulin resistance in female children, suggesting that the effects may be sex-specific.

As mentioned in the literature review, thyroid hormones are involved in the regulation of multiple metabolic pathways in human, including carbohydrate, protein and lipid metabolism and therefore contribute to the regulation of growth [241]. There has recently been an emerging view in the literature regarding the potential interaction between impaired thyroid function and bone/mineral metabolism, and hence body height [249]. This association was highlighted by studies investigating the relationship between congenital hypothyroidism and height in later life. The most recent study was conducted in 760 neonates of whom 208 was
diagnosed with congenital hypothyroidism in their neonatal screening test. The results of this study suggested that the height percentile of the hypothyroid group was significantly lower than that of the euthyroid group at all ages between birth and 5 years of age at which the children were assessed. Importantly, this occurred even though the former group was being treated with L-thyroxine, to correct the hypothyroidism [250]. In this previous study, the height percentile of 5-year old hypothyroid female children was 3% less than healthy females while the difference in male children was 9%. This finding therefore provides evidence that altered thyroid function in early life may have different impacts on males and females. Although the negative influence of neonatal hypothyroidism on height has been reported in many studies, whether thyroid function at birth is associated with height in early childhood has not been addressed in population-based studies. In the present study, I found that neonatal TSH levels at birth were inversely related to either height or height z-score of 5-years-old male children, but this relationship was not present in female children. It is worth noting that several studies in the literature have suggested that L-thyroxine treatment in children with congenital hypothyroidism is effective in improving height in childhood [251-253]. This finding raises the question of whether the iodine fortification implemented in Australia in 2009 has resulted in improved thyroid function of children, and hence helped to counteract any impact of reduced thyroid hormone exposure in the fetal/early neonatal period on height of children. The higher iodine intakes in children since 2009 may also have impacted on relationships between TSH at birth and metabolic markers later in childhood, and thus the results of this study. However, to date there is no study about the impact of iodine fortification thyroid function on infants and children, or their subsequent metabolic health outcomes.

Thyroid disorders in any stage of life may lead to long-lasting impaired mental and physical development [46]. Therefore, the ability to predict thyroid status in later life from thyroid function during the perinatal period could help clinicians to identify and manage individuals who may be at risk of impaired neurodevelopmental outcomes as a result of poor thyroid
function, and put in place preventative strategies. Almost all studies investigating the relationship between the thyroid status in early life and later life have focused on the differences in TSH levels between participants with thyroid dysfunction and euthyroid participants. Pakkila and colleagues have recently conducted a large prospective, population-based cohort in Finland to investigate the relationship between maternal thyroid status in early pregnancy and thyroid function in their adolescent children [254]. The outcome of this study suggested that male adolescent children of hypothyroid mothers, as reflected by TSH and fT4 levels in early pregnancy, had higher TSH levels than those of euthyroid mothers. Previously, Leonardi and colleagues also reported that individuals with higher than normal serum TSH levels at the time of neonatal screening, were at higher risk of persistent subclinical hypothyroidism during childhood compared to those with neonatal TSH levels within the normal range [255]. However, no previous studies have reported the relationship between TSH levels at birth and later in childhood. Our study presents the novel finding that perinatal TSH levels in male children were positively correlated with TSH levels at 5 years of age, suggesting that thyroid function in early life could be predictive of thyroid status later in childhood. Consequently, TSH level at 5 years of age may be confounder of the relationship between neonatal TSH level and the markers of metabolic health. In line with this suggestion, the significant relationships between neonatal TSH level and glucose level, HOMA-IR and height in male children no longer persisted after adjustment of 5-year TSH levels in the analysis.

Taken together, the findings of this study suggest that the markers of metabolic health including glucose levels, HOMA-IR, and height/height z-score as well as TSH level of male children at 5 years of age may be related to TSH levels measured immediately after birth, however whether this is a causal relationship remains to be determined. This study also highlights the need for further investigation to determine whether there is a relationship
between TSH status at birth and metabolic health in children in larger study population, and to confirm whether this is restricted to males.

6.6.2 The association between markers of current thyroid function and metabolic health at 5 years of age

The present study found no evidence to support a relationship between current thyroid function/iodine nutrition and body composition/insulin sensitivity in a healthy population of children at 5 years of age. This is different from previous findings from a number of studies involving obese and overweight children, which have consistently found a positive correlation between TSH levels and either BMI or BMI z-score [138, 238-240]. However, the majority of children in the current study were in the normal weight/BMI range, and findings in the general paediatric population have been less consistent. Ittermann and colleagues [137] reported a positive correlation between BMI and plasma TSH levels in a population of 6435 German children from 3 to 10 years of age, while a similar study conducted in 243 school age children in Spain showed no association [139]. The inconsistent outcomes may be caused by the difference in the thyroid status of population assessed; the former study was conducted in the general childhood population irrespective of their thyroid status, while the latter study only included euthyroid children.

In contrast to previous studies, which have typically included children across a wide age range, the present study only included children between 5 and 6 years of age. This is likely to be important given that children in each stage of life have different lifestyle, physical activity and dietary patterns [256-258]. There is also a considerable variation in body composition (BMI, height, weight and body fat mass) at different periods during childhood [259]. The relationship between thyroid function and weight/metabolic status would therefore also reasonably be expected to vary according to the child’s age. A study conducted in 13691 Indian children from 5 to 8 years of age showed that TSH level was positively correlated with
BMI across the entire population [136]. However, when the population was divided into age-specific groups, the relationship was not present in the 5 year old age group. In line with this, I also found no relationship between TSH level and BMI of children at 5 years of age. Therefore, further studies investigating the association between thyroid function and BMI/BMI z-score should take the age of the children at the time of assessment into account.

Compared to the number of studies involved in BMI, far fewer studies have investigated the relationship between thyroid function and body fat mass of children. No association between TSH level and body fat mass was found in a Spanish population of 234 school age children aged between 5 and 9 years [139]. The findings of the present study support these findings and suggested that that TSH level was unrelated to body fat mass of children at 5 years of age even when the sex of the children was taken into account. The current study therefore provides little evidence to support a role of thyroid function either in the aetiology of obesity or as a reliable maker of body composition in young children.

As emphasised in literature review, there is a paucity of population-based studies investigating the correlation between thyroid function and insulin resistance in children. A study in 240 obese and overweight pre-pubertal children conducted by Brufani and colleagues reported that TSH level was negatively associated with both fasting insulin level and insulin sensitivity index [138]. This finding indicated that higher TSH level was a predictor of lower insulin sensitivity. However, a population based study in children from 2-18 years of age demonstrated that TSH concentration was positively associated with fasting insulin level [260]. The same study also found a positive association between TSH level and HOMA-IR, but did not examine the relationship in specific-age groups. The present study is the first to assess the association between thyroid status and insulin sensitivity within a narrow age range. The findings of this study indicated that TSH level was not associated with either fasting insulin levels or HOMA-IR in children at 5 years of age.
Although Tg is considered as a useful biomarker of iodine deficiency, particularly in children [76, 77], no previous studies have assessed the relationship between Tg level and measures of metabolic health in children. In the present study, I found no correlation between Tg level and either BMI z-score, body fat mass or insulin resistance, in either the whole population or when relationships were assessed separately in males and females.

6.6.3 Limitations of the present study

It is important to note that the present study only included small number of participants (100 children) and information on history of thyroid disease, physical activity and dietary intake which may affect Tg/TSH level as well as body composition parameters of children, was not available for these individuals at the time of analysis. Further studies with larger sample sizes should take these assessments into account in order to provide more robust evidence about the relationship between Tg level and metabolic health of young children.

Furthermore, although BIS has many advantages i.e. non-invasive, low-cost and portable method [261], the measurement of body composition by BIS may be less reliable than other measures, e.g. air-displacement plethsmography (ADP) or dual X-ray absorptiometry (DXA), and further studies in this area which use these techniques to assess body fat mass will be of importance in further exploring the potential role of iodine nutrition in body fat deposition in children.

The addition of iodine in salt for bread making may confound the assessment about the relationship between thyroid function at birth and metabolic health at 5 years of age. This is because the iodine fortification in place since 2009 may correct the effects of iodine deficiency earlier in childhood [262], and therefore any impact of lower thyroid status earlier in development on later metabolic health.
An experimental study conducted in a rat model suggested potential impacts of DHA supplementation during the suckling period on thyroid function [263]. However, there was no difference between the control and DHA groups in either neonatal TSH or TSH/Tg concentrations at 5 years of age in this study. Furthermore, when correlations were examined separately in the DHA groups and placebo group, there were no relationships between neonatal or current thyroid function and BMI, body composition and insulin sensitivity of children at 5 years of age in either treatment group (data not shown).

### 6.6.4 Summary

The present study has provided novel insights into the relationship between neonatal thyroid function and metabolic health of young children. The results suggested that glucose level, HOMA-IR and height z-score of children at 5 years of age were related to TSH level at birth, as least in male infants. Furthermore, the study also provided evidence that current thyroid function was not associated with metabolic markers, e.g. BMI z-score, body fat mass and insulin resistance in healthy 5-year-old children, in either males or females. While these findings provide new information about the potential relationship between markers of iodine nutrition/thyroid status in early life and markers of metabolic health, it is important to acknowledge further studies with larger sample sizes are required to replicate the findings.
CHAPTER 7
CHAPTER 7  SUMMARY AND CONCLUSIONS

Iodine plays a critical role in mental and physical development during fetal life and the first two years after birth [264]. The importance of iodine in early life has been emphasised in many experimental and epidemiological studies regarding to the impacts of iodine deficient status during pregnancy on child development. Almost all of these studies have highlighted that an inadequate iodine intake by pregnant women leads to impaired neurodevelopment of their children in later life [8].

Iodine deficiency is still considered as a significant health problem worldwide [52]. The high prevalence of iodine deficiency was found not only in developing but also in developed countries, including Australia [265]. This led to calls for a program of iodine fortification in salt and other food vehicles in order to improve iodine intake of population. It is worth noting that surveillance data on which the appropriate amount of iodine fortification required to restore iodine sufficiency were conducted in children. However, vulnerable populations, including pregnant women, lactating women and infants, have higher iodine requirements than children and adults. Therefore, whether iodine fortification improved iodine status of these populations to levels indicative of iodine sufficiency remained unclear. This concern was confirmed by the inconsistent findings in many population-based studies determining maternal iodine status during pregnancy and lactation as well as iodine status of infants after the commencement of iodine fortification [116, 117, 226].

Iodine deficiency was not considered as a major public health problem in Australian in the 1990s [109]. However, an Australian National Iodine Nutrition Study conducted in 1708 school-children conducted in the early 2000s provided evidence of the resurgence of iodine deficiency in the Australian population [107]. As a result, mandatory iodine fortification in salt (25-65mg iodine/kg salt) used in bread making was introduced in Australia in late 2009.
Since then, very few studies to date have focused on the impact of mandatory iodine fortification on the iodine status of pregnant and lactating women or their infants.

The findings presented in Chapter 4 of this thesis have added to the limited data regarding to iodine status of these populations in Australia. I found that the UICs lactating women after the introduction of mandatory iodine fortification were at levels indicative of iodine sufficient status, irrespective of iodine supplements during pregnancy. However, the iodine status of pregnant women who are not consuming iodine supplements may be suboptimal as indicated by a borderline UIC level. These data provided evidence that the introduction of mandatory iodine fortification of bread flour has been successful in elevating maternal iodine intake of lactating women, but pregnant women may still require iodine supplements to gain iodine sufficient status during gestation period. Interestingly, although numerous experimental and epidemiological studies had emphasised the importance of adequate iodine intake in infancy for ensuring optimal development of the child [266], no previous studies had determined iodine status of Australian infants either pre or post fortification period. The study in Chapter 4 is the first to assess the iodine status of infants in Australia and reported that median UICs of infants post fortification in South Australia was indicative of iodine sufficient status. It is also acknowledged that the study needs to be repeated in other regions of Australia in order to determine whether the results obtained for the iodine status Australian pregnant and lactating women and infants post iodine fortification are applicable to other states and territories. Furthermore, almost all studies, including the present study, which have reported UICs of both mothers and their infants at the same time have shown the UIC to be significantly higher in the infants, compared to their mother. Therefore, future studies focussed on establishing the recommended level of iodine supplementation for breast feeding women should take both UIC and breast milk iodine concentration into account to avoid the potential for excess iodine being supplied to breast-fed infants.
As highlighted in literature, breast milk is the sole iodine source of breastfed infants. Prior to this thesis, however there had been limited attempts to assess breast milk iodine concentrations (BMICs), largely due to the lack of accurate and precise methods using for routine analysis. In Chapter 3 of this thesis, I developed and validated a new method for assessing iodine concentrations in human breast milk samples. This method was then applied to assess BMICs in breast milk samples collected both before and after the introduction of mandatory iodine fortification in Australia in Chapter 5. The primary aims of this Chapter were to determine breast milk iodine level in lactating women post fortification in South Australia and compare these to iodine level in breast milk of women from the same region of South Australia before mandatory iodine fortification was introduced. The results in this Chapter confirmed that the median BMIC in women pre fortification was at the borderline iodine deficiency, with almost 50% of the women having BMICs below this cut-off. This suggested that these women may not have been able to meet the iodine requirements of their breastfed infants. This Chapter also provided some of the first data of breast milk iodine levels post fortification, and I found that lactating mothers post fortification had median BMICs well above the cut-off for iodine deficiency, both at birth and 3-months postpartum suggesting that they would have the capacity to supply adequate to their infants. The results of the comparison of breast milk iodine level at birth in the sub-populations of non-iodine-supplemented mothers conducted in the study highlighted that iodine fortification alone was successful in improving the median iodine content in breast milk, and hence the iodine nutrition intake of infants in South Australia.

The prevalence of obesity and overweight is increasing worldwide. A huge amount of effort has gone into determining risks, in an effort to control of the current obesity endemic. Prior to the results of this thesis, very few human studies had investigated the possible relationship between iodine nutrition/thyroid function in early infancy and current thyroid function on markers of metabolic health in childhood. One previous study had reported that maternal
thyroid function in early pregnancy was significantly correlated with BMI and body fatness of children at 6 years of age [64]; and one rodent study demonstrated an adverse impact of hypothyroidism during perinatal period on insulin sensitively later life [163]. Chapter 6 of this thesis reported for the first time the relationship between neonatal thyroid function, as reflected by TSH levels at birth, and measures of metabolic health later in childhood. I found that higher TSH levels at birth was associated with higher fasting glucose levels, higher HOMA-IR (indicative of increased insulin resistance) but lower height z-score in 5-year-old male children. Interestingly, these relationships were not present in females, which highlighted a potential sex-specific relationship between perinatal thyroid function and growth and metabolic health outcomes in early childhood. The findings in Chapter 6 indicated that there were no relationships between current thyroid function and metabolic markers in children at 5-years of age. Interestingly, the results obtained in Chapter 6 also demonstrated that there was a strong positive relationship between neonatal TSH levels and TSH/Tg levels at 5 years of age in male children. However, the small sample size and lack of data on iodine intake in the children are 2 major limitations of the study. A prospective cohort study with a larger sample size is required in order to determine whether the measures of thyroid function at birth are predictive of long-term thyroid function and whether this could influence later growth /metabolic health outcomes of children. Similarly, a well-conducted and appropriately powered randomised double controlled trial is ideally needed to assess whether improving the thyroid function of hypothyroid infants via iodine supplementation could potentially improve their metabolic health outcomes in later life.

In conclusion, the studies in this thesis are the first to report the success of mandatory iodine fortification in improving the iodine status of pregnant and lactating women and their infants in South Australia. I have also demonstrated a significant increase in the iodine content in breast milk of mothers after the commencement of mandatory iodine fortification of bread flour in 2009, compared to mothers pre fortification. The BMICs measured in the women in
the post fortification sample suggest that the majority of Australian women post iodine fortification can supply adequate iodine for their breastfed infants. Furthermore, this thesis has reported in the first time that thyroid function at birth, reflected by neonatal TSH levels, was associated with glucose level, insulin resistance and height of male children in early life, but no correlations were found between current TSH/Tg levels and metabolic health markers i.e. BMI, body fat mass and insulin resistant at 5 years of age.
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