THE UNIVERSITY OF ADELAIDE

Doctor of Philosophy Thesis

The Influence of Micronutrients on Placental Development and Pregnancy Outcome

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This thesis is submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the

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Declaration of Authorship

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program.

Signed

Rebecca L. WILSON

Date

Good luck happens when opportunity and hard work collide

Abstract

The Influence of Micronutrients on Placental Development and Pregnancy Outcome

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Appropriate placental development and function is intricately associated with pregnancy success. Indeed, placental function is essential for adequate fetal development *in utero*, as well as orchestrating maternal physiological adaptations to pregnancy. However, the incidence of pregnancy complications that are characterised by placental dysfunction is increasing. Whilst a significant amount is understood about the down-stream effects of placental dysfunction, the causes are poorly understood. Inadequate maternal micronutrient status has been identified as a risk factor for adverse pregnancy outcome; even small perturbations in micronutrient homeostasis can have significant consequences for cellular and physiological pathways. The overall goal of this research was to further the understanding of how maternal micronutrient status during pregnancy influences placental development and pregnancy outcome with a focus on trace elements: zinc, copper and selenium, as well as calcium and vitamin D.

Initially, a systematic review of the literature identified possible associations between maternal zinc deficiency and hypertensive disorders of pregnancy, as well as the delivery of small-for-gestational age or low birthweight infants. Based on these results, an in-depth analysis of the effects of zinc deficiency on placental morphogenesis and maternal haemodynamic changes during pregnancy was conducted in a mouse model. This study confirmed maternal zinc status to be a key determinant of fetal growth, the effects of limited

zinc on fetal growth restriction were likely to be mediated through adverse placental development and that altered placental function was associated with improper maternal cardiovascular adaptations to pregnancy.

Next, a large-scale human epidemiological study was undertaken focusing on maternal trace elements, calcium and vitamin D status and risk of pregnancy complications in the **SC**reening f**O**r **P**regnancy **E**ndpoints (SCOPE) study. Of particular interest, this research showed a clear association between plasma copper in early pregnancy and the risk of developing any pregnancy complication with the data suggesting a possible mechanistic role for elevated free copper and inflammatory responses within the placenta. Furthermore, markers of calcium status were associated with inappropriate placental function and reduced fetal growth. This highlighted the importance of calcium in pregnancy and offered a hypothesis for the disparate published findings on the association between vitamin D deficiency and adverse pregnancy outcomes.

Finally, one possible mechanism by which micronutrient deficiencies may impact placental morphogenesis is through altered epigenetic programming. Optimisation of visualisation techniques: immunohistochemistry and immuno-fluorescence, to study DNA methylation in different cell types offers a unique avenue in which to study how micronutrients influence the epigenome. The research presented confirmed that DNA methylation markers differed between different placenta cell types and that these patterns of localization are dynamic across gestation. These changes are likely to impact placental function and offer some insight into possible changes found in the epigenome in pregnancies complicated by placental dysfunction.

In summary, this PhD research offers a unique perspective on the importance of adequate nutrition during pregnancy and a platform for future endeavours seeking to understand how micronutrients support placental development and pregnancy outcome.

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Publications Arising from this Thesis

1. **Wilson RL**, Grieger J, Bianco-Miotto T, Roberts CT. Association between maternal zinc status, dietary zinc intake and pregnancy complications: a systematic review. ***Nutrients*** 8(10), 1-28 (2016).

2. **Wilson RL**, Leemaqz S, Goh Z, McAninch D, Jankovic-Karasoulos T, Leghi G, Phillips J, Colafella K, Cuong T, O'Leary S *et al.* Zinc is a critical regulator of placental morphogenesis and maternal hemodynamics during pregnancy in mice. ***Scientific Reports*** 7(1), 1-14 (2017).

3. **Wilson RL**, Bianco-Miotto T, Leemaqz S, Grzeskowiak L, Dekker G, Roberts CT. Early pregnancy maternal trace mineral status and the association with adverse pregnancy outcome in a cohort of Australian women. ***Journal of Trace Elements in Medicine and Biology*** 46, 103-109 (2017).

4. **Wilson RL**, Leviton A, Leemaqz S, Anderson P, Grieger J, Verburg P, McCowan L, Dekker G, Bianco-Miotto T, Roberts CT. Vitamin D levels in an Australian & New Zealand cohort and the association with pregnancy outcome. ***Accepted BMC Pregnancy and Childbirth May 2018***.

5. **Wilson RL**, Leviton A, Bianco-Miotto T, Jesudason S, O'Leary S, Anderson P, Dekker G, Roberts CT. Maternal calcium homeostasis is associated with uteroplacental dysfunction in a large prospective cohort of pregnant South Australian women. ***To be submitted June 2018***.

Contents

List of Figures

List of Tables

Chapter 1

Introduction

1.1 The placenta is a key regulator of pregnancy success

Pregnancy is a dynamic state characterised by major changes to maternal physiology and anatomy in order to accommodate the growth of the fetus and placenta. Adjustments in nutrient metabolism are key to supporting not only the fetus but also the mother and deficiencies in certain micronutrients, either through reduced dietary intakes or intestinal absorption, can have dire consequences on pregnancy outcome [1]. Pregnancy complications including preeclampsia, gestational hypertension, intrauterine growth restriction (IUGR) and preterm birth affect one in five first pregnancies. These pregnancy complications are strongly associated with pathology of the placenta and predict lifelong morbidity and mortality for both mother and child [2]. Although a lot is understood about the down-stream effects of placental dysfunction, the causes of improper placental development are poorly understood.

The placenta is a transient organ, unique to pregnancy. It functions as an interface involved in the exchange of gases, nutrients and wastes between the fetus and mother [3], an endocrine organ responsible for producing numerous hormones [4] and as a barrier to protect the semi-allogeneic fetus from the maternal immune system [5]. Correct placental development is essential to placental function and pregnancy outcome. Through a combination of apoptosis [6-8], destruction of the extracellular matrix and smooth muscle de-differentiation, the maternal spiral arteries are remodelled from normally small, high-resistance, low-flow muscular arteries in the decidua into flaccid, low

resistance, high flow distended vessels capable of supplying adequate blood flow to the rapidly growing fetus (Figure 1.1). However, in pregnancies complicated by placental dysfunction, defective placentation sees absent or incomplete remodelling of the maternal spiral arteries [9].

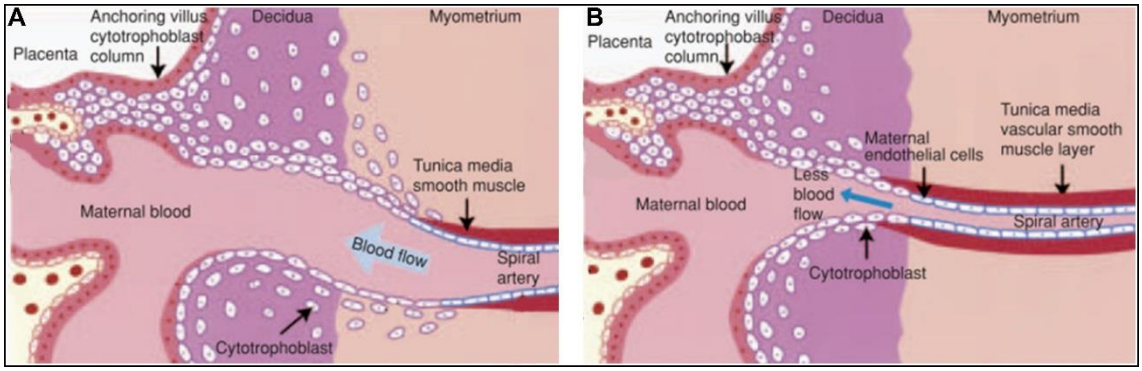

FIGURE 1.1. Adapted from Karumanchi et al., [10]. Schematic representation of spiral artery remodelling and maternal blood flow during early placentation. (A) In normal placental development, fetal cytotrophoblast cells invade the maternal spiral arteries transforming them into flaccid high-flow vessels. (B) Shallow invasion of the maternal spiral arteries by fetal cytotrophoblasts leads to defective transformation of the spiral arteries and placental ischemia which is often a characteristic of pregnancies complicated by preeclampsia and IUGR.

Abnormal, shallow placentation results in inadequate uteroplacental blood flow [11] and the onset of placental ischemia. This not only reduces the delivery of oxygen and nutrients to the fetus and hence reduces fetal growth, but also results in increased cellular and oxidative stress [12]. Excessive placental oxidative stress has since been shown to be associated with the secretion of factors into maternal circulation resulting in wide spread endothelial dysfunction and many of the pathophysiological characteristics of preeclampsia [10]. More recently, it has been hypothesised that increased syncytiotrophoblast stress may also contribute to the development of preeclampsia [13]. Syncytiotrophoblasts form the surface epithelium of the fetal placental villi and coordinate nutrient and oxygen transfer to fetal circulation as well as hormone synthesis and secretion into maternal circulation to orchestrate maternal adaptations to pregnancy. While the causes of syncytiotrophoblast stress, as well as defective placentation remain to be elucidated, it is likely to be due to a combination of

genetic and environmental stimuli in which maternal nutrition is likely to play a major role [14].

1.2 The role of micronutrients in successful pregnancies

It is important that pregnant women retain adequate levels of essential vitamins and minerals in order to support the rapid growth of the fetus. Collectively known as micronutrients, these dietary components support virtually all aspects of cellular and metabolic activity including cell proliferation, apoptosis and differentiation, as well as tissue growth and homeostasis [15]. In terms of pregnancy, there is an exhaustive amount of literature looking at associations between deficiencies in vitamin D, folate, vitamin B_{12}, iodine, iron, zinc and selenium and pregnancy complications [15]. Consequently, many of these vitamins and minerals are included in pregnancy supplements and increased dietary consumption is recommended for pregnant women [16, 17].

Severe micronutrient deficiencies are relatively uncommon in the developed world [18]. However, suboptimal micronutrient status may alter the risk of chronic disease [19] and adverse pregnancy outcomes. This is because many physiological pathways can be disrupted by even the smallest perturbations in micronutrient homeostasis which can then have more significant consequences on health. For example, the one-carbon metabolic pathway, is integral to the production of methyl-donors which are then utilised in numerous biosynthetic reactions including DNA synthesis and epigenetic modifications of the genome (Figure 1.2) [20].

The cyclic nature of the one-carbon metabolic pathway relies heavily on a number of micronutrients including folate, vitamin B_{12} and B_6, as well as choline as cofactors [20]. Furthermore, there are micronutrients like zinc which are structurally integral for enzymes active in the pathway. For example, betaine-homocysteine methyl transferse (BHMT), which catalyses the conversion of homocysteine to methionine, is a zinc metalloenzyme and activates

homocysteine using a zinc ion [21]. Deficiencies in folate [22, 23], vitamin B_{12} [24, 25] and zinc [26] have all been associated with disruption to the one-carbon network and consequently the production of methyl-donors which has knock-on consequences for growth and development in pregnancy. Indeed reduced availability of methyl-donors has been hypothesised to be a mechanism behind inadequate placental development, placental insufficiency and ultimately IUGR [27].

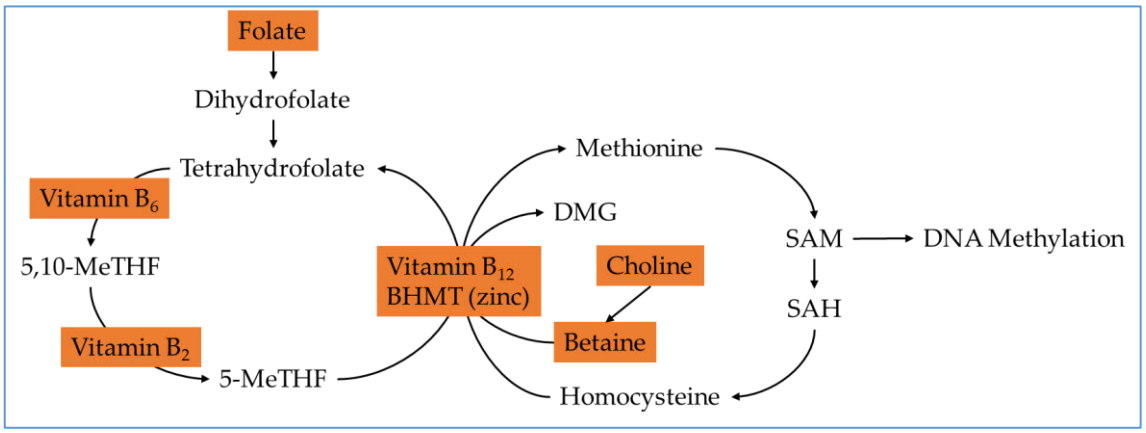

FIGURE 1.2. Adapted from Furness et al., [28]. Schematic representation of the one-carbon metabolism pathway with nutrients highlighted in orange. Vitamin B6 is a cofactor in the conversion of tetrahydrofolate to 5,10-methylene tetrahydrofolate (5,10-MeTHF) and vitamin B2 is a precursor for another cofactor which is involved in the conversion of 5,10-MeTHF to 5-methyl tetrahydrofolate (5-MeTHF). 5-MeTHF donates its methyl group to homocysteine with the aid of vitamin B12 and betaine-homocysteine methyl transferse (BHMT), a zinc metalloprotein, producing methionine which then is converted to s-adenosylmethionine (SAM), the universal methyl-donor. De-methylation of SAM forms s-adenyosylhomocysteine (SAH) and acceptance of the methyl group by DNA causes DNA methylation and epigenetic modifications. Betaine is another nutrient which serves as a methyl donor to homocysteine for the conversion to methionine.

Having identified the possible consequences of reduced zinc availability in the production of methyl-donors, I first undertook a systematic review of the literature on studies which aimed to assess the association between maternal zinc deficiency in pregnancy and placenta-related pregnancy complications. The results are presented in Chapter 2 of this thesis and indicated a possible relationship between zinc deficiency in pregnancy and the development of hypertensive disorders of pregnancy, as well as the delivery of small-for-gestational age or low birthweight infants. Given that both preeclampsia and

IUGR are associated with pathologies of the placenta, we hypothesised a mechanistic connection between reduced zinc availability, placental morphogenesis, fetal growth and the maternal adaptation to pregnancy.

Animal models of zinc deficiency have shown that zinc is a key nutrient in all facets of pregnancy from embryogenesis right through to lactation. Severe zinc restriction modelled by almost completely removing zinc from the diet (<1 mg/kg feed) 3-5 days prior to ovulation causes epigenetic defects in the oocytes and ultimately leads to decreased *in vitro* fertilisation potential [29] and reduced implantation rates [30]. The teratogenic nature of zinc deficiency is also highlighted by affects to skeletal and brain development of the offspring *in utero* [31-33], as well as to significantly higher rates of fetal loss and a reduction in fetal and placental weights [30]. However, the effects on placental development and function are largely unknown. With this in mind, I developed a mouse model to characterise the effects of marginal maternal zinc deficiency on the placenta with a predominant focus on placental morphogenesis and oxidative stress (Chapter 3). This was in combination with analysis of the effects of zinc deficiency on changes in maternal blood pressure across pregnancy.

1.3 Interactions between zinc and other plasma minerals and adverse pregnancy outcomes

Given the diverse range of biological functions for which zinc is required, it is not surprising that inadequate intake of zinc in the maternal diet is associated with adverse pregnancy outcomes and poor infant development. Although severe zinc deficiency is relatively rare, mild to moderate zinc deficiency is estimated to be common throughout the world [34] and overlaps with deficiencies in a number of other micronutrients. Multiple micronutrient deficiencies exist because of interactions which can affect both absorption and bioavailability and are highly dependent on the relative concentrations of the nutrients [35]. Micronutrient interactions can be synergistic for example vitamin

C and iron [36], or antagonistic as in the case of vitamin A and zinc [37]. Therefore, in situations where dietary nutritional intakes are poor and in combination with micronutrient interactions, multiple micronutrient deficiencies exist.

Minerals and trace elements like zinc, copper, iron and calcium share similar chemical properties and therefore can compete for similar uptake mechanisms and proteins [35]. Indeed, there is accumulating evidence which shows a clear connection between zinc, iron, copper and calcium metabolism. For example, severe copper deficiency results in anaemia due to changes in iron metabolism [38]. This is thought to be driven by the multi-copper protein ceruloplasmin which is capable of stimulating iron efflux from the liver [39]. Iron supplementation has been shown to reduce circulating zinc concentrations and vice-versa [40] while there is some evidence to suggest calcium supplementation also has a negative effect on both zinc and iron absorption [41, 42].

In terms of placental transfer of nutrients to the fetus, McArdle *et al*., showed in a series of publications, the essential interactions between copper and iron in the placenta [43]. Furthermore, my own research using our mouse model also showed a possible effect of maternal zinc deficiency on iron transport to the fetus. Yet, there are very few human population studies which have undertaken an assessment of multiple micronutrient deficiencies in pregnancy and their effects on pregnancy outcome [44-47]. With this in mind, I utilised the extensive **SC**reening f**O**r **P**regnancy Endpoints (SCOPE) database and biobank to look at circulating plasma trace elements in early pregnancy and the associations with adverse pregnancy outcomes with particular focus on: zinc, selenium, iron and copper (Chapter 4). A very clear association between high plasma copper in early pregnancy and the risk of developing any pregnancy complication was found with the data suggesting a possible mechanistic role for elevated free copper and inflammatory responses within the placenta. Associations between plasma zinc and selenium and an increased risk of any pregnancy complication were also

observed and has now provided the basis for on-going laboratory experiments into how these mineral profiles affect placental cell function.

1.4 The synergistic relationship between vitamin D and calcium in pregnancy

The inorganic nature of minerals means they retain their chemical structure and can passively move throughout the body and into cells. Vitamins on the other hand are organic compounds, harder to absorb from the diet and are often utilised in metabolic pathways to inhibit or promote cell function [48]. Furthermore, these metabolic pathways can also interact with one another. For example, the one-carbon metabolic pathway which utilises folate, creates the methyl-donors which are required for vitamin D metabolism [27]. Deficiencies in folate can therefore affect vitamin D metabolism resulting in functional vitamin D deficiency. Classically, vitamin D has been associated with calcium homeostasis and bone mineralisation. However, vitamin D plays important roles in other important cellular functions such as regulating immune function, modulating cell proliferation and differentiation and disease prevention [49]. Furthermore, vitamin D has the ability to regulate placental physiology. By the third trimester, serum levels of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active form of the vitamin, increase as much as two-fold when compared to non-pregnant or post-partum levels [50-52]. Indicating the importance of high levels of vitamin D within the mother particularly in late gestation.

The consequences of vitamin D deficiency in pregnancy have been extensively studied and numerous investigations have been conducted on whether vitamin D supplementation can prevent pregnancy complications [53]. However, a recent Cochrane review of vitamin D supplementation and the prevention of a number of adverse pregnancy outcomes, including preeclampsia and fetal growth restriction, concluded that there was no significant reduction in the rates of these complications with vitamin D supplementation [54]. My own

study found higher levels of vitamin D at 15±1 weeks' gestation were associated with a protective effect on developing gestational diabetes mellitus and is presented in Chapter 5. However, there was no significant associations between vitamin D deficiency and other pregnancy complications including preeclampsia and preterm birth. Given the heterogeneous nature of the literature that reports associations with vitamin D deficiency and pregnancy outcome [55], this was not overly surprising.

Vitamin D is necessary to maintain calcium homeostasis and along with parathyroid hormone functions to increase plasma calcium levels [56] through increasing intestinal absorption of calcium, increasing kidney reabsorption and mobilising calcium from bone [57]. Calcium availability during pregnancy is extremely important for fetal bone growth and vitamin D deficiency sees increased demineralisation of maternal bone in order to maintain adequate transfer of calcium to the fetus [58]. Yet many studies on vitamin D in pregnancy have failed to consider calcium status and may explain the inconsistencies in the literature regarding vitamin D deficiency and adverse pregnancy outcomes. Indeed, Chapter 6 of this thesis shows that while vitamin D deficiency was not associated with reduced fetal growth, higher urinary excretion of calcium, a marker for higher calcium status, was protective against uteroplacental dysfunction and SGA. However, while there is a vast amount of literature showing associations between micronutrients and pregnancy complications, the mechanisms are poorly understood. Environmental factors such as maternal diet are known to impact the epigenome and in particular, DNA methylation, has often been shown to be associated with environmental exposures. Thus, it is possible that mechanistically, micronutrient deficiencies may result in altered epigenetic programming of organs like the placenta during pregnancy that affect pregnancy outcome.

1.5 Methylation status of the placenta – the next frontier

Epigenetics refers to the study of molecular modifications to chromatin structure and accessibility which alters gene expression without altering the underlying DNA sequence. Such modifications provide a mechanistic link between nutrition and disease [20]. The most widely studied modification is DNA methylation which as previously described, relies on the one-carbon metabolic pathway in order to generate 5-methylcytosine and maintain methylation status. Micronutrient intake is imperative to this process in order to maintain the cyclical pattern of one-carbon metabolism and the study of nutri-epigenomics is emerging as a new field of biological research, particularly in the context of the developmental origins of health and disease (DoHAD) hypothesis [59]. This is because DNA methylation patterns are prone to change, particularly during reprogramming events like gametogenesis and early embryonic development [60].

Key to determining the importance of epigenetics in placental dysfunction is first understanding how modifications influence normal placental development. In terms of placental development, the placental genome is hypomethylated compared to other healthy tissues [61, 62]. It is unclear as to why this is however, given the placenta comprises numerous different cell types, studying DNA methylation in this organ is complicated [63]. To address this, I aimed to characterise global DNA methylation patterns in placental tissue across gestation using immunohistochemistry and immunofluorescence (Chapter 7). This was in order to visually determine if DNA methylation differed depending on the cell type within the placenta. Furthermore, I wanted to characterise how levels of 5-hydroxy-methylcytosine, a derivative of 5-methylcytosine which is produced during demethylation [64] differed in the different placenta cell types. The findings in this study showed levels of both 5-methylcytosine and 5-hydroxymethylcytosine changed across gestation as well as between different

trophoblast cell types, providing solid evidence that epigenetic profiles can differ significantly between difference cell types within a complex tissue. Such information and optimised protocols can now be used in future experiments determining how these different methylation modifications may be altered in response to environmental exposures like maternal diet and what are the consequences for placenta cell function.

1.6 Summary

The research presented in this PhD focuses on a number of key micronutrients, their interactions and pregnancy outcome offering a unique perspective into the importance of adequate nutrition in pregnancy. Through the combination of an animal model and *ex vivo* and *in vitro* human analyses, it provides novel insights into both the mechanistic effects of micronutrient deficiencies to fetal growth *in utero*, as well as a translational perspective. Furthermore, it provides a platform for future nutritional research to broaden endeavours from a focus on one specific nutrient to multiple micronutrients which, although increasing the complexity, ultimately enhances our understanding for future recommendations for pregnant women.

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Chapter 2

Association between Maternal Zinc Status, Dietary Zinc Intake and Pregnancy Complications: A Systematic Review

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ABSTRACT

Adequate zinc stores in the body are extremely important during periods of accelerated growth. However, zinc deficiency is common in developing countries and low maternal circulating zinc concentrations have previously been associated with pregnancy complications. We reviewed current literature assessing circulating zinc and dietary zinc intake during pregnancy and the associations with preeclampsia (PE); spontaneous preterm birth (sPTB); low birthweight (LBW); and gestational diabetes (GDM). Searches of MEDLINE; CINAHL and Scopus databases identified 639 articles and 64 studies were reviewed. In 10 out of 16 studies a difference was reported with respect to circulating zinc between women who gave birth to a LBW infant (≤2500 g) and those who gave birth to an infant of adequate weight (>2500 g), particularly in populations where inadequate zinc intake is prevalent. In 16 of our 33 studies an association was found between hypertensive disorders of pregnancy and circulating zinc; particularly in women with severe PE (blood pressure ≥160/110 mmHg). No association between maternal zinc

status and sPTB or GDM was seen; however; direct comparisons between the studies was difficult. Furthermore; only a small number of studies were based on women from populations where there is a high risk of zinc deficiency. Therefore; the link between maternal zinc status and pregnancy success in these populations cannot be established. Future studies should focus on those vulnerable to zinc deficiency and include dietary zinc intake as a measure of zinc status.

2.1 Introduction

Adequate maternal nutrition, particularly before and during pregnancy, is imperative to the health of both the mother and child [1,2]. Poor nutrition in pregnancy may lead to inappropriate nutrient partitioning between the mother and fetus, which can be deleterious to the health of both [3]. Each year, 3.5 million deaths in women and children are attributed to undernutrition [4]. Zinc deficiency is predicted to be responsible for 1% of all deaths globally and 4.4% of deaths in children aged 6 months to 5 years [5]. The World Health Organization (WHO) prioritized minimizing zinc deficiency in developing nations as part of the Millennium Development Goal 1: to eradicate extreme poverty and hunger [6]. Therefore, understanding the effects of zinc deficiency on pregnancy and fetal growth is very important.

Zinc is an essential component of over 1000 proteins including antioxidant enzymes, metalloenzymes, zinc-binding factors and zinc transporters. These are required for a variety of biological processes including carbohydrate and protein metabolism, DNA and RNA synthesis, cellular replication and differentiation, and hormone regulation [7–10]. The importance of zinc to the growth of the fetus is demonstrated by the active transport of zinc across the placenta into the fetal circulation resulting in higher cord blood concentrations compared to those in the maternal circulation [11–14]. Rodent models of severe maternal zinc deficiency show increased rates of fetal loss and congenital malformations in the surviving fetuses [15] as well as reduced fetal growth [16–18], lower implantation

rates and impaired placental growth [19], all highlighting the teratogenic effects of zinc deficiency in pregnancy.

Diet is the main factor that determines zinc status [20]. In the United States and Australia, an additional 2–4 mg zinc per day is recommended for pregnant women compared to non-pregnant women [21,22]. It is widely acknowledged that many pregnant women do not meet this recommendation [23–25], particularly in developing countries where diets are often plant-based. Grains and legumes contain a significant amount of phytic acid and phytate binding of zinc limits its absorption in the small intestine, contributing to zinc deficiency [22]. Estimates based on bioavailability of zinc, physiological requirements and predicted zinc absorption suggest the prevalence of zinc deficiency to range from 4% (European countries including the United Kingdom, Sweden, Germany and France) to 73% in Bangladesh, India and Nepal [26]. A more recent evaluation, based on similar estimates, also predicted inadequate zinc intakes in over 25% in populations in Southeast Asia and Africa [27].

A recent Cochrane review assessed the effects of zinc supplementation versus no supplementation (with or without placebo) on the success of pregnancy in 21 randomized controlled trials (RCTs) [28]. It was concluded that zinc supplementation reduced the risk of spontaneous preterm birth (sPTB) by 14% (RR: 0.86, 95% CI: 0.76–0.97; 16 RCTs) but there was no effect on other outcomes such as stillbirth/neonatal death, birthweight and pregnancy-induced hypertension [28]. However, this review did not include the effects of zinc supplementation on reducing the risk of gestational diabetes (GDM) and analysis of maternal circulating zinc concentrations provides evidence that low maternal zinc may be associated with GDM, as well as preeclampsia (PE), gestational hypertension (GH), sPTB and infant birthweight [24,29]. The association between serum zinc and PE has been reviewed recently [30] but there has been no extensive review that has assessed maternal zinc concentrations with respect to a range of pregnancy complications. Here, we review the current literature based

on observational studies assessing the association between maternal zinc status and a number of pregnancy complications in order to determine whether maternal circulating zinc or dietary zinc intake are important factors associated with pregnancy outcome.

2.2 Methods

2.2.1 Eligibility Criteria

Studies included human prospective cohorts, case-control, longitudinal and cross-sectional studies assessing maternal circulating zinc concentrations and pregnancy complications including PE, eclampsia, GH, GDM, small for gestational age (SGA), intrauterine growth restriction (IUGR; <10th percentile), low-birthweight (LBW; ≤2500 g) and sPTB. Only studies that measured maternal circulating zinc during pregnancy or at delivery and/or dietary zinc intake at these times were included. Studies that assessed zinc concentrations in placenta, amniotic fluid, in offspring (post-natally), cord blood only and breast milk were excluded. There were no restrictions imposed on age of women included in the studies or on any other population characteristic such as race or body mass index (BMI). Given the heterogeneity of the observational strategies, a meta-analysis was not possible.

2.2.2 Information Sources and Search

The search strategy and procedure was guided by the PRISMA statement [31]. Potential studies were located through electronic databases [Ovid Medline (1946–present), CINAHL (1937–present) and Scopus (1995–present)], as well as manual searches of references in review articles and relevant articles known by the authors. Limits included full text articles written in English and published in academic journals. The last search was performed on August 25, 2016. Search terms and MeSH headings in the title, abstract, and index terms, were initially identified in Medline and subsequent key words were used for the remaining

databases (Appendix A). Briefly, the search included the following: zinc; dietary zinc; zinc intake; plasma zinc; serum zinc; preeclampsia; eclampsia; gestational hypertension; gestational diabetes mellitus; fetal macrosomia; small for gestational age; intrauterine growth restriction; low birthweight; preterm birth.

2.2.3 Data Collection

An independent search of the literature was performed in April 2015 and again in August 2016. Titles and abstracts were examined independently by two of the authors who documented reasons for excluding full text articles. Any differences between the two reviewers were clarified; a third reviewer resolved any disagreements. If an article appeared in duplicate from two or three of the databases, only the search containing the most relevant and useful information was included. For each eligible study, the following data was extracted: author, year and country of publication; inclusion/exclusion criteria; sample size; zinc measure including sample type, collection time during pregnancy and method of analysis and pregnancy outcome. Most studies did not report on exclusion/inclusion criteria; these were therefore not included in the results table. Values determining zinc status were all converted to μg/L for easier comparisons between studies (Appendix B).

2.3 Results

Figure 2.1 outlines the literature search and selection of studies. We identified 635 citations after searching Medline (OVID), CINAHL and Scopus databases. A further seven were added by the authors. After screening the title and abstract, 116 full text papers were read. Of these, 67 studies met the inclusion criteria, including 29 on SGA/LBW (Table 2.1), 34 on hypertensive disorders of pregnancy (Table 2.2), 11 on sPTB (Table 2.3) and 9 on GDM (Table 2.4). Eleven studies assessed multiple pregnancy outcomes and are included in the relevant

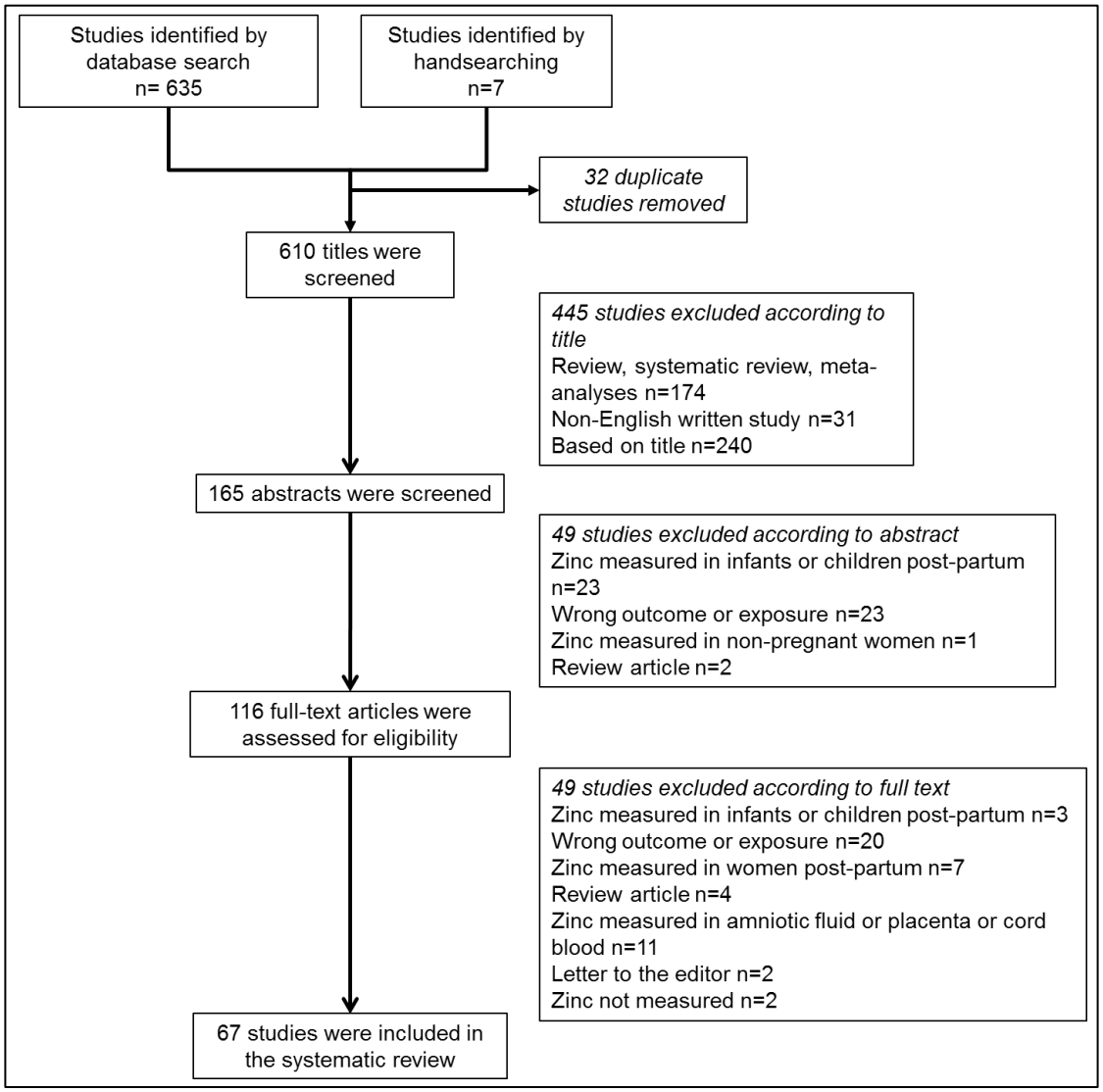

FIGURE 2.3. Flow diagram of the search strategy used in this review including the relevant number of papers at each point.

pregnancy outcome tables. Table 2.5 summarizes all included studies and whether there was a positive, negative or no association between zinc status and the pregnancy complication. The included studies were tabulated based on those that measured dietary zinc intake, then those that measured serum/plasma zinc. Globally, the average percentage of people affected by inadequate zinc intake is estimated to be 17.3% [27]. As dietary consumption of zinc is most influential on zinc status, studies that measured circulating zinc were further categorized based on whether they sampled from countries where inadequate zinc intake has been predicted to affect <17% or ≥17% of the population. We did not limit the studies to a specific period during gestation when zinc was measured and this

information was not provided in eight studies [32–39]. However, zinc concentrations decline across gestation due to a combination of factors including hemodilution and increased fetal demand [40,41] and this made direct comparison of the studies difficult.

2.3.1 Infant Birthweight

There were four studies that assessed dietary zinc intake and birthweight with three based on women from countries where the estimated prevalence of low dietary zinc intake is <17% (Table 2.1) [42–45]. Lower zinc intake was reported in women from the United Kingdom (UK) who gave birth to an SGA infant compared to those who gave birth to an appropriate-for-gestational-age (AGA) infant (SGA: mean (SEM) 11.3 (0.5) vs. AGA 13.0 (0.6) mg/day, $p < 0.05$) [45]. This was similar to another study of Indian women that reported lower zinc intakes in women who delivered an infant weighing <2500 g compared to those who delivered an infant that was ≥2500 g [42]. Logistic regression analysis in one study from the United States reported daily zinc intake <6 mg/day to be associated with a 2-fold increase in the risk of delivering a LBW infant (aOR: 2.01, 95% CI: 1.11–3.66) [44] although dietary zinc intakes <median were not found to be associated with LBW in another study of American women (OR: 1.4, 95% CI: 0.9–2.1) [43]. While both studies used a 24 h recall questionnaire to determine zinc intakes, there were differences in ethnicity of the women studied as Neggers et al., [43] predominantly studied African-American women as opposed to Scholl et al. who studied Caucasian women [44].

Twelve studies were identified that measured maternal circulating zinc in countries where inadequate zinc intake is predicted to be <17%, and looked at the association with birthweight (Table 2.1) [38,46–56]. Only one study, based on 3817 women in China, reported a 3.4-fold increase in the risk of delivering a LBW infant with serum zinc <560 μg/L (adjusted RR: 3.41, 95% CI: 1.97, 5.91) [56]. This is in contrary to two studies that reported significantly higher zinc concentrations

in women with an SGA infant in the third trimester [47,55]. However, these findings were based on a relatively small number of women: 40–51 pregnant women including 10–16 women with SGA. Conversely, another study, which followed 476 women of whom 39 gave birth to an SGA infant, found the incidence of LBW to be 8 times higher in women with serum zinc in the lowest quartile (457.5–797.4 μg/L) compared to the highest (1039.2–1660.1 μg/L) (8.2, 95% CI: 2.4–27.5) [52]. The remaining eight studies found no differences in maternal zinc concentrations between women with a SGA infant and those with an uncomplicated pregnancy. However, one study found a positive correlation between maternal zinc status and birthweight ($r = 0.632$, $p < 0.001$) [50].

The association between maternal circulating zinc and birthweight was assessed in 14 studies based on women where inadequate dietary zinc intake was predicted to affect ≥17% of the population [57–69], of which 7 reported a significant association (Table 2.1) [57,59–61,66–68]. All three of the studies based on women from Africa reported serum/plasma zinc on average 72–333 μg/L lower in women who gave birth to a LBW infant compared to those who gave birth to an appropriate weight infant [57,59,67]. In another study, the risk of delivering a LBW infant was also reported to be 3-fold greater in women with serum zinc levels ≤392.2 μg/L compared to those with levels above this figure (3.07, 95% CI: 1.07–8.97) [67]. Conversely, two other studies reported serum zinc to be 40–172 μg/L higher in women who gave birth to a LBW infant compared to those who gave birth to an appropriate weight infant [66,60]. A further four studies, also based on women from India, reported no association between circulating zinc levels and birthweight [62–64,69] and this was also reported in two studies of Turkish women [58,65]. However, univariate analysis and small sample size in these studies may not provide an accurate assessment of the effects of maternal circulating zinc and birthweight.

TABLE 2.1. Included studies assessing maternal zinc status and birthweight.

Author, Country	Sample Size	Zinc Measure (1) Sample Type (2) Time at Which Gestation Diet was Assessed or Sample Collected (3) Method of Analysis	Outcome of the Study
[42] Simmer, United Kingdom [a]	28 SGA 29 uncomplicated	Dietary zinc intake Third trimester of pregnancy 7 day dietary recall	↓ mean (SEM) dietary intake in the SGA mothers compared to the women with uncomplicated pregnancies. **SGA: 11.3 (0.5) vs. uncomplicated: 13.0 (0.6) mg/day, $p < 0.05$**
[43] Negandhi, India [b]	144 LBW 240 uncomplicated	Dietary zinc intake 26–30 weeks 24 h dietary recall	↓ mean dietary zinc intake in women with a LBW infant compared to those with an uncomplicated pregnancy. **LBW: 5.39 mg/day vs. uncomplicated 6.77 mg/day, $p < 0.001$**
[44] Scholl, United States [c]	115 with zinc intake ≤6 mg/day 699 with zinc intake >6 mg/day	Dietary zinc intake 28 and 36 weeks 24 h dietary recall	2-fold ↓ risk of delivering a LBW infant with dietary zinc intake >6 mg/day. **OR: 2.01, 95% CI: 1.11–3.66**
[45] Neggers, United States [d]	180 LBW 1218 uncomplicated	Dietary zinc intake 18 and 30 weeks 24 h dietary recall using the nutrient data base developed by the University of Minnesota	NS association between low dietary zinc intake (less than median) and risk of LBW. OR: 1.4, 95% CI: 0.9–2.1
Inadequate dietary zinc intake estimated to affect <17% of the studied population			
[46] Wang, China [b]	247 with serum zinc <560 μg/L 2940 with serum zinc ≥560 μg/L	Fasting serum zinc Across gestation Flame AAS	↑ incidence of LBW in the mothers with serum zinc <560 μg/L compared to those with serum zinc ≥560 μg/L. **Adjusted RR: 3.41, 95% CI: 1.97, 5.91**
[47] Voss Jepsen, Denmark [a]	10 SGA 30 uncomplicated	Heparin plasma zinc Collected at 35–41 weeks AAS	↑ mean (SD) plasma zinc between SGA mothers and those with uncomplicated pregnancies. **SGA: 732 (85) vs. uncomplicated: 654 (78) μg/L, $p = 0.03$**

[48] Borella, Italy [a]	16 SGA 35 uncomplicated	Heparin plasma zinc Collected in the third trimester Flame AAS	↑ mean (SD) plasma zinc in SGA women compared to women with uncomplicated pregnancies. **SGA: 685.6 (119.6) vs. uncomplicated: 627.5 (150) μg/L, $p < 0.001$**
[49] Neggers, USA [e]	39 LBW 437 uncomplicated	Serum zinc Collected across gestation Flame AAS	8-fold ↑ prevalence of LBW with serum zinc in the lowest quartile (457.5–797.4 μg/L) compared to the highest (1039.2–1660.1 μg/L). **OR: 8.2, 95% CI:2.4–27.5**
[50] Bro, Denmark [a]	47 SGA and 34 preterm 220 uncomplicated	Serum zinc Collected at delivery Flame AAS	NS mean (SD) serum zinc levels between SGA and women with uncomplicated pregnancies. SGA: 764.7 (119.6) vs. uncomplicated: 679.7 (98) μg/L
[38] Hyvonen-Dabek, Finland [f]	4 SGA 10 uncomplicated	Serum zinc Collection time not specified Particle induced X-ray emission	NS mean (SD) serum zinc in SGA women compared to those with uncomplicated pregnancies. SGA: 1270 (320) vs. uncomplicated: 1150 (220) μg/L
[51] Mistry, UK [a*]	19 SGA 107 uncomplicated	Heparin plasma zinc Collected at 28–32 weeks Inductively coupled plasma mass spectrometry	NS in mean (95% CI) plasma zinc between SGA women and those with uncomplicated pregnancies. SGA: 708.1 (510.4–905.8) vs. uncomplicated: 634.4 (580.5–688.2) μg/L
[52] Tamura, USA [g]	80 SGA 80 uncomplicated	Serum zinc Collected at 18 weeks and 30 weeks Flame AAS	NS in mean (SD) plasma zinc between SGA and women with uncomplicated pregnancies at 18 weeks. SGA: 627 (118) vs. uncomplicated: 667 (98) μg/L NS in mean (SD) plasma zinc between SGA and women with uncomplicated pregnancies at 30 weeks. SGA: 562 (92) vs. uncomplicated: 575 (92) μg/L
[53] Tamura, USA [a]	139 SGA 2038 uncomplicated	Non-fasting heparin plasma zinc Collected at first prenatal visit (6 to 34 weeks) Flame AAS	NS in the prevalence (n (%)) of SGA measured between the lowest quartile and upper 3 quartiles of zinc. Highest: 103 (4.4) vs. lowest: 36 (4.8)
[54] Ghosh, China [a]	22 SGA 38 uncomplicated	Serum zinc Collected within 24 h of delivery AAS	NS in mean (SD) serum zinc levels between SGA and women with uncomplicated pregnancies. SGA: 508.1 (185.9) vs. uncomplicated: 542.3 (162.8) μg/L

[55] Cherry, USA [b]	29 LBW 230 uncomplicated	Heparin plasma zinc Collected across gestation AAS	NS mean (SEM) plasma zinc in mothers with a LBW infant compared to mothers with uncomplicated pregnancies. LBW: 604.9 (22.4) vs. uncomplicated: 577.2 (7.7) μg/L
[56] Bogden, USA [h]	22 LBW 50 uncomplicated	EDTA plasma zinc Collected at delivery Flame AAS	NS mean (SEM) plasma zinc in women with a LBW infant compared to women with uncomplicated pregnancies. LBW: 640 (20) vs. uncomplicated: 620 (20) μg/L
Inadequate dietary zinc intake estimated to affect ≥17% of the studied population			
[57] Atinmo, Nigeria [h]	20 LBW 30 uncomplicated	Heparin plasma zinc Collected at delivery AAS	↓ mean (SD) serum zinc in women with a LBW infant compared to those with uncomplicated pregnancies. **LBW: 663.1 (144.6) vs. uncomplicated: 731.5 (235.6) μg/L, $p < 0.05$**
[58] Abass, Sudan [b]	50 LBW 50 uncomplicated	Serum zinc AAS Atomic absorption spectrometry	↓ median (IQR) serum zinc in women with a LBW infant compared to those with uncomplicated pregnancies. **LBW: 629 (363–968) vs. uncomplicated 962 (846–1257) μg/L, $p < 0.001$**
[59] Rwebembera, Tanzania [c]	81 LBW 84 uncomplicated	EDTA plasma zinc Collected at delivery Flame AAS	3-fold ↓ risk of delivering a LBW infant with serum zinc ≥ 392.2 μg/L **OR: 3.07, 95% CI: 1.07–8.97**
[60] Bahl, India [c]	19 LBW 56 uncomplicated	Serum zinc Collected at delivery Flame AAS	↓ mean (SD) serum zinc in women with a LBW infant compared to those with uncomplicated pregnancies. **LBW: 553 (43) vs. 692 (95) μg/L, $p < 0.001$**
[61] Singh, India [e]	47 LBW 45 uncomplicated	Serum zinc Collected at delivery AAS	↓ mean (SD) serum zinc in women with a LBW infant compared to those with uncomplicated pregnancies. **LBW: 623 (330) vs. uncomplicated: 895 (514) μg/L, $p < 0.001$**
[62] Prema, India [e]	23 LBW 208 uncomplicated	Serum zinc Collected at delivery between 9–11.30 a.m. Flame AAS	↑ mean (SD) serum zinc in mothers with a LBW infant compared to mothers with an uncomplicated pregnancy. **LBW: 660 (162) vs. uncomplicated: 620 (146) μg/L, $p < 0.01$**
[63] Badakhsh, Iran [b]	30 LBW 110 uncomplicated	Serum zinc Collected at delivery AAS	↑ mean (SD) serum zinc in mothers with a LBW infant compared to mothers with an uncomplicated pregnancy. **LBW: 686.2 (204.8) vs. uncomplicated: 514.3 (138.8) μg/L, $p < 0.001$**

[64] Goel, India [a]	20 LBW 25 uncomplicated	Heparin plasma zinc Collected at delivery AAS	NS mean (SD) plasma zinc in women with a LBW infant compared to those with an uncomplicated pregnancy. LBW: 726 (61) vs. uncomplicated: 763 (56) μg/L
[65] Srivastava, India [b]	26 LBW 25 uncomplicated	Heparin plasma zinc Collected at delivery Flame AAS	NS mean (SD) plasma zinc between mothers with a LBW infant and mothers with uncomplicated pregnancies. LBW: 6470 (4860) vs. uncomplicated: 5670 (2490) μg/L
[66] Jeswani, India [a]	10 SGA 25 uncomplicated	Serum zinc Collected at 28–40 weeks AAS	NS mean (SD) serum zinc in SGA women compared to those with uncomplicated pregnancies. SGA: 938 (76.2) vs. uncomplicated: 962.8 (194.8) μg/L
[67] George, India [a]	65 SGA 51 uncomplicated	Heparin plasma zinc Collected before labor between 8–10 a.m. AAS	NS in mean (SD) plasma zinc between SGA and women with uncomplicated pregnancies. SGA: 675 (90) vs. uncomplicated: 706.7 (139) μg/L
[68] Akman, Turkey [f]	22 SGA 34 uncomplicated	Serum zinc Collected at delivery AAS	NS mean (SD) serum zinc between SGA women and women with uncomplicated pregnancies. SGA: 1218 (543) vs. uncomplicated 1038 (343) μg/L
[69] Ozdemir, Turkey [b]	16 LBW 59 uncomplicated	Serum zinc Collected at 38–42 weeks Flame AAS	NS mean (SD) serum zinc between mothers with a LBW infant and mothers with uncomplicated pregnancies. Data represented on graphs

[a] SGA defined as <10th percentile, [b] LBW defined as <2500 g, [c] LBW defined as ≤2000 g, [d] LBW defined as <2750 g, [e] LBW defined as <2000, [f] SGA not defined, [a*] SGA defined as <10th percentile based on customised centiles, [g] SGA defined as <15th percentile, [h] LBW defined as ≤2500 g. **Bold print signifies results that were significantly different**. Abbreviations: AAS: atomic absorption spectrometry; CI: confidence interval; IQR: interquartile range; LBW: low birth weight; NS: non-significant; OR: odds ratio; SD: standard deviation; SEM: standard error of the mean; SGA: small for gestational age.

2.3.2 Hypertensive Disorders of Pregnancy

Only one study assessed dietary zinc intake and the association with hypertensive disorders (Table 2.2) and found no significant differences in dietary zinc intake between 13 women who developed a hypertensive disorder in pregnancy and 44 whose pregnancies remained uncomplicated [70].

Thirteen studies analyzed serum/plasma zinc in women who developed a hypertensive disorder of pregnancy in women residing in countries where inadequate zinc intake is estimated to be low (<17%) (Table 2.2). Three studies reported mean serum/plasma zinc to be on average 120–1200 μg/L lower in women who developed PE compared to women whose pregnancies remained uncomplicated [49,71,72] and included one study that reported a reduction in risk of PE with serum levels above 1360 μg/L after adjusting for maternal age, height and weight before pregnancy (aOR: 0.005, 95% CI: 0.001–0.07) [71]. A further two studies reported circulating zinc to be lower in women who developed severe PE (blood pressure BP ≥ 160/110) compared to women whose pregnancies remained uncomplicated [73,74]. The remaining eight studies, whose sample sizes ranged from 10–271 women with PE/GH and 10–2038 women with an uncomplicated pregnancy, reported no difference in maternal zinc status between women with a hypertensive disorder of pregnancy and those without [38,47,54,75–79].

There were twenty studies that analyzed circulating zinc in women with a hypertensive disorder of pregnancy in populations where inadequate zinc intake is estimated to be ≥17% (Table 2.2) [32–34,36,37,39,80–93]. Ten studies reported mean serum/plasma zinc to be significantly lower in women who developed PE and/or GH [33,34,36,37,39,80–83,93] however, one reported plasma zinc to be higher in women with PE compared to those whose pregnancies remained uncomplicated when measured during the latent phase of labor; with (PE mean (SD): 15.53 (4.92) vs. uncomplicated: 11.93 (3.11) μg/g protein, $p = 0.003$) [89]. These

studies also included three which found circulating zinc to be 80–260 μg/L lower in women who developed severe PE when compared to women whose pregnancies remained uncomplicated [39,37,83]. A further nine studies reported no difference in circulating zinc between women with PE/GH and those whose pregnancies remained uncomplicated.

2.3.3 Spontaneous Preterm Birth

The literature search identified four studies which measured dietary zinc intakes during pregnancy and sPTB with varying conclusions (Table 2.3) [94,95,43,44]. Two of these studies, which analyzed 5738 and 818 women respectively, determined that low zinc intake (≤6 mg/day which is ≤54% of the recommended 11 mg/day [21]) was associated with a more than 2-fold increase in the risk of delivering preterm (aOR: 2.3, 95% CI: 1.2–4.5 and aOR: 1.85 95% CI: 1.09–3.12, respectively), after adjusting for factors such as ethnicity, pre-pregnancy BMI, smoking, alcohol and multivitamin consumption [102,44]. If delivery date was calculated by last menstrual period (LMP), zinc intake below 9 mg/day was associated with a 2.75-fold increased risk in delivering <32 weeks gestation (aOR: 2.75, 95% CI 1.31–5.77) [44]. However, another study reported no association between low dietary zinc intake (less than the median) and the risk of sPTB (OR: 1.1, 95% CI: 0.7–1.7) [43] but mean zinc intake of the women in this study was 14 mg/day, higher than the recommended 11 mg/day, indicating that low zinc intake was not prevalent within this studied population.

When separated based on estimates of inadequate zinc intake, there were three studies which assessed whether there was an association between circulating zinc and sPTB in low-risk populations (Table 2.3). While two showed no significant difference between serum/plasma zinc levels during gestation in women who gave birth preterm and those who gave birth at term [48,54], one study which recruited 3081 women in China found a 2.4-fold increase risk of PTB with serum levels <767 μg/L (aOR: 2.41, 95% CI: 1.57, 3.70) [96].

TABLE 2.2. Included studies assessing maternal zinc status and hypertensive disorders of pregnancy.

Author, Country	Sample Size	Zinc Measure (1) Sample Type (2) Time at Which Gestation Diet was Assessed or Sample Collected (3) Method of Analysis	Outcome of the Study
[70] Tande, United States [a,b]	13 hypertensive (11 PE + 2 GH) 44 uncomplicated	Dietary and supplement intake First 3 months of pregnancy Harvard food frequency questionnaire	NS in mean (SEM) dietary zinc intake between those with and without gestational hypertension. Hypertensive: 16.9 (1.56) vs. uncomplicated: 15.4 (1.03) mg/day
Inadequate dietary zinc intake estimated to affect <17% of the studied population			
[71] Lazebnik, United States [a,b]	17 PE and 14 hypertensive 31 uncomplicated	Plasma zinc Collected within 1 h of delivery AAS	↓ mean (SD) serum zinc in women with PE when compared to women with uncomplicated pregnancies. **PE: 420 (100) vs. uncomplicated: 520 (130) μg/L, $p < 0.05$** NS mean (SD) plasma zinc in hypertensive women compared to those whose pregnancies remained uncomplicated. Hypertensive: 530 (110) vs. uncomplicated: 520 (110) μg/L
[55] Cherry, United States [a]	48 toxemic/ hypertensive 207 uncomplicated	Heparin plasma zinc Collected across gestation AAS	↓ mean (SEM) plasma zinc in women with toxemia/ hypertension compared to women with uncomplicated pregnancies. **Toxemic: 541.5 (16.8) vs. uncomplicated: 590.7 (8) μg/L, p <0.009**
[72] Kim, Korea [a]	29 PE 30 uncomplicated	Serum zinc Collected at delivery Instrumental neutron activation analysis	↓ mean (SEM) serum zinc in mothers with PE compared to women with uncomplicated pregnancies. **PE: 700 (200) vs. uncomplicated: 1900 (500) μg/L, $p < 0.0001$**
[73] Kiilholma, Finland [c,d]	10 mild PE and 10 severe PE 20 uncomplicated	Serum zinc Collected at delivery Particle induced X-ray emission	↓ mean (SD) serum zinc in women with mild and severe PE compared to women with uncomplicated pregnancies. **Mild PE: 510 (70) and severe PE: 370 (10) vs. uncomplicated: 630 (90) μg/L, $p < 0.001$ for both, respectively**

			↓ mean (SD) serum zinc in women with severe PE compared to those with mild PE. **Severe PE: 370 (10) vs. mild PE: 510 (70) μg//L, $p < 0.005$**
[74] Araujo Brito, Brazil [e]	20 mild PE and 24 severe PE 50 uncomplicated	Fasting sodium citrate plasma zinc Collected before delivery Flame AAS	↓ mean (SD) plasma zinc in mothers with severe PE compared to mothers with uncomplicated pregnancies. **Severe PE: 388 (82) vs. uncomplicated: (483 (83) μg/L, $p < 0.05$** NS mean (SD) plasma zinc in women with mild PE compared to women with uncomplicated pregnancies. Mild PE: 500 (94) vs. uncomplicated: (483 (83) μg/L
[75] Magri, Malta [b]	33 GH 110 uncomplicated	Serum zinc Collected in third trimester Electro-thermal AAS	NS in mean (SD) serum zinc between women with GH and women with uncomplicated pregnancies. PE: 606 (80) vs. uncomplicated: 636 (100) μg/L
[76] Fenzl, Croatia [a,b]	30 PE and 30 GH 37 uncomplicated	Fasting serum zinc Collected at the time of diagnosis Flame AAS	NS in mean (SD) serum zinc between both women with PE or GH women and women with uncomplicated pregnancies. PE: 603 (93) and GH: 599 (83) vs. uncomplicated: 578 (93) μg/L
[77] Katz, Israel [d]	43 severe PE 80 uncomplicated	Plasma zinc Collected immediately after delivery Inductively coupled plasma mass spectrometry	NS mean (SD) plasma zinc in mothers with severe PE vs mothers with uncomplicated pregnancies. Severe PE: 685 (875) vs. uncomplicated: 534 (139) μg/L
[38] Hyvonen-Dabek, Finland [f]	10 hypertensive 10 uncomplicated	Serum zinc Collection time not specified Particle induced X-ray emission	NS mean (SD) serum zinc in women with PE compared to women with an uncomplicated pregnancy. PE: 1070 (320) and hypertensive: 1090 (170) vs. uncomplicated: 1150 (220)
[48] Borella, Italy [a]	24 hypertensive 35 uncomplicated	Heparin plasma zinc Collected in the third trimester Flame AAS	NS mean (SD) plasma zinc in the hypertensive women compared to those who remained uncomplicated. Hypertensive: 685.6 (149) vs. uncomplicated: 627.5 (150) μg/L
[78] Mistry, United Kingdom [a]	244 PE 472 uncomplicated	Non-fasting heparin plasma zinc Collected at 15 weeks gestation Inductively coupled plasma mass spectrometry	NS median (interquartile range) plasma zinc in women with PE women compared to those with uncomplicated pregnancies. PE: 579.6 (521.1–638.6) vs. uncomplicated: 575.7 (515.6–641.7) μg/L

[53] Tamura, United States [a]	271 hypertensive 2038 uncomplicated	Non-fasting heparin plasma zinc Collected at first prenatal visit (6 to 34 weeks) Flame AAS	NS in the prevalence (*n* (%)) of hypertension measured between the lowest quartile and upper 3 quartiles of zinc. Highest: 205 (7.9) vs. Lowest: 66 (7.7)
[79] Lao TT, China [a]	28 PE 28 uncomplicated	Heparin plasma zinc Collected after diagnosis, before delivery Flame AAS	NS mean (SD) plasma zinc in women with PE compared to women with uncomplicated pregnancies. PE: 641 (163) vs. uncomplicated: 647 (111) μg/L
Inadequate dietary zinc intake estimated to affect ≥17% of the studied population			
[80] Sarwar, Bangladesh [a]	50 PE 58 uncomplicated	Fasting serum zinc Collected >20 weeks gestation Flame AAS	↓ mean (SEM) serum zinc in mothers with PE compared to mothers with uncomplicated pregnancies. **PE: 770 (50) vs. uncomplicated: 980 (30) μg/L, $p < 0.001$**
[34] Kumru, Turkey [a]	30 PE 30 uncomplicated	Serum zinc Collection time not specified AAS	↓ mean serum zinc in women with PE when compared to women with uncomplicated pregnancies. **Data represented on graphs, $p < 0.001$**
[81] Ilhan, Turkey [a]	21 PE 20 uncomplicated	Serum zinc Collected at 31–38 weeks Flame AAS	↓ mean (SD) serum zinc in women with PE when compared to those with an uncomplicated pregnancy. **PE: 829.4 (289.3) vs. uncomplicated: 1251.9 (242.3) μg/L, $p < 0.001$**
[82] Bakacak, Turkey [a]	38 PE 40 uncomplicated	Fasting serum zinc 32–38 weeks Flame AAS	↓ median (max-min) serum zinc in women with PE when compared to those with an uncomplicated pregnancy. **PE: 812.4 (1106.5–624) vs. uncomplicated: 1084.5 (1385.5–881.2) μg/L, $p < 0.001$**
[36] Farzin, Iran [a]	60 PE 60 uncomplicated	Fasting heparin plasma zinc Collection time not specified Flame AAS	↓ mean (SEM) serum zinc in mothers with PE compared to mothers with uncomplicated pregnancies. **PE: 764.9 (176.2) vs. uncomplicated: 1006.1 (201.2) μg/L, $p < 0.001$**
[83] Al-Jameil, Saudi Arabia [a]	40 PE 40 uncomplicated	Serum zinc Collected in the third trimester Inductively coupled plasma optical emission spectrometry	↓ mean (SD) serum zinc in mothers with PE compared to mothers with uncomplicated pregnancies. **PE: 670 (590) vs. uncomplicated: 1300 (830) μg/L, $p < 0.05$**

[33] Akinloye, Nigeria [a]	49 PE 40 uncomplicated	Serum zinc Collection time not specified Flame AAS	↓ mean (SD) serum zinc between women with PE and women with uncomplicated pregnancies. **PE: 562 (92) vs. uncomplicated: 614 (52) μg/L, $p < 0.05$**
[39] Jain, India [e]	25 mild PE and 25 severe PE 50 uncomplicated	Serum zinc Collection time not specified AAS	↓ mean (SD) serum zinc between women with mild PE and those with uncomplicated pregnancies. **Mild PE: 831 (111) vs. uncomplicated: 1022 (157) μg/L, $p < 0.05$** ↓ mean (SD) serum zinc between women with severe PE and women with uncomplicated pregnancies. **Severe PE: 787 (92) vs. uncomplicated: 1022 (157) μg/L, $p < 0.05$**
[37] Gupta, India [b,e]	47 mild PE and. 18 severe PE and 10 eclamptic 74 uncomplicated	Non-fasting heparin plasma zinc Collection time not specified AAS	↓ mean (SD) serum zinc in mothers with severe PE and eclampsia compared to mothers with uncomplicated pregnancies. **Severe PE: 607 (107) and eclampsia: 607 (171) vs. uncomplicated: 695 (119) μg/L, $p < 0.01$** NS in mean (SD) serum zinc between women with mild PE and women with uncomplicated pregnancies. Mild PE: 684 (134) vs. uncomplicated: 695 (119) μg/L
[84] Bassiouni, Egypt [g,d]	52 PE (28 mild and 24 severe) 20 uncomplicated	Heparin plasma zinc Collected at delivery AAS	NS in mean (SD) plasma zinc in women with mild PE compared to women with uncomplicated pregnancies. **Mild PE: 604.2 (162.7) vs. uncomplicated: 646 (173.7) μg/L** ↓ mean (SD) plasma zinc in women with severe PE compared to the women with uncomplicated pregnancies. **Severe PE: 410.8 (116.5) vs. uncomplicated: 646.0 (173.7 μg/L, $p < 0.001$**
[85] Harma, Turkey [a]	24 PE 44 uncomplicated	Heparin plasma zinc Collected just during the latent phase of labor AAS	↑ mean (SD) plasma zinc levels in women with PE when compared to women with uncomplicated pregnancies. **PE: 15.53 (4.92) vs. uncomplicated: 11.93 (3.11) μg/g protein, $p = 0.003$**
[86] Rafeeinia, Iran [h]	35 PE and 15 severe PE 50 uncomplicated	Fasting serum zinc Collected in the third trimester AAS	NS mean (SD) serum zinc in mothers with PE or severe PE and uncomplicated pregnancies.

			Mild PE: 690 (40) and severe PE: 780 (80) vs. uncomplicated: 720 (40) μg/L
[87] Vafaei, Iran [e]	20 mild PE and 20 severe PE 40 uncomplicated	Serum zinc Collected at 28-40 weeks Auto-analyser	NS mean (SD) serum zinc in either the mild or severe PE women compared to women with uncomplicated pregnancies. Data represented on graphs
[88] Ahsan, Bangladesh [a,i]	44 PE and 33 eclampsia 27 uncomplicated	Serum zinc Collected at 28–42 weeks Flame AAS	NS mean (SD) serum zinc in PE or eclamptic women compared to women with uncomplicated pregnancies. PE: 1045.8 (131) and eclampsia: 915 (131) vs. uncomplicated: 980.4 (131) μg/L
[89] Rathore, India [a]	14 PE 47 uncomplicated	Serum zinc Collected at delivery Flame AAS	NS mean (SD) serum zinc between women with PE and those with uncomplicated pregnancies. PE: 492 (178) vs. uncomplicated: 575 (216) μg/L
[90] Kolusari, Turkey [a]	47 PE 48 uncomplicated	Serum zinc Collected between 29-38 weeks AAS	NS mean (SD) serum zinc between women with PE women and those with uncomplicated pregnancies. PE: 10.6 (4.4) vs. uncomplicated: 12.7 (4.1) μg/L
[91] Atamer, Turkey [a]	32 PE 28 uncomplicated	Fasting serum zinc Collected at 28-29 weeks Flame AAS	NS in mean (SD) serum zinc between women with PE and women with uncomplicated pregnancies. PE: 792 (180) vs. uncomplicated: 1086 (199) μg/L
[92] Adam, Turkey [a]	20 PE 20 uncomplicated	Plasma zinc Collected before the onset of labor Flame AAS	NS mean (SD) plasma zinc in women with PE compared to women with an uncomplicated pregnancy. PE: 313 (47) vs. uncomplicated: 341 (44) μg/L
[93] Vigeh, Iran [a]	31 PE 365 uncomplicated	Heparin plasma zinc Collected at delivery Inductively coupled plasma mass spectrometry	NS mean (SD) plasma zinc between women with PE women and women with uncomplicated pregnancies. PE: 5200 (1444) vs. uncomplicated: 5561 (1057) μg/L
[32] Adeniyi, Nigeria [a]	55 pregnant women	Plasma zinc Collection time not specified AAS	NS mean (SD) plasma zinc in women with PE compared to women with uncomplicated pregnancies. PE: 940 (270) vs. uncomplicated: 970 (230) μg/L

[a] PE defined as high blood pressure (≤140/90 mmHg) after 20 weeks gestation and proteinuria (≥300 mg/24 h), [b] GH defined as high blood pressure (≤140/90 mmHg) after 20 weeks gestation without proteinuria, [c] PE not defined, [d] Severe PE not defined, [e] Mild PE defined as blood pressure ≥140/90 but less than 160/110 mmHg and severe PE defined as ≥160/110 mmHg, [f] PE defined as blood pressure >130/85 and proteinuria ≥1 by dipstick, severe PE defined as blood pressure >160/110, [g] PE defined by the classification proposed by the Paris meeting of the Gestosis Organisation, 1970, [h] PE defined

as blood pressure > 130/85 and proteinuria ≥1 by dipstick, severe PE defined as blood pressure >160/110, [i] eclampsia defined as women diagnosed with PE whom also suffer seizures that cannot be attributed to other causes. **Bold print signifies results that were significantly different**. Abbreviations: AAS: atomic absorption spectrometry; GH: gestational hypertension; PE: preeclampsia; SD: standard deviation; SEM: standard error of the mean.

TABLE 2.3. Included studies assessing maternal zinc status and sPTB.

Author, Country	**Sample Size**	**Zinc Measure (1) Sample Type (2) Time at Which Gestation Diet was Assessed or Sample Collected (3) Method of Analysis**	**Outcome of the Study**
[44] Scholl, United States [a]	115 with zinc intake ≤6 mg/day 699 with zinc intake >6 mg/day	Dietary zinc intake 28 and 36 weeks 24 h dietary recall	2-fold ↓ risk of delivering a preterm infant with dietary zinc intake >6 mg/day. **OR (LMP): 1.85, 95% CI: 1.09–3.12, OR (OE): 2.13, 95% CI: 1.20–3.79** 2.75 to 3.44-fold ↓ risk of delivering a very preterm infant with dietary zinc intake >9 mg/day. **OR (LMP): 2.75, 95% CI: 1.31–5.77, OR (OE): 3.44, 95% CI: 1.39–8.55**
[94] Carmichael, United States [a,b]	413 preterm and 58 early preterm 5267 term	Dietary zinc intake Harvard food frequency questionnaires	2-fold ↓ for preterm birth <32 weeks with zinc intake > 8.0 mg/day compared to 8.0–14.2 mg/day. **OR: 2.3, 95% CI: 1.2–4.5**
[45] Neggers, United States [a]	238 preterm 1160 term	Dietary zinc intake 18 and 30 weeks 24 h dietary recall using the nutrient database developed by the University of Minnesota	NS association between low dietary zinc intake (less than median) and risk of PTB. OR: 1.1, 95% CI: 0.7–1.7
[95] Hsu, Taiwan [c]	28 preterm 423 term	Dietary zinc intake Each trimester 24 h dietary recall	NS in dietary zinc intake between each of the trimesters and in those who delivered preterm versus term. Preterm: 9.6–10.8 mg/day vs. term: 8.90–10.9 mg/day

Inadequate dietary zinc intake estimated to affect <17% of the studied population			
[96] Wang, China [a]	169 preterm 2912 uncomplicated	Fasting serum zinc First and second trimester Flame AAS	↑ risk of preterm birth with serum zinc <767 μg/L and serum zinc between 767-996 μg/L. **aOR: 2.41, 95% CI: 1.57, 3.70; aOR: 1.97, 95% CI: 1.27, 3.05, $p < 0.001$ for both, respectively**
[50] Bro, Denmark [c]	34 preterm 220 uncomplicated	Serum zinc Collected at delivery Flame AAS	NS mean (SD) serum zinc levels in women who delivered preterm compared to term women. Preterm: 666.7 (104.6) vs. term: 679.7 (98) μg/L
[54] Tamura, United States [c]	505 preterm and 136 early preterm 2038 uncomplicated	Non-fasting heparin plasma zinc Collected at first prenatal visit (6 to 34 weeks) Flame AAS	NS in the prevalence or n(%)of PTB measured between the lowest quartile and upper three quartiles of zinc. Highest: 373 (14.5) vs. lowest: 132 (15.3) NS in the prevalence (*n* (%)) of early PTB measured between the lowest quartile and upper three quartiles of zinc. Highest: 107 (4.2) vs. lowest: 29 (3.4)
Inadequate dietary zinc intake estimated to affect ≥17% of the studied population			
[66] Jeswani, India [c]	25 preterm 25 term	Serum zinc Collected at 28–40 weeks AAS	↑ mean (SD) serum zinc in women who delivered preterm women compared to term. **Preterm: 1154.4 (154.1) vs. uncomplicated: 962.8 (194.8) μg/L, $p < 0.01$**
[64] Goel, India [d]	20 preterm 25 term	Heparin plasma zinc Collected at delivery AAS	↑ mean (SD) plasma zinc in mothers who delivered preterm compared to term mothers. **Preterm: 842 (43) vs. term: 744 (51) μg/L, $p < 0.001$**
[60] Bahl, India [a]	10 preterm 97 term	Serum zinc Collected at delivery Flame AAS	NS mean (SD) in women who delivered Preterm that were an appropriate weight for date compared to uncomplicated. Preterm: 627 (212) vs. uncomplicated: 670 (96) μg/L
[65] Srivastava, India [c]	26 preterm 23 term	Heparin plasma zinc Collected at delivery Flame AAS	NS mean (SD) plasma zinc between preterm and term mothers. Preterm: 6350 (2640) vs. term: 6310 (5090) μg/L

[a] PTB defined as <37 weeks gestation, [b] Early PTB defined as <32 weeks gestation, [c] PTB defined as ≤37 weeks gestation, [d] PTB not defined. **Bold print signifies results that were significantly different**. Abbreviations: AAS: atomic absorption spectrometry; aOR: adjusted odds ratio; CI: confidence interval; LMP: last menstrual period; OE: obstetric estimate; PTB: preterm birth; SD: standard deviation.

The association between maternal circulating zinc and sPTB was determined in four studies on populations with inadequate zinc intake ≥17%, all of which sampled women in India (Table 2.3) [61,63,64,69]. Two of the studies reported serum/plasma zinc to be higher in women who delivered preterm compared to those who delivered at term (average 98–1991 μg/L increase) [63,64]. However, no difference in circulating zinc measured at delivery was reported in the remaining two studies [61,69].

2.3.4. Gestational Diabetes Mellitus

Two studies looked at the association between dietary zinc intake and GDM (Table 2.4) [98,97]. One collected data at 24–28 weeks gestation, and found an 11% reduction in the risk of gestational hyperglycaemia with every 1 mg/day increase in dietary zinc intake (aOR: 0.89, 95% CI: 0.82–0.96) [97]. The second, which sampled women at 14–20 weeks' gestation, found no association between maternal dietary zinc intakes below 50% of the recommended daily allowance and GDM (OR: 1.4, 95% CI: 0.6–2.9) [98]. Differences between the studies included when dietary zinc was measured (early versus late second trimester) as well as ethnicity (Italian versus Iranian in which, genetic and cultural differences are likely).

Of the five studies which assessed the association between circulating zinc and GDM in countries where inadequate zinc intake is estimated to be <17%, two, both studying Italian women, reported a significant difference in serum/plasma zinc in women who developed GDM compared to women whose pregnancies remained uncomplicated (Table 2.4) [97,47]. However, while one study reported that serum zinc was negatively associated with the risk of hyperglycemia in pregnancy (aOR: 0.94, 95% CI: 0.91–0.96) [97], the other found that there was in increase in serum zinc in women with GDM compared to women whose pregnancy remained uncomplicated (GDM mean (SD): 766.6 (117.6) vs. uncomplicated: 627.5 (150) μg/L, $p < 0.001$) [47]. Both studies sampled women at

similar times during pregnancy and used atomic absorption spectrometry to quantitate zinc. The remaining three studies found no difference in circulating zinc [35,38,99] however, given the small sample size of women with GDM in these studies (n = 5–46), it is likely they were underpowered and not suitable for the chosen statistical tests.

There were two studies that sampled women from countries where inadequate zinc intake was estimated to be ≥17% and assessed the association between maternal circulating zinc and GDM (Table 2.4) [98,100]. Neither study reported a difference in serum zinc in early pregnancy or at delivery in women with GDM compared to those whose pregnancies remained uncomplicated.

2.4 Discussion

This systematic review assessed whether maternal circulating zinc levels and/or dietary zinc intake were associated with a number of pregnancy complications. Overall, the evidence regarding the association between maternal zinc status and PE/GH, LBW/SGA, sPTB and GDM is weak and heterogeneity between the studies made comparisons difficult. However, systematic analysis of the available literature indicated some trends between maternal zinc status and infant birthweight as well as the development of severe PE (BP ≥160/110 mmHg).

There is consistent evidence in animal models that maternal dietary zinc deficiency during pregnancy reduces fetal growth [16–19]. From the studies that measured maternal zinc intake during pregnancy reviewed here, a possible relationship between low zinc intake (≤54% of the recommended 11 mg/day) and decreased infant birthweight may exist in human populations. Both food frequency questionnaires and 24 h recalls are limited by the preparedness of the participants to accurately record their diets, the food composition tables used and their ability to capture variations within diets [103]. This may explain the conflicting results between studies which assessed dietary zinc intake and the association with infant birthweight, sPTB and GDM. However, three of the four

TABLE 2.4. Included studies assessing maternal zinc status and GDM.

Author, Country	Sample Size	Zinc Measure (1) Sample Type (2) Time at Which Gestation Diet was Assessed or Sample Collected (3) Method of Analysis	Outcome of the Study
[97] Bo, Italy [a,b]	126 GDM and 84 aOGTT 294 uncomplicated	Dietary zinc intake 24–28 weeks Food frequency questionnaire	↓ mean (SD) daily zinc intake between GDM and aOGTT women and women with uncomplicated pregnancies. **GDM: 8.5 (2.4) and aOGTT: 8.7 (2.5) vs. uncomplicated: 9.4 (2.8) mg/day, p = 0.007**
[98] Behboudi-Gandevani S, Iran [a]	72 with GDM 961 uncomplicated	Dietary zinc intake 14–20 weeks Semi-quantitative food frequency questionnaire	NS in mean (SD) daily zinc intake between GDM and those with uncomplicated pregnancies. GDM: 6.91 (3.42) vs. uncomplicated: 10.1 (7.45) mg/day
Inadequate dietary zinc intake estimated to affect <17% of the studied population			
[48] Borella, Italy [a]	18 GDM 35 uncomplicated	Heparin plasma zinc Collected in the third trimester Flame AAS	↑ mean (SD) plasma zinc in GDM women compared to women with uncomplicated pregnancies. **GDM: 766.6 (117.6) vs. uncomplicated: 627.5 (150) μg/L, p <0.001**
[35] Wang, China [a,c]	46 GDM and 98 IGT 90 uncomplicated	Plasma zinc Collection time not specified Inductively coupled plasma atomic emission spectroscopy	NS in mean (SD) plasma zinc between women with IGT and women with uncomplicated pregnancies. IGT: 1080 (270) vs. uncomplicated: 1130 (330) μg/L NS mean (SD) plasma zinc between women with GDM and those with uncomplicated pregnancies. GDM:1020 (190) vs. uncomplicated: 1130 (330) μg/L
[38] Hyvonen-Dabek, Finland [d]	5 GDM 10 uncomplicated	Serum zinc Collection time not specified Particle induced X-ray emission	NS mean (SD) serum zinc in women with GDM compared to women with uncomplicated pregnancies. GDM: 1070 (190) vs. uncomplicated: 1150 (220) μg/L

[99] Wibell, Sweden [d]	20 GDM 13 uncomplicated	Serum zinc Collected across gestation AAS	NS mean (SD) serum zinc between women with GDM and those with uncomplicated pregnancies. GDM: 700 (100) vs. uncomplicated: 700 (80) μg/L
Inadequate dietary zinc intake estimated to affect ≥17% of the studied population			
[98] Behboudi-Gandevani, Iran [a]	72 with GDM 961 uncomplicated	Serum zinc Collected 14–20 weeks Flame AAS	NS mean serum zinc between GDM and women with uncomplicated pregnancies. GDM: 844 (440) vs. uncomplicated: 835 (444) μg/L
[100] Al-Saleh, Kuwait [a]	30 GDM 30 uncomplicated	Serum zinc Collected at delivery Furnace AAS	NS mean (SEM) serum zinc in women with GDM compared to women with uncomplicated pregnancies. GDM: 610.3 (60.1) vs. uncomplicated: 656.2 (241.4) μg/L

[a] GDM defined as high blood glucose levels in pregnant women who have not previously been diagnosed with diabetes which over a 3 h oral glucose tolerance test provided at least two values over the criteria of Carpenter and Coustan, [b] aOGTT defined as high blood glucose levels in pregnant women who have not previously been diagnosed with diabetes which over a 3 h oral glucose tolerance test provided one abnormal value over the criteria of Carpenter and Coustan, [c] IGT defined as women with blood glucose consistently higher than 7.8 mmol/L, [d] GDM diagnosed with an intravenous glucose tolerance test at 30 weeks gestation. **Bold print signifies results that were significantly different**. Abbreviations AAS: atomic absorption spectrometry; aOGTT: abnormal oral glucose tolerance test; BMI: body mass index; GDM: gestational diabetes mellitus; IGT: impaired glucose tolerance; OGTT: oral glucose tolerance test; SD: standard deviation.

TABLE 2.5. Summary of all the studies reviewed and whether zinc status was positively, negatively or not associated with the studied pregnancy complication.

Dietary Zinc Intake				
Total No. Reference	LBW/SGA	Hypertensive Disorders of Pregnancy	sPTB	GDM
9	4	1	4	2
	3 reported a negative association [42-44]	Reported no association [70]	2 reported a negative association [44,93]	1 reported a negative association [96]
	1 reported no association [45]		2 reported no association [45,94]	1 reported no association [97]
Serum/Plasma Zinc				
Total No. Reference	LBW/SGA	Hypertensive Disorders of Pregnancy	sPTB	GDM
58	26	33	7	6
No. where inadequate zinc intake affects <17% of the population				
	12	13	3	4
	2 reported a negative association [46, 49]	5 reported a negative association [55,71–74]	1 reported a positive association [95]	1 reported a positive association [48]
	2 reported a positive association [47,48]	8 reported no association [38,48,53,75–79]	2 reported no association [50,53]	3 reported no association [35,38,98]
	8 reported no association [38,50-56]			
No. where inadequate zinc intake affects ≥17% of the population				
	14	20	4	2
	5 reported a negative association [57-61]	10 reported a negative association [33,34,36,37,39,80–83,100]	2 reported a positive association [64,66]	2 reported no association [97,99]
	2 reported a positive association [63,101]	1 reported a positive association [84]	2 reported no association [60,65]	
	6 reported no association [64-69]	9 reported no association [32,85-92]		

Abbreviations: GDM: gestational diabetes mellitus; LBW: low birth weight; SGA: small for gestational age; sPTB: spontaneous preterm birth.

studies that measured dietary zinc intake in pregnancy and recorded infant birthweight reported a significant reduction in maternal zinc status in those who delivered a LBW/SGA infant [42,44,45]. The relationship between infant

birthweight and maternal serum/plasma zinc is less clear. Plasma measures of zinc are considered preferable over serum as erythrocytes can be a source of zinc contamination within serum samples [22]. However, plasma zinc only accounts for approximately 0.1% of total body zinc [104], is heavily influenced by confounding factors like stress, infection and hormones [105–108,101] and does not directly correlate with dietary zinc intake [109]. This limits how useful measuring circulating zinc is as a biomarker for health and disease. When studies on LBW/SGA that measured maternal circulating zinc were separated based on populations where inadequate zinc intake is predicted to be ≥17%, 7 of the 13 studies reported a difference in serum/plasma zinc between women who delivered LBW/SGA infant and those whose infants were of an appropriate weight. Given the lack of suitable alternatives, particularly in studies of pregnant women, determining zinc status by measuring serum/plasma zinc can still be informative about the importance of zinc to pregnancy, especially if measured in conjunction with dietary zinc intakes.

Other maternal factors such as age, BMI, smoking status and alcohol consumption in pregnancy not only influence pregnancy outcome but also circulating zinc [110,111]. BMI is a significant factor in influencing the risk for developing PE and GH [112,113]. However, only 11 of the 32 studies on PE/GH [33,36,54,75,71,79,80,82,84,88,92] reported on BMI, making it difficult to comment on whether differences in BMI may be influencing the outcomes of the studies included in this review. Despite this, there may be a relationship between maternal circulating zinc levels and the severity of PE. Mean maternal zinc concentrations in women with severe PE (ranging from 388–410 μg/L) [73,74,83] were well below 562.1 μg/L, which is the defined zinc deficiency cut-off [26,114]. In women with mild PE and those with uncomplicated pregnancies, mean maternal zinc concentrations ranged between 684–831 μg/L [37,39,83] and 630–1022 μg/L [37,39,74,83] respectively. A current leading hypothesis relating to the development of PE is increased placental oxidative stress [115]. Zinc itself has

antioxidant capabilities and is an integral structural component of superoxide dismutase, a first line defense antioxidant [116] which has reduced activity in cell lines, animal models and human studies of zinc deficiency [117–121]. Hence, it is possible in pregnancies complicated by PE, that low maternal zinc concentration (<562.1 μg/L) may reduce the potential to combat rises in free radical production and increase the severity of the complication.

Zinc levels in maternal circulation decrease across gestation; this is thought to be due to a combination of increased maternal blood volume and fetal demands [40,122–124], and therefore comparisons between studies which measured zinc in maternal serum or plasma early in pregnancy versus late should be interpreted with caution. Overall, regardless of pregnancy outcome, the majority (31 out of 59 studies which measured maternal circulating zinc) collected samples during labor or at delivery. Physiologically, parturition results in huge changes to maternal hormonal profile with rises in estrogen, oxytocin and prostaglandin required to initiate labor [125]. Furthermore, there is an increase in the production of inflammatory cytokines and a withdrawal of anti-inflammatory cytokines within the gestational tissues [126]. Infection and inflammation decrease plasma zinc [105] and use of the contraceptive pill, which raises estrogen and progesterone levels, also decreases circulating zinc [106,101]. Given that pregnancy itself is likely to confound zinc status, this has implications for interpreting studies that have measured serum/plasma zinc at delivery. In addition, how zinc may be associated with a pregnancy outcome needs to be measured before the pregnancy complication has manifested. Only five studies of 6795 pregnant women in total measured either circulating zinc or dietary zinc intake prior to 20 weeks gestation [44,53,54,79,98]. All found no significant difference in maternal zinc status during this time period between women who developed a pregnancy complication and those who did not, indicating that zinc status in early pregnancy may not be associated with adverse pregnancy outcomes.

Due to the additional demands associated with pregnancy and fetal growth, pregnant women are more vulnerable to multiple nutrient deficiencies [127] and this is potentially another cofounding factor when assessing the association between maternal zinc status and pregnancy outcome. This is because nutrients can interact with each other in both a positive (e.g., vitamin A and zinc [128]) and negative manner (e.g., calcium or iron and zinc [129,130]). A number of studies reviewed here measured serum/plasma concentrations of other nutrients as well as zinc, including copper [35,97], iron [75,98], selenium [51,88], magnesium [95,99] and lead [78]. While circulating zinc levels were not different for the pregnancy outcomes studied in these articles, those of other micronutrients were. Serum copper concentrations were found to be higher in women with GDM or those who delivered an SGA infant when compared to women with an uncomplicated pregnancy in two studies [97,35]. Furthermore, serum iron was higher in women with PE and GDM compared to women whose pregnancies were uncomplicated [75,98]. Two other studies found selenium to be lower in the serum of women with PE or those who delivered an SGA infant compared to women with an uncomplicated pregnancy [88,51]. Therefore, it is important to consider other nutritional factors that may influence pregnancy outcome as well as micronutrient ratios in order to fully understand the importance of micronutrient status on pregnancy success.

Finally, the lack of studies identified in this review analyzing truly zinc deficient women, nor those in populations at high risk of zinc deficiency, is a major limitation in determining the effects of zinc on pregnancy outcome. Only 8 of the 64 studies reported mean circulating zinc below 562.1 μg/L [49,50,74,72,60,83,87,91] and there were very few studies based on women in countries where inadequate zinc intake is predicted to be prevalent like South-East Asia and parts of Africa [26,27]. The majority of studies were based on populations in the United States and Europe where zinc deficiency is estimated to only affect 3.9%–12.7% of the population [26]. Therefore, there is the potential

that the results from this review may be skewed given the lack of evidence based on women living in areas predicted to be at high risk of zinc deficiency.

2.5 Conclusions

The current review has explored the connection between maternal zinc status and pregnancy complications including hypertensive disorders of pregnancy, infant birthweight, spontaneous preterm birth (sPTB) and gestational diabetes mellitus (GDM). While it appears that there may be a relationship between maternal dietary zinc intake and infant birthweight and the development of severe PE, there is little evidence to suggest an association between zinc and sPTB or GDM. However, heterogeneity in the studies identified in this review reflects real uncertainty in the evidence linking zinc deficiency and pregnancy complications and therefore this warrants further study, particularly in developing countries whose populations are at increased risk of zinc deficiency. If we are to continue to reduce preventable deaths of newborns and children under the age of five [6], understanding the importance of micronutrients like zinc in child development, particularly in utero, will greatly increase the likelihood of success. Future studies need to focus on women more vulnerable to zinc deficiency in pregnancy in order to fully determine the effects of zinc status on pregnancy outcome.

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2.6 Appendices

2.6.1 Appendix A

Outline of the search terms and MeSH headings identified in the Medline search and used for the remaining database searches.

Search strategy: MEDLINE (OVID).

	Searches	Results
1	exp Zinc/ or zinc.mp.	108695
2	plasma zinc.mp.	1365
3	zinc intake.mp.	679
4	dietary zinc.mp.	1158
5	serum zinc.mp.	2083
6	1 or 2 or 3 or 4 or 5	108695
7	preterm birth.mp. or exp Premature Birth/	14642
8	premature birth.mp. or Premature Birth/	11131
9	small for gestational age.mp.	9255
10	exp Infant, Small for Gestational Age/	5977
11	gestational hypertension.mp. or exp Hypertension, Pregnancy-Induced/	32078
12	pre?eclampsia.mp. or exp Pre-Eclampsia/	30863
13	exp Pre-Eclampsia/ or exp Eclampsia/ or eclampsia.mp.	32471
14	exp HELLP Syndrome/ or HELPP syndrome.mp.	1613
15	gestational diabetes.mp. or exp Diabetes, Gestational/	11382
16	fetal macrosomia.mp. or exp Fetal Macrosomia/	2369
17	7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16	69148
18	infant, low birth weight.mp. or Infant, Low Birth Weight/	16571
19	infant, very low birth weight.mp. or exp Infant, Very Low Birth Weight/	8491
20	17 or 18 or 19	89771
21	6 and 20	380
22	limit 21 to (english language and full text and humans)	165

2.6.2 Appendix B

List of conversion factors used to convert all measures of zinc to μg/L.

Units	Conversion
μg/100 mL or μg/dL	Multiply 10
μmol/L or μM	Divide 0.153
mg/L	Multiply 1000
μg/mL	Multiply 1000

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ii. permission is granted for the candidate in include the publication in the thesis; and

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Chapter 3

Zinc is a critical regulator of placental morphogenesis and maternal hemodynamics during pregnancy in mice

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ABSTRACT

Zinc is an essential micronutrient in pregnancy and zinc deficiency impairs fetal growth. We used a mouse model of moderate zinc deficiency to investigate the physiological mechanisms by which zinc is important to placental morphogenesis and the maternal blood pressure changes during pregnancy. A 26% reduction in circulating zinc (P=0.005) was exhibited in mice fed a moderately zinc-deficient diet. Zinc deficiency in pregnancy resulted in an 8% reduction in both near term fetal and placental weights (both P<0.0001) indicative of disrupted placental development and function. Detailed morphological analysis confirmed changes to the placental labyrinth microstructure. Continuous monitoring of maternal mean arterial pressure (MAP) revealed a late gestation decrease in the zinc-deficient dams. Differential expression of a number of regulatory genes within maternal kidneys supported observations on MA*P* changes in

gestation. Increased MA*P* late in gestation is required to maintain perfusion of multiple placentas within rodent pregnancies. Decreased MA*P* within the zinc-deficient dams implies reduced blood flow and nutrient delivery to the placenta. These findings show that adequate zinc status is required for correct placental morphogenesis and appropriate maternal blood pressure adaptations to pregnancy. We conclude that insufficient maternal zinc intake from before and during pregnancy is likely to impact *in utero* programming of offspring growth and development largely through effects to the placenta and maternal cardiovascular system.

3.1 Introduction

During pregnancy, the placental vasculature provides the interface between the fetus and the mother for exchange of nutrients and wastes. Adequate placental function underpins normal fetal development [1]. Defects in placental development and function are implicated in a number of clinical pregnancy complications. These include preeclampsia (PE) [2], a common hypertensive disorder of pregnancy [3] and fetal growth restriction (FGR) [4-7], defined as birth weight, adjusted for gestational age, of ≤5th percentile [3]. Together these conditions pose a lifelong risk of morbidity and mortality for both the mother and infant. Increased placental oxidative stress is hypothesised to be an underlying cause of pathogenesis in pregnancies complicated by PE and FGR [8]. While the precise mechanisms remain unclear, it is highly likely that micronutrient deficiencies play a pivotal role.

Zinc is extremely important during the accelerated fetal growth phase characteristic of late gestation. It is an essential element in more than 1000 proteins required for biological functions including antioxidant defence, cell signalling and gene expression [9-12]. In development, zinc is not only important for the action of transcription factors and the enzymes which catalyse the synthesis of DNA and RNA, but also as a component of the accessory proteins

that regulate the activation of key development genes [13]. Increased fetal loss and high rates of congenital malformation in several organs of surviving fetuses are characteristics of severe zinc deficiency in pregnancy [14,15]. Compromised DNA integrity and increased oxidative stress are likely drivers of impaired tissue and physiological function when zinc intake is insufficient, since zinc is an essential component of copper-zinc superoxide dismutase (Cu/Zn-SOD) [16], and DNA-repair mechanisms such as p53 [17,18].

In rodents, maternal zinc deficiency consistently causes reduced fetal growth evident from mid-gestation to near term [19-23]. The mechanisms by which placental development and function contributes to impaired fetal growth in these models is largely unknown. Maternal hemodynamic adaptations to pregnancy are essential for optimal placental development and function, since adequate blood flow to the placenta underpins normal fetal growth [24]. Reduced uterine blood flow to the placenta not only constrains delivery of nutrients to the fetus and increases fetal hypoxia but also has implications for production of factors which modulate placental vascular growth [25]. We hypothesised that reduced fetal growth associated with maternal zinc deficiency is modulated by altered placental development and function and aimed to characterise the effect of moderate maternal zinc deficiency on placental morphogenesis and maternal cardiovascular adaptations to pregnancy, in particular maternal blood pressure.

3.2 Results

3.2.1 Moderate Dietary Zinc Restriction Reduces Circulating Zinc and Liver Metallothionein Concentrations

To confirm that moderate dietary zinc restriction reduced maternal zinc status in C57BL/6J female mice fed a zinc-deficient diet (containing 10 mg/kg zinc) compared to a zinc-replete diet (containing 40 mg/kg zinc), we analysed maternal plasma zinc concentrations, liver metallothionein expression and placental and

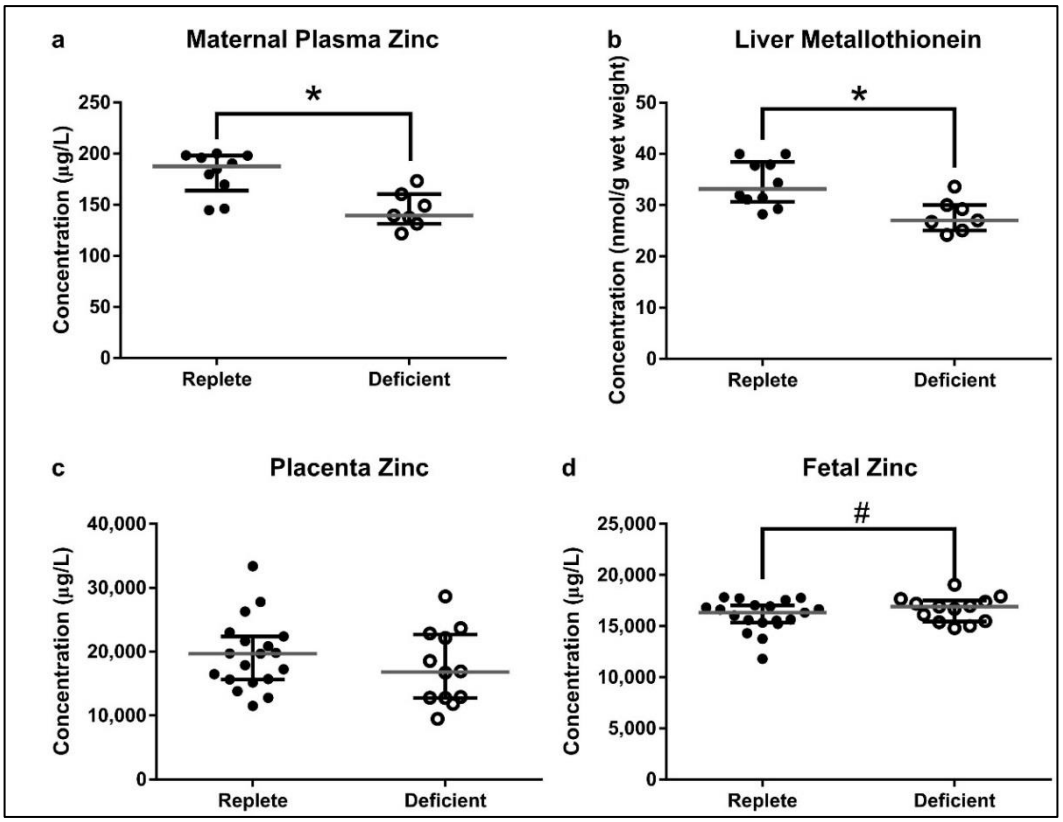

FIGURE 3.1. Lower zinc in the diets of the zinc-deficient mice reduced both short-term and long-term zinc stores. At GD18.5, a reduction in circulating zinc levels (a) as well as liver metallothionein (b) was observed in the zinc-deficient dams. Placental tissue zinc however remained similar between the two diet groups (c) and there was a trend for an increase in zinc content of fetuses from zinc-deficient dams (d). Data are median and interquartile range (n = 10 zinc-replete and 7 zinc-deficient [a & b] and n = 19 zinc-replete and 13 zinc-deficient [c & d]). Statistical significance was determined using Mann-Whitney Test on data based on an average litter size of 7.00. **P*<0.05, #*P*=0.08.

fetal zinc content. At gestational day (GD) 18.5, there was a 26% decrease in circulating zinc in the zinc-deficient dams when compared to the zinc-replete dams (Fig. 3.1a; P = 0.005) and long term zinc status, as measured by liver metallothionein, was also reduced by 19% (Fig. 3.1b; P<0.0001). There was no difference in maternal food consumption across the experimental period (mean 7 day food consumption per cage containing 4 mice (SEM) deficient: 92.4 (1.29) vs. replete: 90.6 (1.86) g; P = 0.45), confirming reduced zinc status was not a consequence of anorexia. Placental zinc concentrations remained similar between the two diet groups (Fig. 3.1c; P = 0.59) and there was a trend for an increase in

zinc concentrations in fetuses from the zinc-deficient dams (Fig. 3.1d; $P = 0.08$), suggesting active mechanisms for sequestering zinc into gestational tissues. Circulating levels of phosphorus, sulphur and potassium were slightly higher in the zinc-deficient dams (Supplementary Table S3.1) but was likely due to minor differences in the diet compositions (Supplementary Table S3.2) and these nutrients did not differ in placental or fetal tissues (Supplementary Table S3.1).

3.2.2 Moderate Maternal Zinc Deficiency Impairs Fetal Growth

To investigate the impact of zinc deficiency during pregnancy on placental development, we first compared reproductive outcomes in zinc-deficient and zinc-replete dams. At GD0.5 the zinc-deficient dams did not differ in weight compared to the zinc-replete dams (Fig. 3.2a; $P = 0.24$). However, at GD18.5 (24 hours before birth) they were 3% lighter compared to the zinc-replete (Fig. 3.2b; $P = 0.044$). The proportion of mated mice exhibiting viable pregnancies in late gestation was not different (data not shown) and litter size was not significantly different between the two diet groups (median [IQR] zinc-deficient: 7 [5, 8] vs. zinc-replete: 8 [6, 8.75]; $P = 0.36$), indicating no impact on fertility or fecundity. The lighter maternal body weight in the zinc-deficient dams was largely accounted for by an 8% reduction in both fetal and placental weights (Fig. 3.2c-d; both $P < 0.0001$) as maternal carcass weight (maternal weight minus fetal and placental weights combined) did not differ between the two diet groups (Fig. 3.2e; $P = 0.24$). The fetal-placental weight ratio did not significantly differ (Fig. 3.2f; $P = 0.21$), indicating similar nutrient transport efficiencies between the placentas. The importance of zinc to growth and development was highlighted in the postnatal phase, with pups from the zinc-deficient mothers 32% lighter at weaning compared to pups born to control dams (Supplementary Fig. S3.1). Survival rates to weaning were similar regardless of zinc status (survival at weaning; zinc-deficient: 30 out of 40 (75%) vs. zinc-replete: 20 out of 27 (74%); χ^2 analysis; $P = 1$).

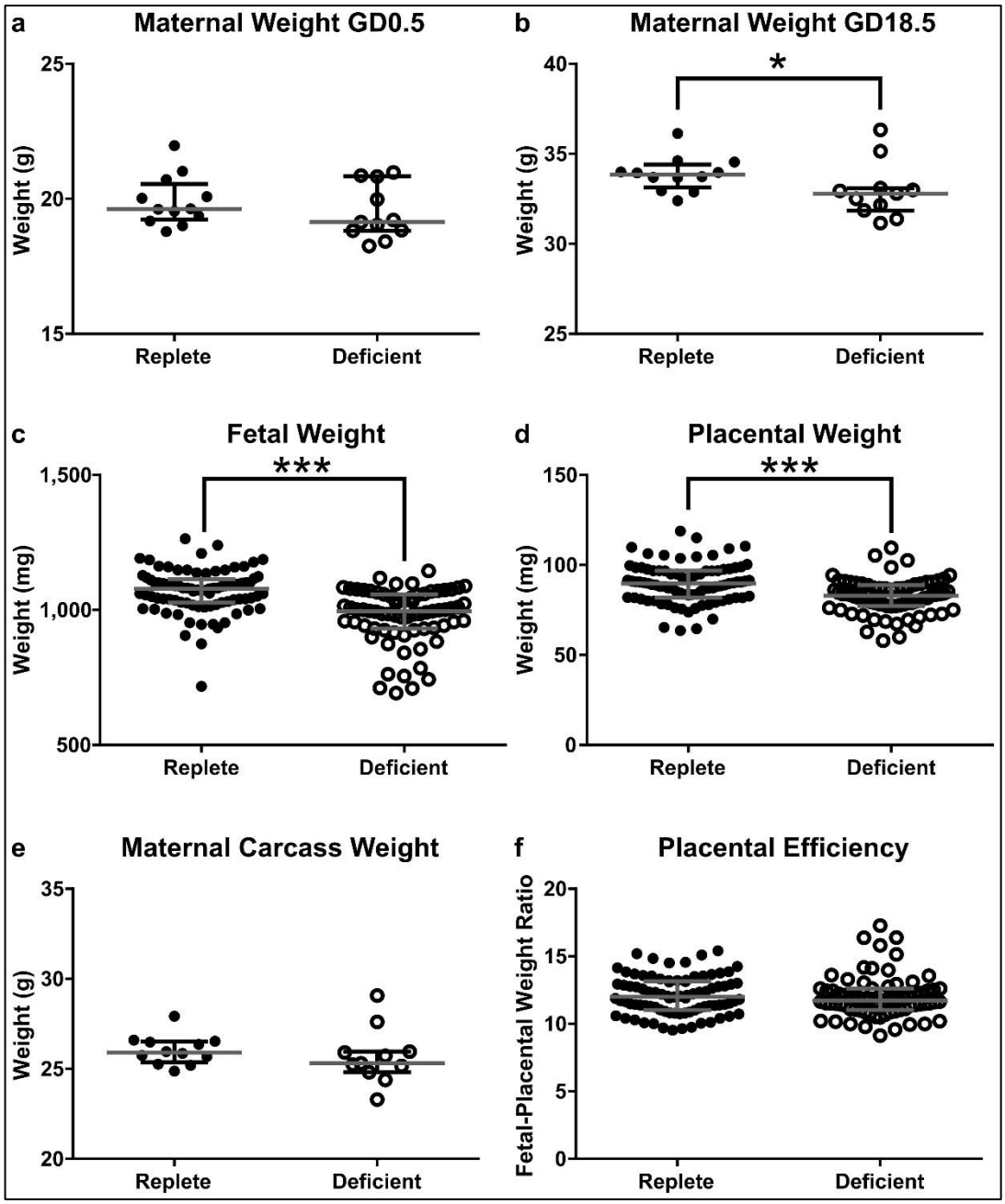

FIGURE 3.2. Maternal zinc deficiency altered reproductive outcome, measured at GD18.5. No significant difference in maternal weight was observed between the zinc-replete and zinc-deficient dams at mating (a). At GD18.5, maternal weight of the zinc-deficient dams was reduced compared to the replete (b). This was largely due to a decrease in both fetal (c) and placental (d) weight as maternal carcass weight (e) was not significantly different. Placental efficiency, measured by the fetal-placental weight ratio was not different between the two diet groups (f). Data are median and interquartile range (n = 12 zinc-replete and 11 zinc-deficient). Statistical significance was determined using Mann-Whitney Test on data based on an average litter size of 7.00. *P<0.05, ***P<0.001.

3.2.3 Effect of Maternal Zinc Deficiency on Placental Morphology

Having confirmed that moderate zinc deficiency in pregnancy resulted in reduced placental weight, we analysed placental morphology at GD18.5 as FGR is often associated with poor placental morphogenesis, and underpins future placental function [26]. Total placental mid-sagittal cross sectional area was not significantly different between the two diet groups despite a reduction in placental weight (median [IQR], deficient: 7.17 [5.88, 7.35] vs. replete: 6.71 [6.18, 7.01] mm2; $P = 0.21$). Furthermore, there was no difference in the mid-sagittal cross sectional area of the labyrinth zone; the region responsible for nutrient and waste exchange (Fig. 3.3a; $P = 0.69$), despite an 8% reduction in labyrinth zone weight in placentas from zinc-deficient dams (Fig. 3.3b, $P = 0.012$). This implied a disproportionate shift in placental architecture, as commonly occurs in an adaptive response to nutritional perturbation [27], and was supported by detailed immunohistochemical (IHC) analysis. There are three main compartments which comprise the labyrinth zone; the fetal capillaries (FC: fetal circulation), maternal blood space (MBS: maternal circulation) and the trophoblasts which act as a barrier between the FC and MBS, to coordinate nutrient and waste exchange (Fig. 3.3c). In the placentas of zinc-deficient dams, both trophoblast volume and trophoblast barrier thickness were reduced by 16% (Fig. 3.3d-e; $P = 0.007$ and $P = 0.016$, respectively), there was a 17% increase in the volume density of FC (Figure 3.3f; $P = 0.02$) and 10% increase in surface area density (Figure 3.3g; $P = 0.028$) compared to placentas from zinc-replete dams. Together these results indicate a compensatory mechanism in the zinc-deficient placentas that nevertheless was unable to rescue fetal growth.

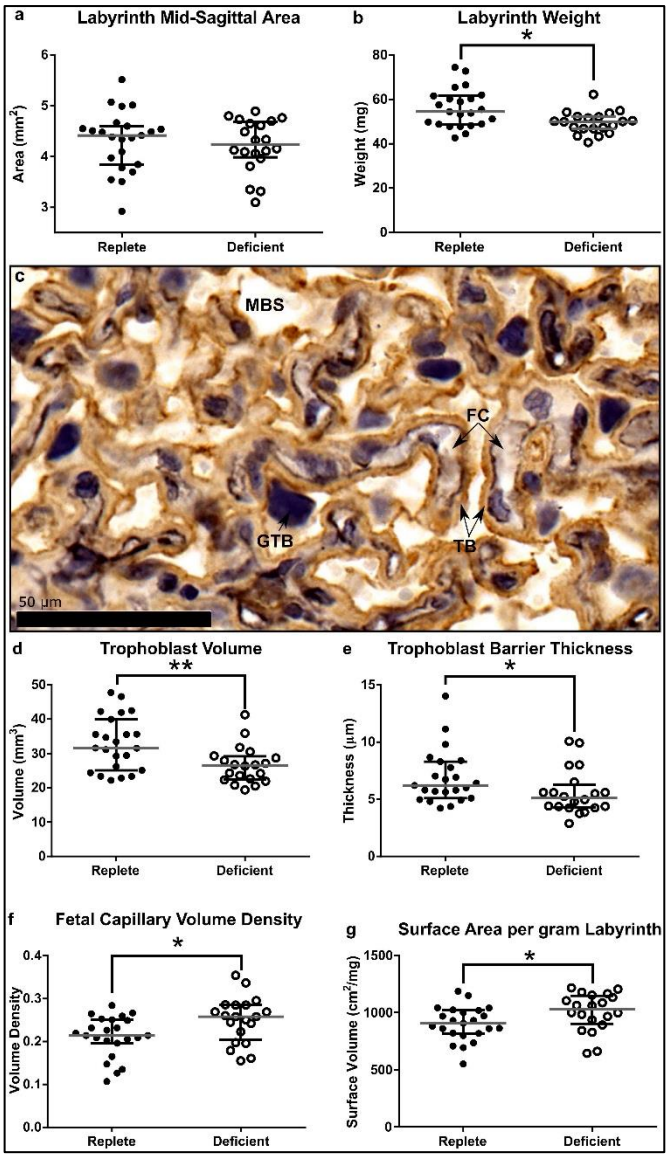

FIGURE 3.3. Maternal zinc deficiency during pregnancy resulted in changes to the placental architecture likely to affect fetal growth. Despite no significant different in the labyrinth zone mid-sagittal cross sectional area (a), labyrinth zone weight was reduced in the zinc-deficient placentas at GD18.5 (b). Double labelling immunohistochemistry was used to identify the fetal capillaries (FC) trophoblasts (TB) and maternal blood space (MBS) within the labyrinth zone (c). Analysis revealed decreases in the trophoblast volume (d) as well as trophoblast barrier thickness (e) in the placentas from zinc-deficient dams. An increase in fetal capillary volume density (f) and surface volume (g) was also found. Data are median and interquartile range (n = 23 and 21 placentas from 12 zinc-replete and 11 zinc-deficient dams, respectively). Statistical significance was determined using Mann-Whitney Test on data based on an average litter size of 7.00. *P<0.05, **P<0.01. GTB: giant trophoblast cells.

3.2.4 Placental Iron Transport is Reduced by Zinc Deficiency

Zinc is extremely important in regulating the expression of a number of development genes crucial for fetal growth [13]. Conservation of zinc within the placentas and fetuses of the zinc-deficient dams suggested reduced zinc in the gestational tissues was not driving the reduction in fetal growth. A microarray analysis was used to identify pathways within the placenta that may be disrupted by maternal zinc deficiency however, only eight genes were identified to be potentially differentially expressed (Supplementary Table S3.3). An 8.5-fold increase in the expression of *Transferrin Receptor* (*Tfrc*) in placentas from zinc-deficient dams was identified (Fig. 3.4a and Supplementary Table S3.3) and qPCR validation in an independent cohort of placentas confirmed an increase in gene expression (Fig. 3.4b; P<0.0001). Furthermore, there was a 36% increase in the expression of *Tfrc* in maternal kidney tissue from the same cohort of mice (Fig. 3.4c; $P = 0.019$). IHC staining showed Tfrc protein localised to the apical surface of the trophoblast cells within the placental labyrinth (Fig. 4d) and Western blot analysis confirmed a 32% increase in protein expression in zinc-deficient placentas compared to zinc-replete (Fig. 3.4e and 3.4f; $P = 0.022$). Increased *Tfrc* expression was most likely a response to decreased placental and fetal iron concentration in the tissues from zinc-deficient dams (Supplementary Table S3.1). A 24% ($P = 0.048$) and 20% ($P = 0.001$) decrease in the zinc-deficient dams in placental and fetal iron, respectively, indicated disruption to placental iron transport despite maternal circulating iron remaining similar between the two diet groups (Table S3.1; $P = 0.42$). These data suggest a possible perturbation in the expression of Transferrin (Trf) protein which binds Tfrc to move predominantly iron into cells. However, using qPCR, we did not observe differential expression of *Trf* in either placentas or kidneys between the two diet groups (Fig. 3.4g and 3.4h; $P = 0.12$ and $P = 0.82$, respectively).

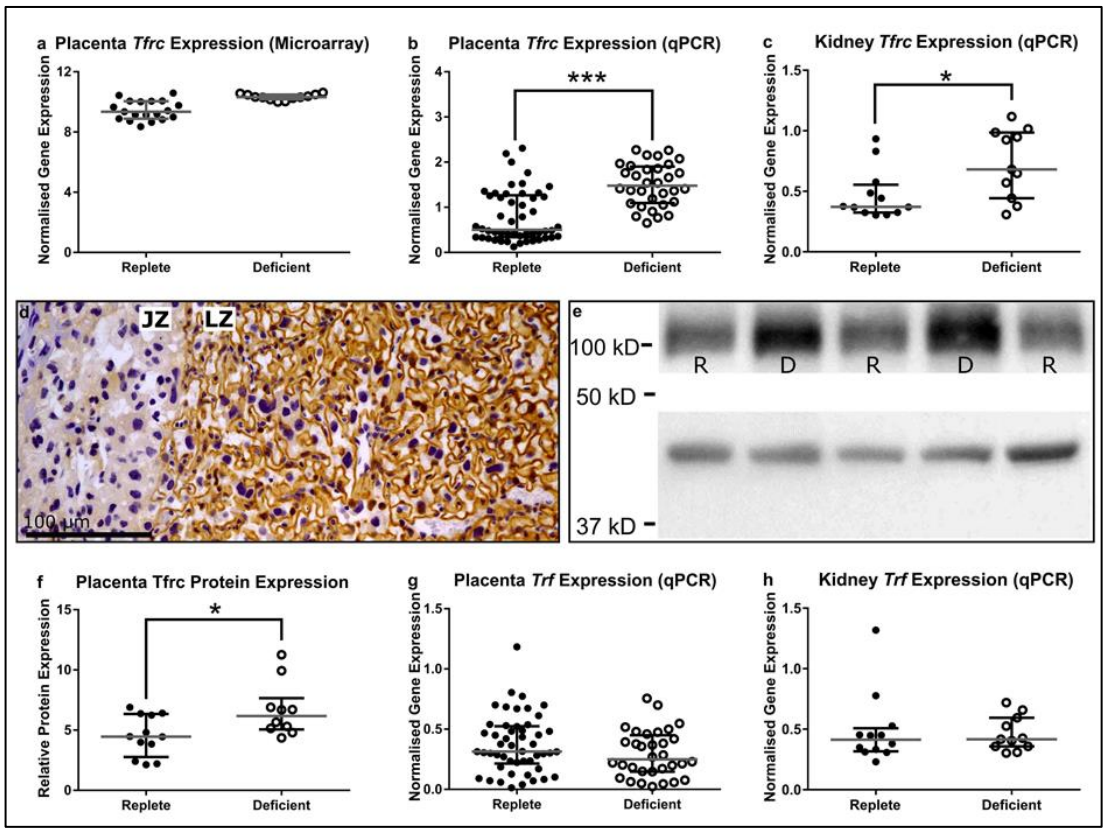

FIGURE 3.4. Microarray analysis revealed an 8.5-fold increase in gene expression of *transferrin receptor* (*Tfrc*) in the placentas of the zinc-deficient dams (a). This increase was validated and confirmed in an independent cohort of placental samples using qPCR (b). qPCR was also used to measure *Tfrc* expression within kidney tissue at GD18.5 and was also increased in tissue collected from zinc-deficient dams (c). Immunohistochemical analysis of Tfrc protein showed localisation to the apical surface of the trophoblast cells within the labyrinth zone (LZ) of the placenta (d). Tfrc protein expression was analysed using Western blot and compared to β-actin expression (e); Tfrc band at ~100kD and β-actin at ~42kD, (full image in supplementary information). This revealed an increased expression of Tfrc protein in placentas from zinc-deficient dams (f). *Transferrin* (*Trf*) gene expression within the placenta and kidney was also quantified by qPCR but did not differ between the two diet groups (g and h). Data are median and interquartile range (n = 19 and 12 placentas [a]; 50 and 32 placentas [b and g]; 12 and 11 kidneys [c and h] and 12 and 10 placentas [e and f] from 12 zinc-replete and 11 zinc-deficient dams, respectively). Statistical significance was determined using Mann-Whitney Test. **P*<0.05, ****P*<0.001. JZ: junctional zone.

3.2.5 DNA Damage Caused by Oxidative Stress is Elevated by Zinc Deficiency

In vitro and *in vivo* models of both zinc and iron deficiency reveal increased oxidative stress and DNA damage [28-32] as well as increased oxidative stress within the placenta in pregnancies complicated by FGR [33,34]. We investigated whether disrupted placental function in this model of zinc deficiency was associated with increased oxidative stress. Placental expression of 4-hydroxynonenal (4HNE; a product of lipid peroxidation and marker of oxidative stress) was largely localized to the giant trophoblasts within the labyrinth zone (Fig. 3.5a) however, Western blot quantification of 4HNE expression revealed no difference in relative protein expression between placentas from zinc-deficient and zinc-replete dams (Fig. 3.5b; $P = 0.72$). There was also no difference in the expression of Cu/Zn-SOD between placentas from the two diet groups (Figure 3.5c-d; $P = 0.28$). This indicates that neither increased lipid peroxidation nor reduced SOD expression was a characteristic of placental dysfunction in this model. However, increased DNA damage within the placental labyrinth zone in the placentas from the zinc-deficient dams as indicated by increased nuclei stained positively for 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Fig. 3.5e-f; $P<0.0001$) suggests a reduction in cellular integrity mediated by increased oxidative stress which would contribute to placental dysfunction. 8-OHdG staining was localised predominantly within giant trophoblasts as well as within glycogen cells present in the junctional zone.

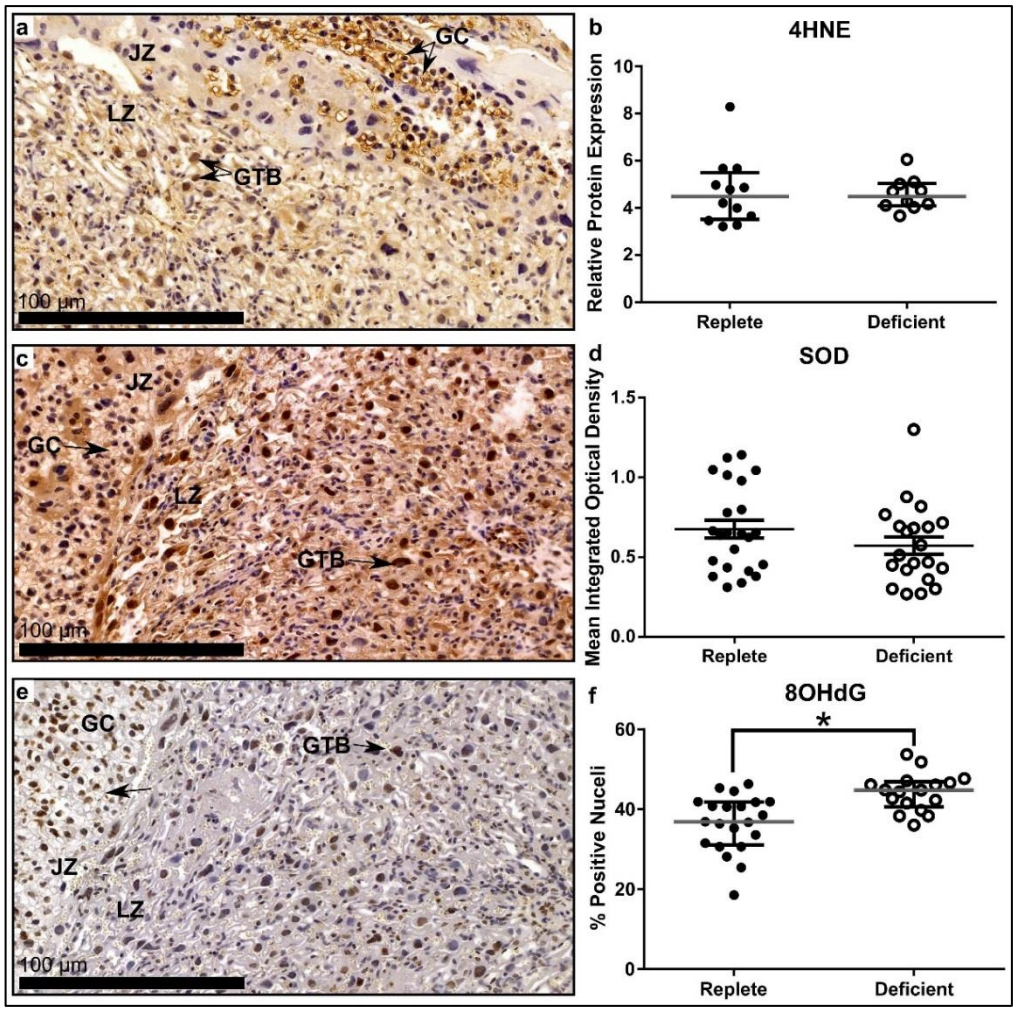

FIGURE 3.5. Maternal zinc deficiency resulted in increased DNA damage caused by oxidative stress within the placenta at GD18.5. 4-hydoxynoneal (4HNE) expression was localisation to the glycogen cells (GC) present in the junctional zone (JZ) and giant trophoblast cells (GTB) within the labyrinth zone (LZ) of the placenta (a). Western blot analysis showed no difference in 4HNE expression between the two diet groups (b). Expression of Cu/Zn-Superoxide Dismutase (SOD) was found throughout the placenta (c) but expression was not different between placentas collected from a zinc-deficient or zinc-replete dams (d). 8-hydroxy-deoxyguanosine (8OHdG) was also expressed by the GTB cells and GC within the junctional zone (e). There was a statistically significant increase in the percentage of positive cells in the labyrinth zone of the placentas from zinc-deficient dams indicating an increase in DNA damage caused by oxidative stress (f). Data are median and interquartile range (n = 12 and 10 placentas [b] and 23 and 21 placentas [d & f] from 12 zinc-replete and 11 zinc-deficient dams, respectively). Statistical significance was determined using Mann-Whitney Test. *P<0.05.

3.2.6 Maternal Zinc Deficiency Alters Maternal Blood Pressure Profile During Pregnancy and Lactation

Adequate blood flow to the placenta is crucial for the maintenance of fetal growth and an exponential increase in placental blood flow is necessary for transport of oxygen, water and nutrients in late gestation [25]. Maternal cardiovascular adaptations to pregnancy are crucial in maintaining appropriate placental blood flow so we sought to investigate whether zinc deficiency in pregnancy altered maternal blood pressure. Continuous measurements of mean arterial pressure (MAP), systolic blood pressure (SBP) and diastolic blood pressure (DBP) prior to pregnancy, within pregnancy and across lactation revealed that blood pressure was disturbed without adequate zinc intake (Fig. 3.6a and Supplementary Figure S3.2a). Across the five days prior to mating, average 24 h MA*P* was elevated in the zinc-deficient dams compared to zinc-replete dams (Fig. 3.6a), driven by increased DB*P* during this period (Supplementary Fig. 3.2a). 24 h MAP, SPB and DB*P* were also elevated in the zinc-deficient dams during lactation. Within pregnancy, regardless of diet, MA*P* was different between days 11-19 of pregnancy compared to days 1-5. Between days 6-10 of pregnancy, 24 h MA*P* was elevated in the zinc-replete dams but decreased in deficient dams. This resulted in lower 24 h MA*P* between days 11-19 of pregnancy in the zinc-deficient dams compared to zinc-replete dams (Fig. 3.6a).Further analysis of diurnal MA*P* pattern during the day (0700 h – 1850 h) and night (1900 h – 0650 h) revealed differences in MA*P* during pregnancy were driven by differences in MA*P* during the day when mice are at rest and not during the night when mice are active (Supplementary Fig. S3.2b). Average 24 h pulse pressure; the difference between SB*P* and DBP, was lower in the zinc-deficient dams across the whole experimental period when compared to the zinc-replete dams (Fig. 3.6b). This along with the fact that 24 h heart rate (HR) was also decreased in the zinc-

deficient dams (Supplementary Fig. S3.2c) indicates a potential reduction in cardiac output and reduced organ perfusion.

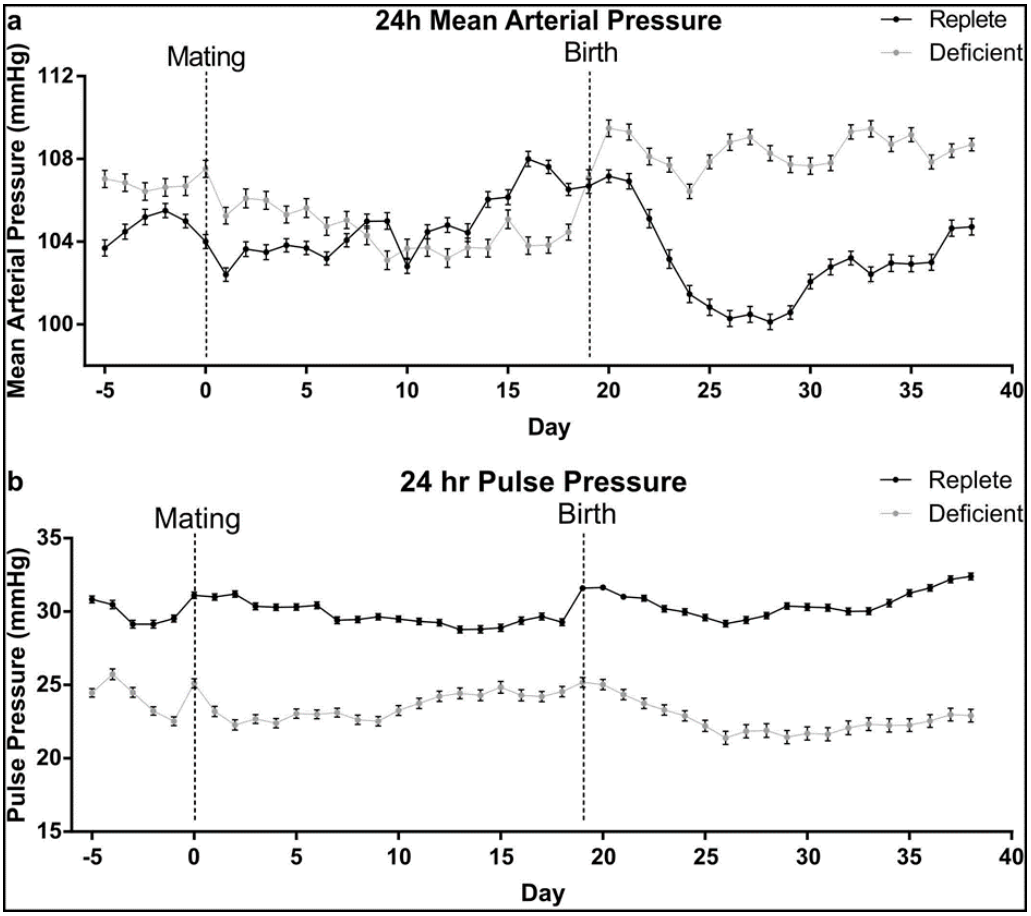

FIGURE 3.6. Maternal zinc deficiency changed the blood and pulse pressure profile of dams before pregnancy, during pregnancy and in lactation. Prior to pregnancy and during lactation, 24 hour mean arterial pressure (MAP) was significantly elevated in the zinc-deficient dams. (a) During pregnancy, from days GD0 to GD5, MA*P* was significantly elevated in the zinc-deficient dams. However, across GD6 to GD10, MA*P* began to decrease in the zinc-deficient dams while an increase was observed in the zinc-replete dams. This resulted in significantly lower MA*P* in the zinc-deficient dams between GD11 to GD19 of pregnancy. (b) Pulse pressure, which is the difference between systolic and diastolic blood pressures, was significantly lower in the zinc deficient animals across the whole experimental period. Each data point represents the average 24 h MA*P* or pulse pressure for each diet grou*P* ± SEM. n = 4 zinc-replete and 4 zinc-deficient dams. Statistical differences were determined using a general additive model.

3.2.7 Maternal Zinc Deficiency Effects Renal Gene Expression and Structure

To investigate whether changes in maternal blood pressure late in gestation may be influenced by effects on aspects relating to renal function, we analysed kidney gene expression and morphology. qPCR analysis of kidney tissue at GD18.5 identified a number of differentially expressed genes in zinc-deficient and zinc-replete dams associated with blood pressure regulation (Supplementary Table S3.4). In particular, a 16% reduction in kidney expression of *angiotensin converting enzyme* (*Ace*) ($P = 0.031$) and a 44% reduction in *inositol 1,4,5-triphosphate receptor type 2* (*Itpr2*) ($P = 0.05$) in the zinc-deficient dams occurred in association with reduced blood pressure observed at GD18.5 in mice implanted with blood pressure monitors. Ace protein expression in the kidneys was localised to the apical brush-borders of the proximal convoluted tubules and a 57% reduction in Ace protein expression in the kidneys of the zinc-deficient dams was demonstrated when compared to the zinc-replete dams (Fig. 3.7a-c; $P < 0.01$). Zinc deficiency also caused changes to glomerular morphology which can have implications for blood pressure. An 11% reduction in glomeruli basement membrane (GBM) thickness was observed in the zinc-deficient dams, compared to the zinc-replete dams at GD18.5 (Fig. 3.7d-f; $P < 0.01$), which would affect glomerular filtration capabilities and therefore maternal blood pressure. Overall, this data is consistent with blood pressure measurements in late gestation. Given that placental blood flow is dependent upon maternal hemodynamic alterations across gestation, the effects of zinc deficiency on maternal blood pressure are likely to contribute to placental dysfunction.

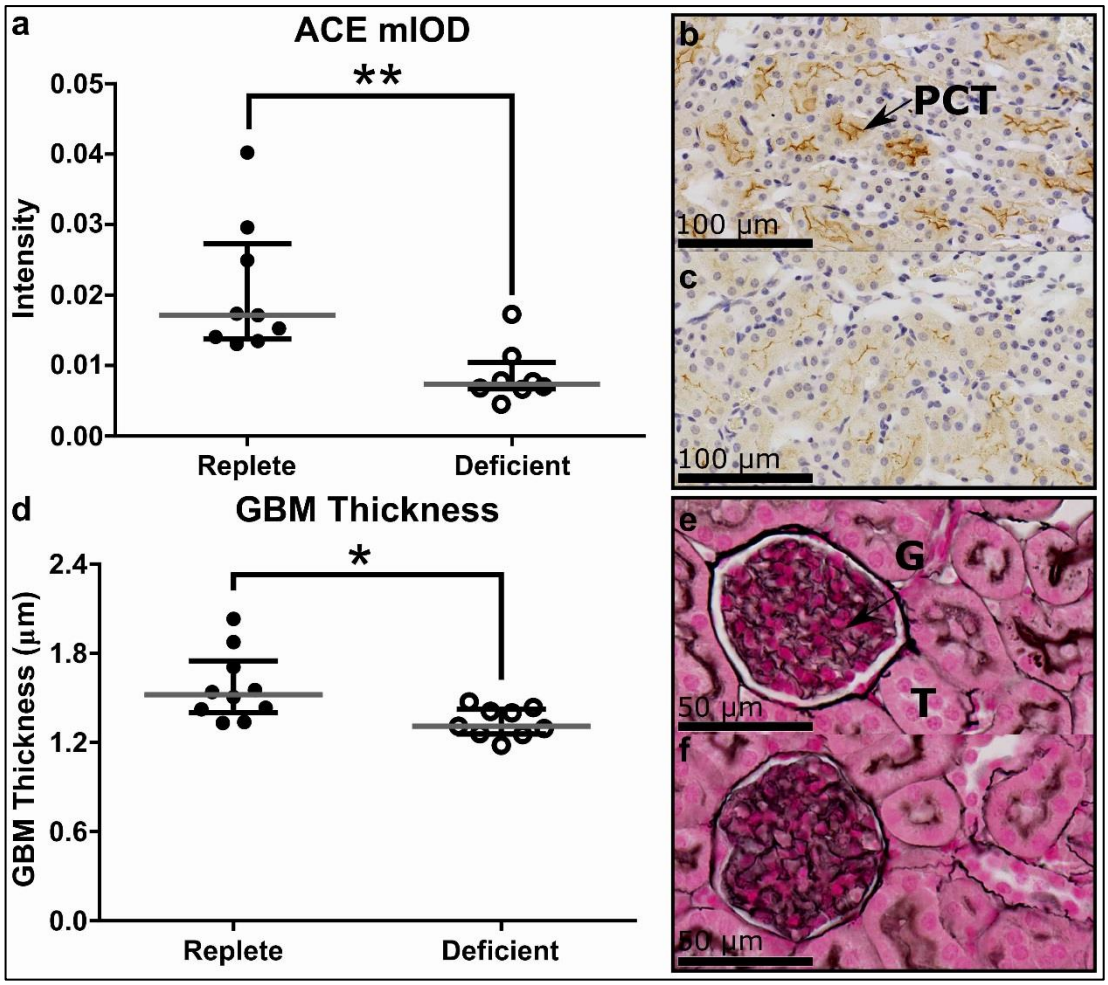

FIGURE 3.7. Effects of marginal zinc deficiency on renal parameters at GD18.5. Decreased gene expression of *angiotensin converting enzyme* (*Ace*) was associated with a decrease in Ace protein expression within the kidneys of zinc-deficient dams (a). Representative image of Ace expression in a zinc-replete (b) and zinc-deficient (c) kidney section. Ace protein was localised to the proximal convoluted tubules (PCT). Marginal maternal zinc deficiency also decreased glomeruli basement membrane thickness (d). Representative images of a glomerulus in a zinc-replete (e) and zinc-deficient (f) kidney section. Data are median and interquartile range (n = 10 zinc-replete and 9 zinc-deficient dams). Statistical significance was determined using Mann-Whitney Test. **P*<0.05, ***P*<0.01. G: glomerulus. T: tubule.

3.2.8 Maternal Zinc Deficiency Alters Immunological Parameters in Late Gestation

In addition to renal function, maternal blood pressure in pregnancy is influenced by immune cells, particularly T cells and natural killer (NK) cells [35]. These cells impact maternal hemodynamics and placental development by suppressing

inflammatory mediators in the implantation site [36] and promoting appropriate uterine vascular adaptation [37]. Spleen hypotrophy, as indicated by a 50% decrease in maternal spleen weight as a percentage of carcass weight, was observed in the zinc-deficient dams compared to zinc-replete dams at GD18.5 (Supplementary Fig. S3.3a; $P = 0.01$). There was also a significant reduction in liver, kidney and lung weights as a percentage of carcass weight in the zinc-deficient dams suggesting aberrant maternal adaptation to pregnancy (Supplementary Fig. S3.3b-d; $P<0.05$ for all). The failure to exhibit the spleen hypertrophy typical of late gestation [38,39] is consistent with an impaired immune response to pregnancy. Histological analysis of the spleen at GD18.5 showed no difference in the percentage area of white pulp, the splenic region populated by lymphocytes, between the two diet groups (Supplementary Fig. S3.4a). However, there was a decrease in the absolute number of cells, calculated based on splenic weight, within the white pulp in the spleens of the zinc-deficient animals compared to the zinc-replete indicative of a reduced lymphocyte pool (Supplementary Fig. S3.4b; $P = 0.051$). We investigated changes to immune cell populations within the spleen at GD18.5 using qPCR to analyse marker genes and found a 41% reduction in the expression of *Fasl*, a marker expressed by activated T cells and NK cells and a 1.26-fold increase in the expression of *Il10*, which is expressed by regulatory T cells and type 2 T helper cells, in the zinc-deficient dams (Supplementary Fig. S3.5a,b; $P = 0.043$ for both, respectively). Furthermore, a trend toward elevated *Ifna1* (Supplementary Fig. S3.5c; $P = 0.07$) expression was observed in the spleens of zinc-deficient dams compared to controls. This data indicates a possible imbalance in the residual lymphocyte subpopulations [40] which would be expected to contribute to the cardiovascular and placental anomalies seen in the zinc-deficient dams. Maternal immune adaptations to pregnancy are crucial to placental development during the implantation period and an altered immune balance may contribute to the

mechanisms through which zinc deficiency disturbs placental morphogenesis and fetal growth.

3.3 Discussion

Correct placental morphogenesis underpins pregnancy success and offspring phenotype, through tight regulation of transport of nutrients, gases and waste between the mother and fetus. This study confirms that maternal zinc status is a key determinant of fetal growth and shows that the effects of limited zinc are likely to be mediated through adverse placental development and function. Additionally, we show that altered placental development is associated with and likely to be a consequence of combined effects of altered maternal cardiovascular adaptations to pregnancy and altered renal function and immune changes. An interaction between zinc and other micronutrients within the placenta has also been identified, demonstrating the existence of micronutrient bioavailability interactions within this complex physiological network.

Reduced fetal growth has been described in a number of rodent models assessing the effect of marginal and severe dietary zinc restriction during pregnancy [19-23]. In our model, reduced fetal growth at GD18.5 was associated with an 8% reduction in placental weight, a common phenotype of growth restriction in humans and animals [26,41]. We sought to further investigate perturbations in placental development by defining impact on the placental architecture which regulates nutrient transport and fetal growth. A lower placental weight was reported at GD12.5 in a model of severe zinc restriction (<1 mg/kg) [21]. Unlike this earlier study, we did not observe a difference in labyrinth zone area at GD18.5 but IHC analysis of labyrinth zone composition demonstrated a change in placental structure indicative of a compensatory mechanism. Nutrients transfer from the maternal circulation to the fetus requires passage through a layer of trophoblast cells which surround the fetal capillaries [42]. Increases to the exchange surface area and the volume density of fetal

capillaries, as well as decreases in the trophoblast barrier thickness of the zinc-deficient placentas suggest an increase in the transfer efficiency of nutrients per gram of placenta. However, the reduction in total placental size and thus labyrinthine volume is likely to elicit a greater effect on the transport of nutrients to the fetus than the difference in the magnitude of nutrient transfer per gram of placenta. This is supported by the observed similar placental efficiency between dietary zinc groups, as measured by the fetal-placental weight ratio, indicating these compensatory changes to the labyrinth zone structure are insufficient to rescue fetal growth.

The structure of the placenta at term is determined by events that occur much earlier in placental morphogenesis to drive differentiation of various trophoblast cell lineages and mesenchymal cells from stem cells in a defined stochastic relationship [26,43]. Alterations in gross placental structure as well as to the ultrastructure of the labyrinthine zone can confer either a functional advantage or disadvantage for nutrient transfer and fetal growth. Changes in developmental patterning that result in altered surface area, barrier thickness and fetal capillary volume can in part be influenced by the maternal immune adaptation to pregnancy, and the capacity of immune cells to support or limit early placental development[44]. Since the spleen is a key source of NK cells and T lymphocyte recruited into the uterine decidua and gestational tissues [35], the reduced reservoir of splenic lymphocytes and the altered gene expression in spleen cells found in zinc-deficient females suggests that the immune response is aberrant in zinc-deficient mice. This is consistent with observations of the impact of zinc on T cell responses in non-pregnant animals [45-47]. Given the critical impacts of lymphocytes on maternal vascular remodeling and systemic cardiovascular adaptations to accommodate pregnancy [35] the effects of zinc deficiency mediated via immune cells may contribute to placental dysmorphogenesis.

In pregnancy, placental function and the maternal circulatory system are intricately dependent upon one another. Perturbations in placental function are likely to influence the maternal circulation and vice-versa [24]. Increases in blood pressure late in gestation are a characteristic of rodent pregnancies as indicated by the blood pressure profile in the zinc-replete dams of this experiment and by others [48]. This presumably facilitates maintenance of adequate perfusion of multiple placental beds as mice are litter-bearing species. Reduced uterine blood flow can also be used as a predictor of FGR in human pregnancies [49,50] as transport of oxygen and nutrients needs to keep pace with fetal growth and this can only occur with adequate blood flow to the placenta. The decrease in blood pressure during late pregnancy, as well as reduced pulse pressure which was observed in the zinc-deficient dams is likely to result in reduced blood flow to the placenta, impairing nutrient delivery. The renin-angiotensin system (RAS) is one of the major physiological regulators of blood pressure mediated by the kidneys. Zinc is important to the function of a number of the RAS enzymes which catalyse the production of vasoconstrictors thereby increasing blood pressure [51]. Ace is a zinc metalloenzyme and chelation of zinc inhibits Ace functionality [52]. We also identified a reduction in *Itpr2* expression which is a protein that controls vascular tone and is thought to alter the responsiveness of cells to components of the renin-angiotensin system [53]. These data support the importance of zinc in correct functioning of the RAS, particularly in the kidneys, and therefore blood pressure control which is necessary to maintain adequate placental perfusion and transplacental nutrient exchange.

Not only is blood flow a crucial determinant of placental function and fetal growth, but it also has implications for oxidative stress. Fluctuations in the perfusion of the placenta and therefore oxygenation is a major inducer of oxidative stress within the placenta which can severely affect placental transport capabilities [54] and increased oxidative stress has been characterised in the placenta of human pregnancies complicated by FGR [33,34]. Zinc itself is capable

of acting as an antioxidant but is also required as a structural component of a number of first line defence antioxidant molecules [55,56]. Studies have shown that zinc deficiency leads to increased oxidative stress *in vitro* as well as in plasma of *in vivo* animal models [28-30]. Furthermore, zinc concentrations have also been shown to be positively correlated with superoxide dismutase concentrations in cord blood [57]. Zinc deficiency in this model did not increase the amount of lipid peroxidation within the placentas which is not entirely surprising as a similar result in human FGR placentas has been documented [34,58] and may reflect the placenta's ability to rapidly metabolise 4HNE [59]. Furthermore, there did not appear to be a disruption to the expression of Cu/Zn-SOD in the near term placenta. However, conservation of zinc within the gestational tissues may have ameliorated any effect on Cu/Zn-SOD expression or function characterised in other studies [60,61]. There was however, a significant elevation in DNA damage, particularly within the giant trophoblast cells present within the labyrinth zone of the placentas suggesting possible disruption to DNA repair mechanisms within these cells similar to that previously seen in cell culture models of zinc deficiency [17,18]. The giant trophoblast cells within the labyrinth zone function to produce hormones such as placental lactogen II and are positioned in order to facilitate delivery of hormones and nutrients into the fetal circulation [62,63]. Consequently, increased DNA damage would likely reduce cellular integrity which could then effect aspects of giant trophoblast function and nutrient delivery, potentially amplifying already poor nutrient delivery from the maternal circulation resulting in fetal growth restriction.

There may also be a strong relationship between increased DNA damage in the placentas and the reduction of both placental and fetal iron in the zinc-deficient dams. Iron deficiency results in impaired mitochondrial function and increased oxidative stress in a number of different tissues [31,32] hence it is reasonable to assume a reduction in placental iron is influencing oxidative stress pathways in a similar manner. Perturbed iron transport in the placentas also

suggested a shift in iron transport out of the placenta occurring as part of a response to conserve zinc. Similar to our study, placental Tfrc expression has been shown to be inversely related to fetal liver iron concentrations [64] suggesting a dialogue between the fetus and placenta which mediates iron transport. However, how this may be mediating zinc conservation within the gestational tissues - so why iron is reduced is unknown. Iron is known to be extremely important in supporting cell growth and metabolism [65,66] and therefore reduced transport to the fetus would contribute to growth reduction. Previous studies have shown an accumulation of iron in tissues including the placenta and fetus in zinc deficient animal models [67,68]. The disparity with the current results likely reflects that previous studies utilised models of severe zinc restriction (<0.5 mg/kg). Nevertheless, this new data further highlights a strong association between zinc and iron transport in the placenta worthy of further investigation.

In conclusion, this study describes the effects of moderate maternal zinc deficiency on placental development and function, as well as responses of the maternal cardiovascular system to reduced zinc. Furthermore, the work implicates mechanistic pathways involving altered kidney and immune function by which inadequate zinc intake and stores during pregnancy impact on offspring phenotype. Zinc deficiency contributes substantially to the predicted 3.5 million deaths per year that are attributed to child and maternal undernutrition [69]. Reducing the prevalence of zinc deficiency in developing countries was a key aspect of the Millennium Development Goal 1 [70]. Our findings highlight the importance of adequate zinc status in pregnancy as deficiency impairs fetal growth. Since the trajectory of placental development is set in very early pregnancy, our data point to a need for further research to define the most critical window for zinc effects in pregnancy, and to develop strategies to reduce the impact of zinc deficiency in vulnerable women.

3.4 Materials and Methods

3.4.1 Animals and diets

Seven-week-old C57Bl/6J female mice (Laboratory Animal Services, University of Adelaide) were fed either a zinc replete diet containing 40 mg/kg zinc (Modified AIN93G, SF09-093; Specialty Feeds) or a marginally zinc deficient diet containing 10 mg/kg zinc (Zinc modified AIN93G, SF14-087; Specialty Feeds) as determined by inductively coupled plasma atomic emission spectrometry (ICP-MS). Animals were housed and maintained at Laboratory Animal Services, University of Adelaide on a 12 h: 12 h light-dark cycle with both water and food provided *ad libitum*. After six weeks on the respective diets, females were randomly allocated to either blood pressure monitoring by radio-telemetry (Cohort 1; n = 8) or analysis of placental and fetal growth (Cohort 2; n = 30). Mice in Cohort 2 were mated with a C57BL/6J male fed standard chow (Specialty Feeds). The presence of a vaginal copulatory plug was designated gestational day 0.5 (GD0.5). Given the difficulty associated with implanting the radio-telemetry probe into very small mice, the mice in Cohort 1 were maintained on respective diets until 21 g (17-18 weeks-old), when radio-telemetry surgery was performed. All animals were maintained on diets until conclusion of experiments. Animal use complied with the Australian Code of Practice for the Care and Use of Animals and ethics approval was obtained from the University of Adelaide Ethics Committee (Approval #M-2014-84).

3.4.2 Elemental Analysis

Elemental analysis on maternal plasma, placental and fetal tissues was undertaken to determine the effect of dietary zinc restriction on calcium, potassium, magnesium, sodium, sulphur and phosphorus, determined using inductively-coupled plasma optical emission spectrometry (ICP-OES) (Agilent 5100 ICP-OES; CSIRO Analytical Services, South Australia) and zinc, iron,

copper, and selenium determined by ICP-MS (Agilent 7700 ICP-MS). Details of the sample preparation procedure are provided in Supplementary Materials and Methods. Disruptions to long term zinc stores were analysed by measuring liver metallothionein at GD18.5 and 21 days post-birth using a 109Cd/heme affinity assay [71].

3.4.3 Placental and Fetal Characteristics

Post-mortem was performed on the dams in cohort 2 between 1000-1200 h on GD18.5, described in full in Supplementary Materials and Methods. Maternal, placental and fetal tissue was collected and the number of total, viable and resorbing implantation sites was counted. From each dam, 2 placentas were fixed and 5 μm thickness full-face sections were stained with Masson's Trichrome following standard protocols in order to determine the mid sagittal total, labyrinth zone and junctional zone cross sectional areas. In depth analysis of labyrinth structure was performed using immunohistochemistry (IHC) as previously described [72,73] and details of antibodies and dilutions used are provided in Supplementary Table S5. Microarray, qPCR, IHC and Western blot analysis were employed to analyse gene and protein parameters and are detailed in Supplementary Materials and Methods. Microarray data have been deposited to NCBI GEO under the accession GSE97112.

3.4.4 Radio-telemetry surgery, blood pressure monitoring and kidney function

Dams in cohort 1 were anaesthetised (2.5% isofluorane and 1 L/minute O2, then maintained at 1.5% isofluorane and ½ L/minute O2; AttaneTM Isoflurane, Bomac) and a radio-telemetry probe (PAC10, Data Sciences International) was inserted into the left carotid artery. The probe body was placed subcutaneously on the right flank. Mice recovered for 10 days, then MAP and HR were monitored

for 10 seconds every 10 minutes for 5 days prior to the dams being placed with males. The presence of a copulatory plug designated GD0.5 and MAP and HR continued to be measured through pregnancy, birth and lactation. Pups were counted on the day of birth and weighed at 3, 5 and 7 days post-birth and at weaning. 21 days post-birth, dams and pups were killed and post-mortems performed between 1000-1200 h to collect maternal and pup tissues as described in Supplementary Materials and Methods.

To further investigate the effects of zinc deficiency on maternal blood pressure, the left kidney was fixed as per the protocol outlined in Supplementary Materials and Methods. The right kidney was snap-frozen for molecular analyses. 5 μm full-faced, mid-sagittal sections were stained using Masson's Trichrome in order to determine the cortex and medullary zone area. Picrosirius red staining was used to determine collagen deposition and Jones' membrane staining was used to assess glomeruli morphology. These staining procedures as well as details of qPCR gene expression analysis, IHC and Western blot analysis of protein expression are detailed in Supplementary Materials and Methods.

3.4.5 Statistics

All statistical analysis was performed in R (v3.1.1) [74] using Mann-Whitney Test unless otherwise specified. Maternal organ weights, fetal and placental weight were corrected for viable litter size using the *lme* function in the *nlme* package to generate predicted values based on an average litter size of 7, then Mann-Whitney test was used to calculate exact P-values based on the predicted results. Significant differences in 24 h MA*P* and HR between the two diet groups were determined using a generalized additive mixed-model, as detailed in Supplementary Materials and Methods. Results are reported as median and interquartile range unless otherwise stated.

Data Availability

Microarray data have been deposited to NCBI GEO under the accession GSE97112.

Acknowledgments

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3.5 Supplementary Information

S3.5.1 Materials and Methods

Post-mortems and pregnancy parameters

Dams were anaesthetised with an intraperitoneal injection of 0.75 μg/g Avertin (2,2,2-tribromomethanol, *Sigma Aldrich*) in 2-methyl-2-butanol. Blood was collected in a heparinised tube via retro-orbital bleed to which plasma was extracted after centrifugation at 10,000 x g and stored at -80°C. Dams were then killed via cervical dislocation. Placental and fetal tissue was dissected from the uterus, membranes removed and sna*P* frozen for molecular analysis. Two placentas from each dam were randomly selected to be fixed in 4% (wt/vol) paraformaldehyde/2.5% polyvidone-40 (wt/vol) in 70 mM phosphate buffer [pH 7.0] overnight at 4°C, washed in 4 changes of PBS and paraffin-embedded. Maternal tissues including spleen, liver, kidneys, lungs, heart and parametrial fat were also excised with the left kidney and heart being fixed following the same procedure as the placentas.

Elemental analysis of maternal plasma and placental and fetal tissues

Briefly, 200 μL of plasma, approximately 25 mg of placental tissue and the torso of the GD18.5 fetus were digested in Teflon vessels at room temperature in concentrated nitric acid (~70% HNO3) for approximately 2 h before further digestion under pressure at approximately 90°C overnight. The following day, the heat was further increased to approximately 140°C and left a further 24 h. The samples were then diluted with milliQ water and made u*P* to 20 mL to be injected onto the relevant apparatuses for analysis. For ICP-MS, samples were run alongside two internal standards: iridium and rhodium (*Choice Analytical*) at a concentration of 200 ppb and an 8-point calibration, including blank, was carried out between 0.01 μg/L and 100 μg/L. An 8-point calibration (including blank) was also carried out between 500 mg/L and 1000 mg/L for the ICP-OES analysis.

Kidney and spleen morphological analysis

For kidneys, full-face mid-sagittal sections were stained using Masson's Trichrome in order to determine the cortex and medullary zones following standard protocols. Collagen deposition was measured using picrosirius red staining. De-waxed sections were immersed in a solution of 0.1% Direct Red (*Sigma-Aldrich*) and 1.2% picric acid (*Sigma-Aldrich*) in water for 1 h, rinsed in running ta*P* water, counter stained with Wiegert's haematoxylin for 20 mins then dehydrated, cleared and mounted. Slides were scanned using the Nanozoomer 2.0-HT C9600-13 and random systematic sampling of the cortex area was used to select 10 images of each section at 20x magnification using the ND*P* View2 software (*Hamamatsu*). ImageJ Version 1.48 software (*National Institutes of Health*) colour deconvolution plugin was used to measure percentage of collagen. Glomeruli morphology was assessed using Jones Basement Membrane (PAS-M) staining kit (cat. # AR18011-2, *Dako*) following manufacturers protocol. Glomeruli basement membrane thickness, area, perimeter and roundness ((perimeter^2)/(4*π*area)) were calculated using the ND*P* View2 software.

For spleens, frozen sections were fixed for 10 mins in cold methanol, washed in PBS and then stained using hematoxylin and eosin following standard protocols. Slides were scanned using the Nanozoomer 2.0-HT C9600-13. Using the ND*P* View2 software, total spleen area and the areas of red and white pul*P* were measured. Random systematic sampling was used to select 10 images of each section at 80x magnification. The total number of cells in each image was counted using the Particle Analysis tool in the Image J Version 1.48 software.

Placental and Kidney protein expression analysis

Immunohistochemistry and western blots were used to quantitate protein expression of angiotensin converting enzyme (Ace), transferrin receptor, hydroxynonenal (4-HNE), Cu/Zn-Superoxide Dismutase (SOD) and 8-hydroxy-

2'-deoxyguanosine (8-OHdG). Antibodies and dilutions are provided in supplementary table 1 (Table S3.5).

As for placental and kidney morphometric analyses, cut sections were de-waxed, rehydrated according to standard protocols. Where required, antigen retrieval was performed and endogenous peroxidase activity was suppressed by incubating the slides in 3% hydrogen peroxide. Primary antibodies were diluted in 5% serum in PBS and applied overnight at 4°C in a humidified chamber. Negative controls were also included by omitting the primary antibody from the diluent. Secondary antibodies were diluted in 5% serum and slides were incubated for 1 h at room temperature. Primary antibody binding was amplified using streptavadin horseradish peroxidase (strep-HRP) (*Dako*) and detected using DAB (Sigma-Aldrich). Video Image Analysis (VIA) software (*Leading Edge Software*) was used to calculated intensity and percent positivity of DAB staining.

For western blotting, placental and kidney tissue were homogenised in ice-cold RIPA buffer. Protein concentrations were determined using a Bradford Assay (*Bio-rad*). 25 μg of protein was run on a 10% mini PROTEAN™ TGX™ pre-cast gel (Bio-Rad) following manufacturer's protocols and then transferred onto nitrocellulose membranes using the Criterion™ Blotter (*Bio-Rad*). Membranes were incubated overnight a 4°C in a blocking solution containing 3% skim milk in TBS-T (Tris-Buffered Saline containing Tween 20) before being incubated in the relevant primary antibody for 1 h at room temperature. After thorough washing in TBS-T, membranes were further incubated with a secondary antibody conjugated with HR*P* for 1 h at room temperature. Protein bands were visualised by chemiluminescence with ECL (*GE Healthcare*) on the Chemidoc M*P* (*Bio-Rad*). Levels of protein expression were normalised to HSP-90 or β-actin.

RNA extraction, placental microarray analysis and placenta, spleen and kidney qPCR

Placental, spleen and kidney tissues were homogenised using a Powerlyzer with ceramic 1.4 mm beads (*Mo Bio Laboratories, Inc*). Total RNA was extracted using Trizol (Invitrogen) following the manufacturer's instructions and RNA integrity was determined using the Experion (*Bio-Rad*) system.

For placental microarray, 31 placentas from 10 zinc-repletezinc-replete and 7 zinc-deficient dams were analysed as described by [73]. Affymetrix Mouse Gene 2.1 ST array data were checked for quality, RMA background-corrected and quantile normalised in R [74], using the aroma.affymetrix package [75] and v19.0.0 of an Ensembl-derived CDF (http://brainarray.mbni.med.umich.edu/Brainarray/). Detection above background (DABG) was performed by estimating non-specific binding (NSB) properties of the antigenomic probes using a modified version of the MAT background model [76] and obtaining empirical distributions of residuals across 10 bins of fitted values. After training the model on the set of antigenomic probes, fitted NSB estimates were obtained for the PM probes based on their sequence content. Fitted NSB estimates were subtracted from the observed PM intensities on the log2 scale and residuals were compared to the empirical distributions obtained from the antigenomic probes to provide p-values for detection of signal above the background. Probe-level P values were obtained using Fisher's method across the set of arrays, and only PM probes with a p-value < 0.05 were considered as containing "true" signal, giving 51.4% of probes which were able to be used for estimation of expression levels. Differential expression between diet groups was assessed using the package *limma* [77], and all p-values were corrected for multiple testing using the Benjamini-Hochberg method [78] to provide estimates of the false discovery rate (FDR).

The outcome of the microarray analysis was validated using qPCR on RNA samples from all placentas collected at post-mortems (n=72, 50 from zinc-

replete and 32 from zinc-deficient dams). PrimerPCR™ assays (Bio-Rad) specific for mouse *Transferrin Receptor* (*Tfrc*) (cat. # QMMUCID0039655) and *Transferrin* (*Trf*) (cat. # QMMUCID0061477) were purchased along with housekeeping assays for *Tbp* (cat. # QMMUCID0040542) and *Hmbs* (cat # QMMUCID0022816). *Tfrc* and *Trf* gene expression was also quantified in kidney samples from dams collected at GD18.5 (n=23, 12 zinc-replete and 11 zinc-deficient). Extracted RNA was DNase treated using TURBO DNA-free™ (*Ambion*) as per manufacturer's instructions and quality zinc-replete for DNase-treatment was double-checked using PCR for primers specific for mouse genomic DNA. 500 ng of DNase-treated RNA was reverse transcribed using iScript™ Reverse Transcriptase Kit (*Bio-Rad*) following manufacturer's instructions. Each cDNA sample was diluted 1:5 and added to a PCR reaction containing the relevant primer assay and Ssofast Evergreen Supermix (Bio-Rad). qPCR was performed in triplicate following cycling conditions recommended by the manufacturer. Data was analysed using the Bio-Rad CFX Manager Software v3.1 and all gene expression data was normalised to *Tbp* and *Hbms.*

Expression of genes associated with blood pressure zinc-replete and immune function were assessed in the kidneys and spleens of dams collected at GD18.5, respectively (n=17, 10 replete and 7 deficient). RNA from each kidney or spleen was DNAse treated as previously mentioned and 4 μg of DNase-treated RNA was reverse transcribed using iScript™ Advanced cDNA Synthesis Kit (*Bio-Rad*). cDNA was diluted 1:15 and added to a reaction mix containing SsoAdvanced™ SYBR® Green Supermix (Bio-Rad) and then added to PrimePCR™ Pre-eclampsia Tier 1 M384 plates (cat. # 10039501, *Bio-Rad*) for kidney RNA or PrimePCR™ Immune Tier 1 M384 plates (cat. # 10029798, Bio-Rad) for spleen RNA. Plates were run on the CFX384 PCR machine (*Bio-Rad*) with cycling conditions according to the manufacturer's protocol. The Bio-Rad CFX Manager Software v3.1 was used to analyse the data. For kidney gene expression,

data was normalised to *Tbp* and *Gapdh* while spleen gene expression was normalised to *Hprt* and *Gapdh*.

Statistics for radio-telemetry data

A generalized additive mixed model was fitted to the 24 h MA*P* and HR data in order to determine the effects of the diet on blood pressure using the *gamlss R* package in Revolution R Open (RRO) 3.2.0. For 24 h MAP, the model was adjusted for age, weight, log of activity and heart rate using a non-linear time trend using cubic spline. A random effect was also fitted for each dam. The estimated means of the two diet groups across days of gestation were then examined for each pregnancy state (pre-pregnancy, pregnancy; including mating and birth and lactation), as well as within pregnancy based on pivotal placental development events (day 0-5, 6-10 and 11-19).

S3.5.2 Figure and Tables

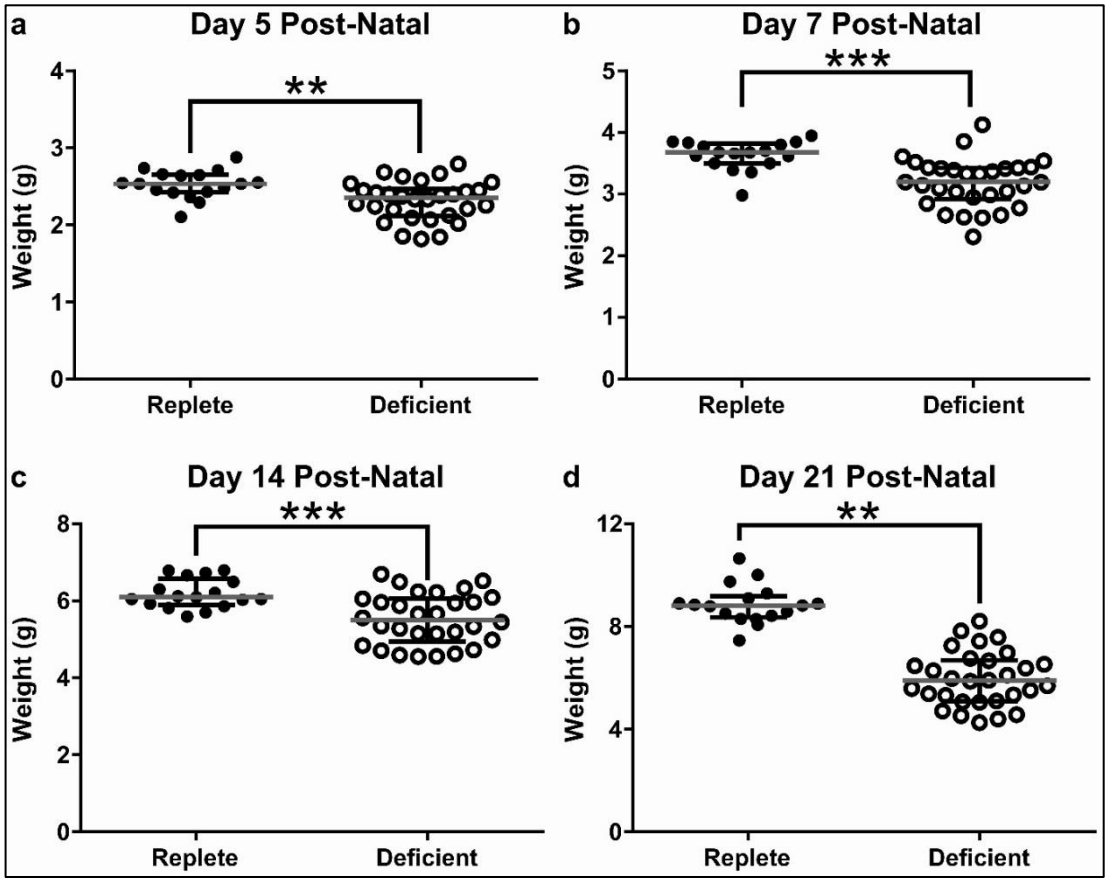

FIGURE S3.1. Maternal zinc deficiency reduced pup weight gain during lactation. Pup weight was significantly lighter in those from the zinc-deficient dams at 3 day post-natal (a), 1 week post-natal (b), 2 weeks post-natal (c) and at weaning, 3 weeks post-natal (d). Data are median and interquartile range (n = 17 and 30 pups from 4 zinc-replete and 6 zinc-deficient dams, respectively). Statistical significance was determined using Mann-Whitney Test on data based on an average litter size of 7.00. $^{**}P<0.01$, $^{***}P<0.001$.

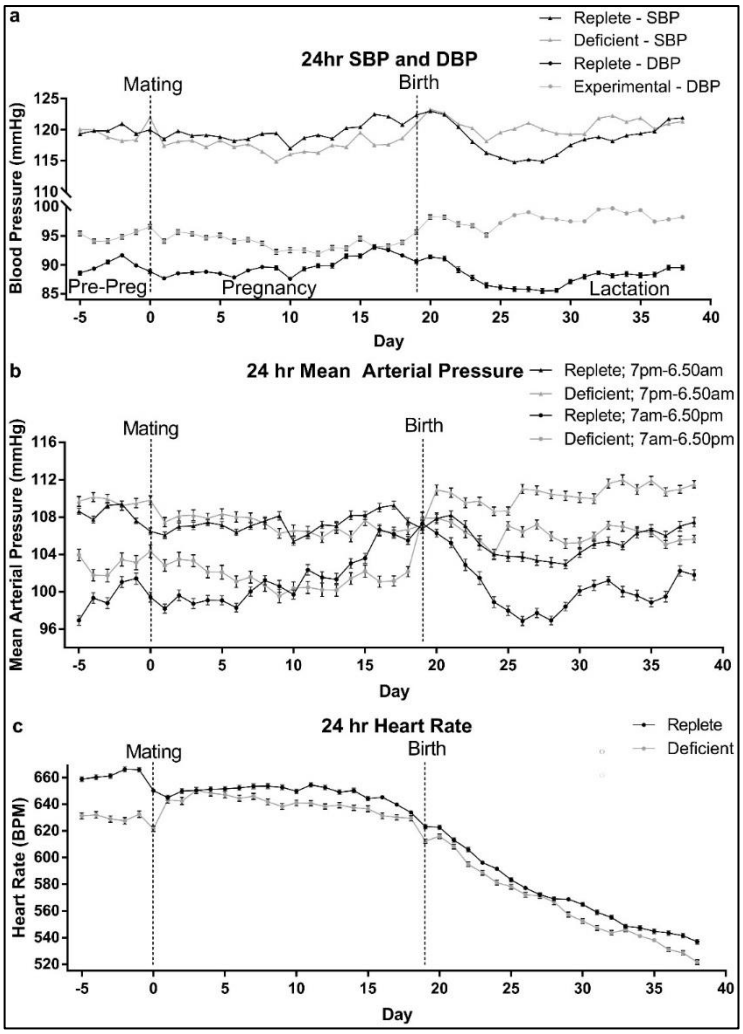

FIGURE S3.2. The effect of zinc on maternal systolic blood pressure (SBP) and diastolic blood pressure (DBP) as well as the influence of the day-night cycle. The SBP and DBP profiles prior to pregnancy, during pregnancy and in lactation were abhorrent within the animals fed the zinc deficient diet compared to those on a zinc replete diet (a). A clear circadian rhythm was observed in both diet groups as evident by higher 24 h mean arterial pressure (MAP) between the hours of 7pm to 6.50am compared to 7am to 6.50pm (b). During pregnancy differences in MAP between the two diet groups were driven by significant differences in MAP between the day light hours (7am-6.50pm). No significant differences between MAP in the night hours (7pm-6.50am) were observed between the two diet groups across pregnancy. 24 h heart rate (HR) was decreased in the zinc-deficient dams across all time periods assessed (c). Each data point represents the average 24 h SBP/DBP (a), MAP (b) or average 24 h HR (c) for each diet group ± SEM. n = 4 zin-replete and 4 zinc-deficient dams. Statistical differences were determined using a general additive model.

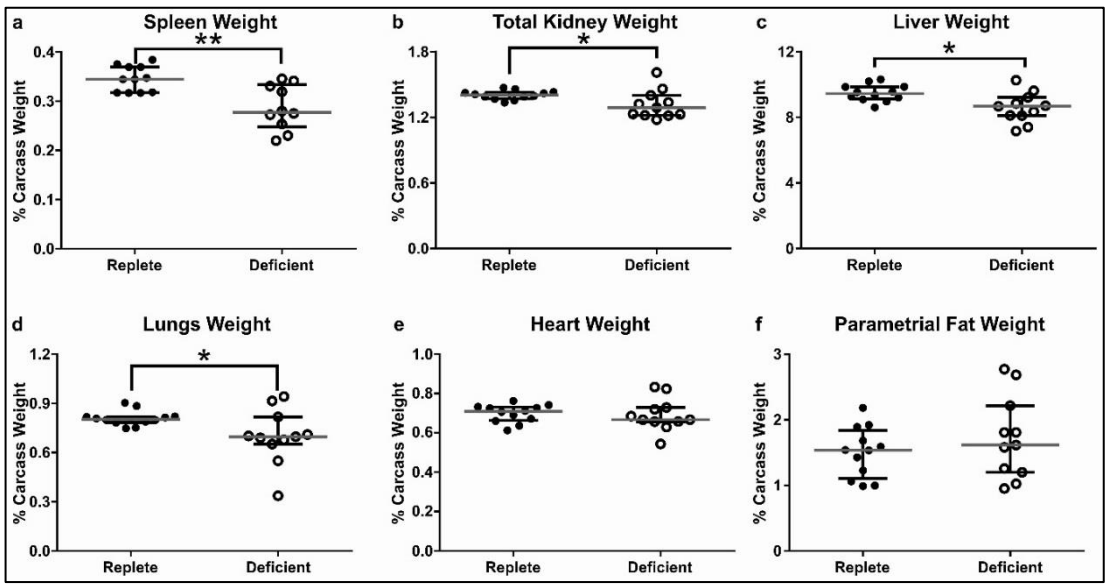

FIGURE S3.3. Liver (a), total kidney (b), spleen (c) and lungs (d) weight as a percentage of carcass weight were all reduced in the zinc-deficient dams at GD18.5. Heart (e) and parametrial fat (f) weight as a percentage of carcass weight did not change between the two diet groups. Data are median and interquartile range (n = 12 zinc-replete and 11 zinc-deficient dams). Statistical significance was determined using Mann-Whitney Test on data based on an average litter size of 7.00. *P<0.05, **P<0.01.

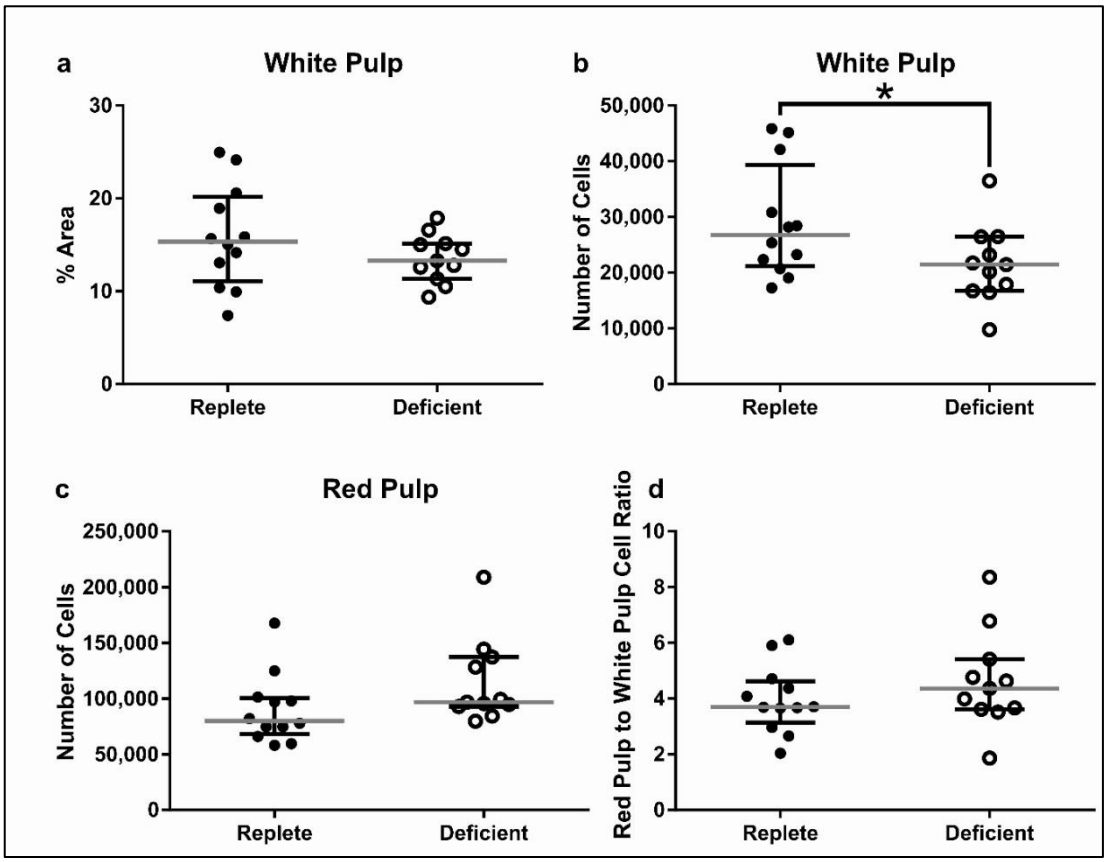

FIGURE S3.4. Histological analysis was used to determine the effects of zinc deficiency on spleen morphology at GD18.5. There was no difference in the percentage area of white pulp within the spleens between the two diet groups (a) but there was a decrease in the number of cell present within the white pulp of the zinc-deficient animals when compared to the zinc-replete animals (b). Analysis of the red pulp revealed no difference in the number of cells present within this area of the spleen (c) nor was there any difference in the red pulp to white pulp cell ratio (d). Data are median and interquartile range (n = 13 zinc-replete and 12 zinc-deficient dams). Statistical significance was determined using Mann-Whitney Test. **P*=0.051.

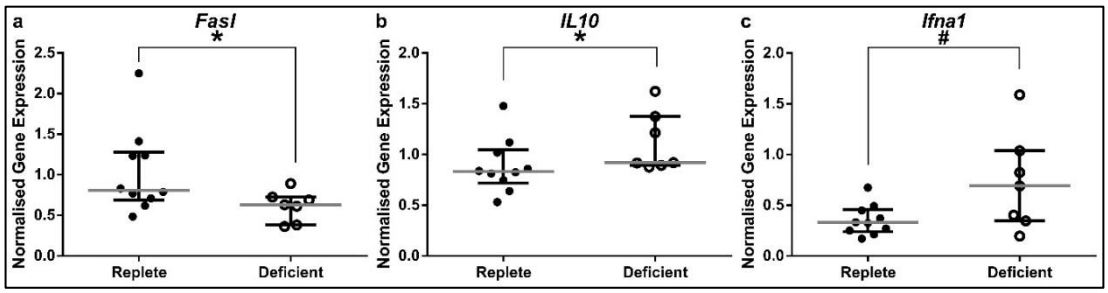

FIGURE S3.5. Maternal zinc deficiency resulted in differential gene expression of a number of cytokines specific for certain T cell populations in the spleens at GD18.5. A decrease in *Fas ligand* (*Fasl*) (A) and an increase in *interleukin 10* (*IL10*) expression (B) was observed in the spleens of the zinc-deficient dams. There was also a trend for an increase in the expression of *interferon alpha 1* (*Ifna1*). Date are median ± interquartile range (n = 10 zinc-replete and 7 zinc-deficient dams). Statistical significance was determined using Mann-Whitney Test. **P*<0.05, #*P* = 0.07.

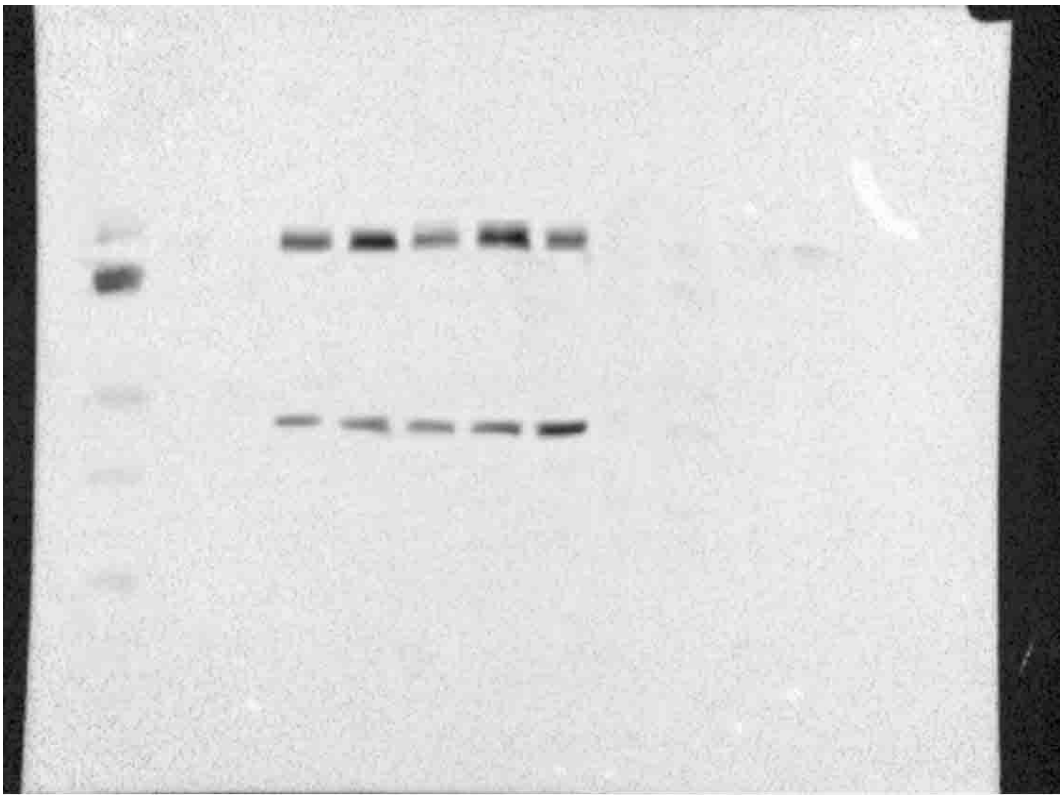

FIGURE S3.6. Full length western blot image of placental Tfrc expression. Tfrc protein band was observed at ~100kD whilst the corresponding β-actin band was observed at ~48kD

TABLE S3.1. Differences in elemental compositions of maternal plasma, placental tissue and fetal tissue

Element	Plasma Content	Placental Content	Fetal Content
Iron (mg/L; mg/kg)	520.02 [493.4-787.1] vs. 457.81 [357.6-635.3]	63.20 [54.6-85.4] vs. 49.60 [45.1-66]*	35.88 [35-39.4] vs. 28.94 [25.6-35]**
Potassium (mg/L; mg/kg)	32.28 [29.7-40.1] vs. 41.90 [34.4-51.6]*	2287.46 [1683.7-2932.7] vs. 2109.85 [1860.1-2685.1]^{NS}	2345.94 [2171.6-2511.4] vs. 2421.07 [2259.4-2494.2]^{NS}
Sulphur (mg/L; mg/kg)	132.12 [120-139.8] vs. 149.90 [140-164]**	1422.56 [910.4-1724] vs. 1299.33 [1109.1-1570.6]^{NS}	1146.06 [1108.7-1190.9] vs. 1139.88 [1123-1160.1]^{NS}
Phosphorus (mg/L; mg/kg)	16.42 [14.9-18] vs. 19.61 [18.6-22.4]**	2148.78 [1655.7-2406.1] vs. 2134.85 [1684.9-3147.5]^{NS}	2552.25 [2336.8-2684.8] vs. 2658.39 [2571-2720.6]^{NS}
Copper (μg/L; μg/kg)	238.29 [207.9-252] vs. 281.02 [234.8-296.2]*	2575.66 [1766.3-3023.9] vs. 3024.66 [1874.7-3734.8]^{NS}	2233.05 [2022.5-2387] vs. 2184.70 [2017.6-2484.3]^{NS}
Sodium (mg/L; mg/kg)	629.53 [606.5-650] vs. 654.81 [641.1-680.1]^{NS}	1118.93 [902.3-1751] vs. 1149.60 [759.8-1449.3]^{NS}	1694.22 [1647.8-1739.6] vs. 1768.22 [1703.1-1808.6]*

Data are presented as median [interquartile range]. n = 10 zinc-replete and 7 zinc-deficient dams and n= 19 and 13 placentas and fetuses from zinc-replete and zinc-deficient dams, respectively. Statistical significance was determined using a Mann-Whitney test. *P<0.0**5,** **P<0.0**1** NS, not significant.

TABLE S3.2. Elemental composition of the zinc-replete and zinc-deficient diets as determined by mass spectrometry

Element	Zinc-replete Diet (40 mg/kg)	Deficient Diet (10 mg/kg)	*P* value
Iron (mg/kg)	41 ± 7.3	35 ± 3.3	NS
Manganese (mg/kg)	10 ± 1.7	17 ± 13.7	NS
Copper (mg/kg)	8 ± 0.2	5 ± 1.2	$P<0.05$
Zinc (mg/kg)	39 ± 0.3	11 ± 1.3	$P<0.001$
Calcium (mg/kg)	6500 ± 141.4	6133 ± 115.5	NS
Magnesium (mg/kg)	715 ± 7.1	660 ± 26.5	NS
Sodium (mg/kg)	1850 ± 14.1	1963 ± 63.5	NS
Potassium (mg/kg)	4700 ±141.4	7267 ± 321.5	$P<0.01$
Phosphorus (mg/kg)	2300 ± 141.4	3600 ± 200.0	$P<0.01$
Sulphur (mg/kg)	3400 ± 0	3733 ± 152.8	NS
Aluminium (mg/kg)	4 ± 0.9	7 ± 0.2	NS

Elemental analysis of the diet composition was performed on two independent samples of zinc-replete chow and three independent samples of zinc-deficient chow and analysed by inductively-coupled plasma atomic emission spectrometry. Data are mean ± standard deviation. Statistical significance was determined using a two-tailed T-test, assuming unequal variances. NS, not significant

TABLE S3.3. Genes differentially expressed between zinc-replete and zinc deficient placentas

Gene (Gene symbol)	Fold change	Deficient Expression	*P* value	FDR
Transferrin Receptor (*Tfrc*)	8.59	↑	8.77E-06	0.112
Sclerostin domain containing 1 (*Sostdc1*)	2.70	↑	4.10E-05	0.182
Elongation of very long chain fatty acids-like 4 (*Elovl4*)	2.57	↑	4.70E-05	0.182
Interferon-related developmental regulator 1 (*Ifrd1*)	1.69	↓	6.95E-05	0.182
RIKEN cDNA 1700023L04 (*1700023L04Rik*)	2.40	↓	7.13E-05	0.182
WD repeat domain 60 (*Wdr60*)	1.67	↓	1.09E-04	0.231
TSC22 domain family, member 3 (*Tsc22d3*)	1.74	↓	1.27E-04	0.232
Glutamate receptor interacting protein 1 (*Grip1*)	2.21	↓	1.67E-04	0.266
Catsper channel auxiliary subunit delta (*Catsperd*)	2.80	↓	2.29E-04	0.324

TABLE S3.4. Effect of zinc deficiency on renal expression of seven genes at GD18.5 as quantified by qPCR

Gene	Normalised Expression (zinc-replete vs. zinc-deficient)	Difference in Deficient	*P* value
Ace	1.15 [1.04-1.75] vs. 0.97 [0.65-1.30]	↓16%	0.031
Actg2	0.26 [0.14-0.52] vs. 0.68 [0.43-1.08]	↑162%	0.011
Aldh3a2	1.36 [1.18-1.50] vs. 1.11 [1.01-1.32]	↓18%	0.031
Braf	1.46 [1.28-1.74] vs. 1.07 [1.03-1.31]	↓27%	0.005
Calcoco1	1.57 [1.26-1.71] vs. 1.01 [0.85-1.09]	↓36%	0.002
Cbx7	1.45 [1.28-1.74] vs. 1.03 [0.76-1.17]	↓29%	0.003
Itpr2	1.03 [0.77-1.37] vs. 0.58 [0.55-1.03]	↓44%	0.05

Data represented as median [IQR]. Gene expression was normalised to *TbP* and *Gapdh*. n = 9 and 7 kidneys from zinc-replete and zinc-deficient dams, respectively. Statistical significance was determined using a Mann-Whitney test.

TABLE S3.5. Antibodies and dilutions used for immunohistochemistry and western blots

Antigen	Supplier	Species	Clone	IHC Dilution	Antigen Retrieval	Western Blot Dilution
Vimentin	Dako	Monoclonal Mouse	Vim 3B4	1/10	Yes, Pronase	
Pan-cytokeratin	Chemicon	Monoclonal Mouse	AE1/AE3	1/100	Yes, Pronase	
ACE	Santa Cruz	Polyclonal Rabbit	H-170	1/50	Yes, Citrate	1/250
Tfrc	Thermo Fisher	Monoclonal Mouse	H68.4	1/500	Yes, Pronase	1/1500
4-HNE	Alpha Diagnostics	Polyclonal Rabbit		1/500	Yes, 0.1% Triton and 0.1% Tween 20 in TBS	1/1000
8-OHdG	JaICa	Monoclonal Mouse	N45.1	1/100	Yes, Citrate	
Cu/Zn-SOD	Sigma	Polyclonal Rabbit	SOD1	1/2000	Yes, Citrate	
HSP-90	Santa Cruz	Polyclonal Rabbit	H-114			1/2000
β-actin	Santa Cruz	Polyclonal Rabbit				1/1000

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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
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Chapter 4

Early pregnancy maternal trace mineral status and the association with adverse pregnancy outcome in a cohort of Australian women

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ABSTRACT

Maternal micronutrient deficiencies in pregnancy can have profound effects on fetal development and pregnancy outcome. Plasma trace minerals including copper, zinc, selenium and iron have been shown to be extremely important in supporting reproduction. We sought to determine whether there is an association between maternal trace mineral status in early pregnancy and pregnancy complications using a prospective cohort study of 1065 pregnant Australian women who were recruited as part of the Screening for Pregnancy Endpoints (SCOPE) study in Adelaide. Copper, zinc, selenium and iron present in the plasma were measured using mass spectrometry in samples collected at 15±1 weeks' gestation. After adjusting for covariates, women with lower plasma copper (<27.9 μmol/L and 27.9-32.5 μmol/L) had decreased risk for any pregnancy complication when compared with women with high plasma copper (>32.5 μmol/L) (aRR = 0.87; 95% CI = 0.76, 0.99 and aRR = 0.88; 95% CI = 0.78, 1.00,

respectively). This was also observed when adjusting for plasma zinc and selenium status (aRR = 0.86; 95% CI = 0.74, 1.00). Combined low copper and zinc status was also associated with a reduced risk of any pregnancy complication as compared with high copper and zinc status (aRR = 0.80; 95% CI = 0.70, 0.93). These results provide justification for further work into elucidating the mechanistic role of trace elements in early pregnancy, as well as their interactions in supporting successful pregnancy outcomes.

4.1 Introduction

Maternal nutrient stores and diet supply all the macro- and micro-nutrients to support optimal fetal growth essential for successful pregnancy [1]. Hence, it is not surprising that maternal deficiencies in key micronutrients can have profound effects on fetal development and pregnancy outcome [2]. Pregnancy complications including preeclampsia (PE), gestational diabetes mellitus (GDM), spontaneous preterm birth (sPTB) and fetal growth restriction (FGR) together affect 25% of first pregnancies and predict lifelong morbidity and mortality for both the mother and infant [3]. Furthermore, micronutrient deficiencies which tend to be associated with decreased consumption of foods rich in micronutrients, have also been associated with the development of PE, GDM, sPTB, FGR, as well as gestational hypertension (GH) [2].

Extensive investigations into micronutrient deficiencies have focused on those common within pregnant populations including folate and vitamin D [4]. However, evidence is emerging about the importance of trace minerals like iron, zinc and copper in supporting successful pregnancy [5]. It is known that trace minerals are crucial for the maintenance of cell proliferation and function with severe deficiencies in copper and zinc during pregnancy having been shown to have a teratogenic effect on the fetus [6]. This is likely driven by a reduction in the activity of key enzymes which require these metals structurally in order to function, as well as compromised oxidant defence systems [6]. It is also important

to acknowledge the importance of micronutrients in mediating inflammation and the immune response. Animal models of iron, copper and zinc deficiencies have been shown to be associated with compromised immunity and increased susceptibility to infection [7]. Pregnancy complications including PE and FGR have been associated with increased oxidative stress and circulating markers of inflammation [8, 9] and therefore there may be a causal connection between micronutrient deficiencies in pregnancy and the development of pregnancy complications mediated by oxidative stress and inflammation.

The association between trace minerals and pregnancy have been described previously [10-17], however many of these studies have been conducted in late pregnancy or at term. Given that many of these pregnancy complications originate in early gestation, it is important to also understand how micronutrient status in the first trimester is associated with adverse pregnancy outcomes. Marginal micronutrient deficiencies early in pregnancy may lead to more severe deficiencies later in pregnancy due to increased metabolic demands from the rapidly growing placenta and fetus. Thus we aimed to determine whether deficiencies in the trace minerals copper, zinc, selenium and iron at 15±1 weeks' gestation may be associated with a number of pregnancy complications. As copper and zinc share similar electro-chemical properties and biological pathways [18], we also explored interactions between zinc and copper status in early pregnancy and their relationship with adverse pregnancy outcomes with the goal of better understanding how these minerals may be important to successful pregnancy.

4.2 Methods

4.2.1 Study Participants

Plasma samples were obtained from Adelaide participants recruited as part of the international prospective Screening for Pregnancy Endpoints (SCOPE) study. Nulliparous women carrying a singleton pregnancy were recruited at 15±1

weeks' gestation from the Lyell McEwin Hospital, Adelaide, Australia between November 2004 and September 2008. Ethics approval was gained from the University of Adelaide ethics committee and all women provided written consent (approval no: REC 1712/5/2008). At recruitment, women were interviewed by a research midwife and asked questions on maternal demographics and had physical measurements recorded. These included age, body mass index (BMI) and smoking status [19]. Biochemical markers were also measured at 15±1 weeks' gestation and included plasma C-reactive protein (CRP) [20]. Women were not eligible to participate in the study if they suffered from a pre-existing medical condition or had obstetric history which placed them at high risk of developing PE, sPTB or delivering a small-for-gestational age (SGA) infant. Those who had suffered three or more miscarriages or had undergone three or more pregnancy terminations were also excluded.

Uncomplicated pregnancies were defined as those without any pregnancy disorder and included normotensive women who delivered an appropriate weight for gestational age infant at term (≥37 weeks' gestation) [21]. GH was diagnosed as the development of high blood pressure (systolic blood press ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg) on at least two occasions after 20 weeks' gestation. PE was defined as GH in conjunction with proteinuria (24 h urinary protein ≥300 mg or a spot urine protein:creatinine ratio ≥30 mg/mmol creatinine or urine dipstick protein ≥2) or any multisystem complication of PE or uteroplacental dysfunction [22]. GDM was diagnosed according to the International Associations of Diabetes and Pregnancy Study Groups criteria [23]. sPTB was defined as the spontaneous onset of labour at <37 weeks' gestation. SGA was defined as birth weight below the 10th customised centile adjusted for maternal height, booking weight, ethnicity, delivery gestation and infant sex.

4.2.2 Elemental Analysis

Venous blood samples were provided at 15±1 weeks' gestation and collected into heparinised tubes in order to obtain plasma samples. Plasma samples were then analysed using inductively-coupled plasma mass spectrometry (ICP-MS) (Agilent 7700 ICP-MS) (Agilent 5100 ICP-OES; *CSIRO Analytical Services, South Australia*) to measure the concentrations of copper, selenium, iron and zinc. Prior to analysis, 250 μL of plasma was digested in concentrated nitric acid (~70% HNO_3) in sealed Teflon containers for approximately 48 hours and then diluted. Samples were run alongside two internal standards: iridium and rhodium (*Choice Analytical*) at a concentration of 200 ppb and an 8-point calibration, including blank, was carried out between 0.01 μg/L and 100 μg/L. There were 47 (4.4%) women with plasma iron levels below the detection limit of 7.16 μmol/L and were therefore assigned 7.15 μmol/L.

4.2.3 Statistical Analysis

All statistical analysis was performed in R (v3.1.1) [24]. Baseline characteristics were tested for normality using the Shapiro-Wilk test and summarised according to pregnancy outcome. Fisher's exact tests were performed for categorical variables and Welch's *t* test for continuous variables comparing women with each pregnancy complication to all other women. Plasma copper, zinc, selenium and iron were expressed as mean (± standard deviation: SD) and compared between each pregnancy complication and all women using a non-parametric Mann-Whitney *U*. Spearman's correlations were used to examine the relationship between each of the trace minerals with each other, as well as with circulating CRP; a marker of inflammation.

In order to assess the effects of each trace mineral on pregnancy outcome, plasma copper, zinc, selenium and iron were divided into tertiles based on their distribution amongst all women in this study. Relative risks (RR) and 95% confidence intervals (CIs) of pregnancy complications from any complication,

PE, GH, GDM, sPTB and SGA for copper, zinc, selenium and iron were examined using multivariable Poisson regression with robust variance estimation. Multivariable adjustment was made for maternal age, maternal BMI and smoking status at 15±1 weeks' gestation (yes compared to no) as covariates. Maternal socioeconomic status, determined by assigning the New Zealand socioeconomic index score (SEI) [25], was initially included in the adjusted model but did not change any effects observed and was subsequently removed in the final analyses. The final analyses were also repeated for copper, zinc and selenium adjusting for each other as well as covariates previously mentioned.

4.3 Results

Of the 1164 SCOPE women recruited in Adelaide, 1065 (91%) plasma samples from 15±1 weeks' gestation were available for analysis of trace minerals. These included 558 (52%) women whose pregnancies were uncomplicated, 85 (8%) who later developed PE, 108 (10%) who were diagnosed with GH, 51 (5%) who were diagnosed with GDM, 65 (6%) who delivered spontaneously preterm and 134 (13%) who delivered an SGA infant. Mean maternal age and BMI for all women whose plasma was analysed was 24±5 years and 27.01±6.52 kg/m^2 (Table 4.1). Compared to those whose pregnancies were uncomplicated, women who went on to have a pregnancy complication had a higher BMI in early pregnancy but there was no difference in maternal age, smoking status or use of supplements at 15±1 weeks' gestation.

Plasma trace minerals in all women ranged from 10.3 to 52.99 μmol/L for copper, 3.24 to 34.70 μmol/L for zinc, 0.253 to 1.785 μmol/L for selenium and 7.14 to 72.60 μg/L for iron. Mean plasma copper at 15±1 weeks' gestation was higher in women who went onto have a pregnancy complication when compared to those whose pregnancies remained uncomplicated (Table 4.1; P<0.001). Moderate differences in circulating levels of zinc, selenium and iron were also observed in

the women who later developed a pregnancy complication compared to those who did not (Table 4.1).

TABLE 4.1 Participant characteristics of the Adelaide SCOPE cohort in which plasma trace minerals were measured at 15±1 weeks' gestation.

	All (*n* = 1065)	**Uncomplicated (*n* = 558)**	**Complicated (*n* = 507)**	***P* value**
Age *yrs*, mean (SD) [range]	23.71 (5) [14-43]	23.59 (5) [14-40]	23.85 (5) [15-43]	0.399
BMI kg/m^2, mean (SD) [range]	27.01 (6.52) [16.2-58.5]	26.30 (5.86) [16.2-49.7]	27.77 (7.12) [16.4-58.5]	**0.000**
Smoking status, *n* (%)				0.063
No	651 (61)	352 (54)	299 (46)	
Quit	152 (14)	85 (56)	67 (47)	
Smoking	262 (25)	121 (46)	141 (54)	
Education, *n* (%)				0.410
No Secondary	422 (40)	211 (50)	211 (50)	
Secondary	608 (57)	327 (54)	381 (46)	
Tertiary	35 (3)	20 (57)	15 (43)	
Supplement Use, *n* (%)				0.535
No	105 (10)	51 (49)	53 (51)	
Yes	960 (90)	506 (53)	454 (47)	
Plasma Cu *µmol/L*, mean (SD) [range]	30.30 (5.49) [10.30-52.99]	29.61 (5.18) [14.76-47.73]	31.07 (5.72) [10.30-52.99]	**0.000**
Plasma Zn *µmol/L*, mean (SD) [range]	9.39 (2.32) [3.24-34.70]	9.28 (2.11) [4.83-21.36]	9.51 (2.52) [3.24-34.70]	0.077
Plasma Se *µmol/L*, mean (SD) [range]	0.919 (0.151) [0.253-1.785]	71.96 (11.06) [0.544-1.380]	73.36 (12.74) [0.253-1.785]	0.055
Plasma Fe *µmol/L*, mean (SD) [range]	19.06 (7.77) [7.14-72.60]	19.49 (7.90) [7.14-71.06]	18.61 (7.61) [7.14-72.60]	0.053

Supplement use was defined as using any form of mineral or vitamin supplement at 15±1 weeks' gestation.
P values for continuous variables were determined using Welch t-test, categorical variables a Fisher's exact test and plasma trace elements a Mann-Witney U test comparing women with a pregnancy complication to those whose pregnancies remained uncomplicated. BMI: body mass index; Cu: Copper; Zn: Zinc; Se: Selenium; Fe: Iron

Circulating copper was positively correlated with both zinc (Supplementary Fig. S4.1A; R^2=0.263) and selenium (Supplementary Fig. S4.1B; R^2=0.303) but negatively correlated with iron (Supplementary Fig. S4.1C; R^2=-0.156). Iron on the other hand, was positively correlated with zinc (Supplementary Fig. S4.1E; R^2=0.107) and selenium (Supplementary Fig. S4.1F; R^2=0.204).

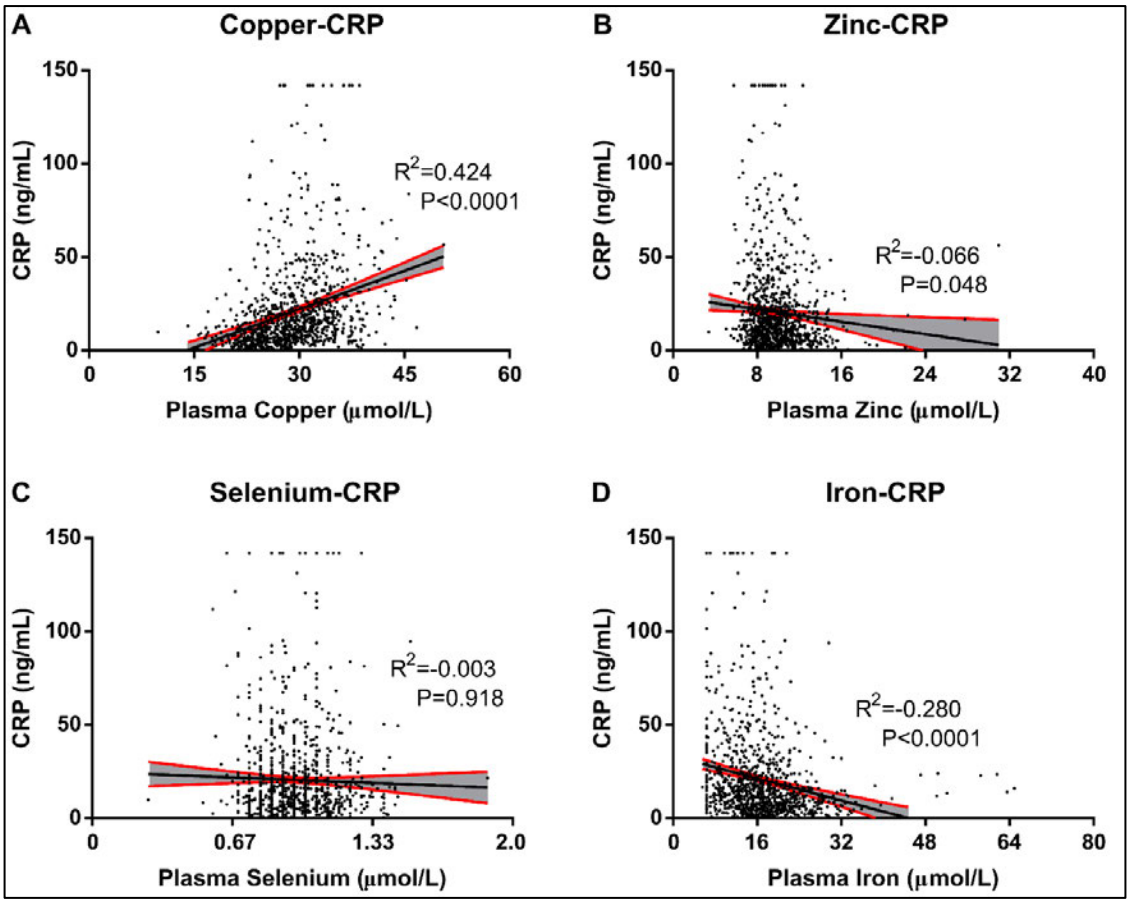

FIGURE 4.4. Spearman's correlations between plasma trace minerals at 15±1 weeks' gestation and serum CRP at 15±1 weeks' gestation. A significant positive correlation was seen between plasma copper and CRP (A), whilst significant negative correlations were observed between plasma zinc (B) and plasma iron (D) with CRP. No correlation was found between plasma selenium and CRP (C).

A significant positive correlation between plasma copper and plasma CRP was observed at 15±1 weeks' gestation (Fig. 4.1A: R2= 0.423, P<0.001) while there was an inverse, albeit not as strong, correlation between plasma zinc and plasma iron and CRP (Fig. 4.1B: R2=-0.066, P=0.048 and Fig. 4.1D R2= -0.274, P<0.001, respectively). When BMI was categorised as underweight (≤20 kg/m^2), normal weight (20.1-24.9 kg/m^2), overweight (25-29.9 kg/m^2) and obese (≥30 kg/m^2), compared with normal weight women, plasma zinc, selenium and iron was lower in the obese women (Fig. 4.2). Plasma copper on the other hand was significantly higher in the obese women when compared to underweight, normal weight and overweight women (Fig. 4.2).

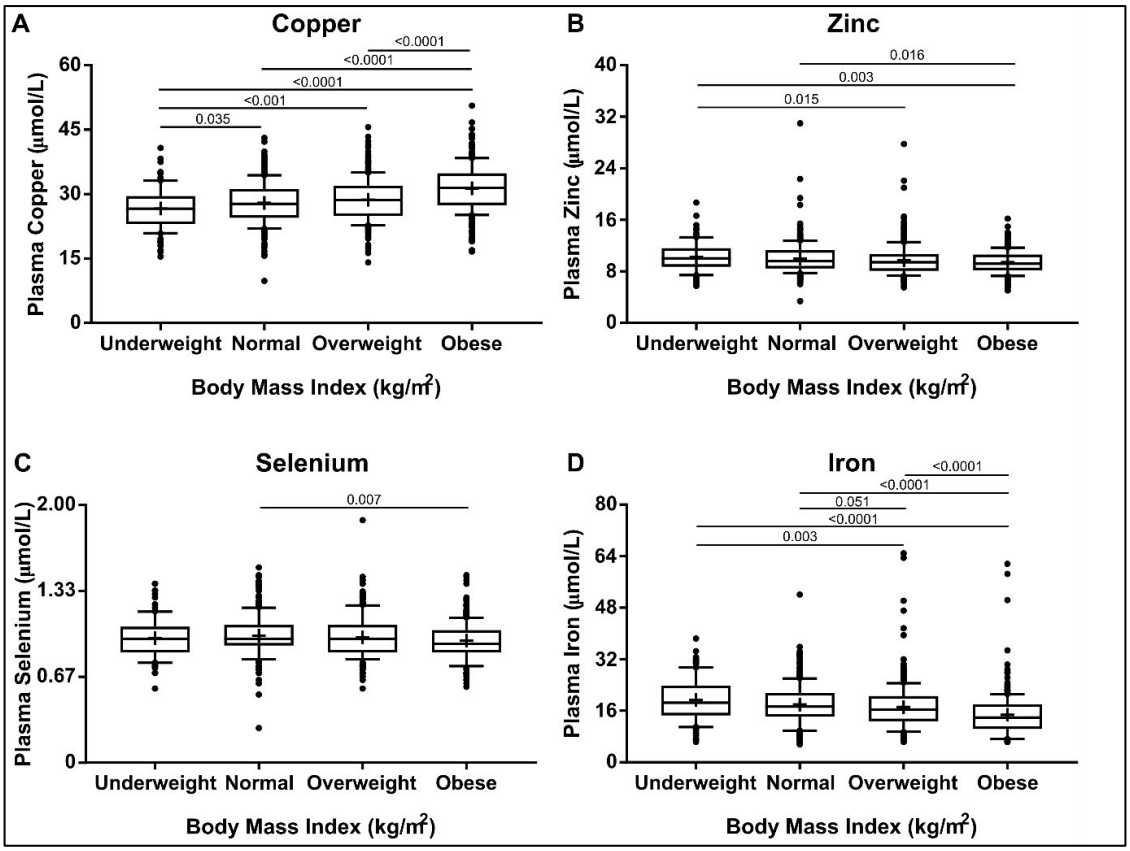

FIGURE 4.5. Relationship between plasma trace minerals at 15±1 weeks' gestation and maternal body mass index (BMI). Compared with normal weight (BMI 20.1-24.9) women, plasma copper was lower in lean (BMI≤20) women, higher in obese (BMI≥30) and no different in overweight (BMI 25-29.9) (A). Plasma zinc (B), plasma selenium (C) and plasma iron also did not change between normal weight and overweight women, however, were significantly lower in the obese women when compared to normal weight women. Box plots represent median: 10th-90th centile. Groups compared using a Dunn's test with a Bonferroni post-hoc test.

The adjusted relative risks (aRR) for each pregnancy complication with plasma copper, zinc, selenium and iron categories based on population tertiles are shown in Table 4.2. Women with lower plasma copper (1st and 2nd tertile) had decreased risk for any pregnancy complication combined compared with women with high plasma copper (3rd tertile) (aRR = 0.77; 95% CI = 0.66, 0.91 and aRR = 0.81; 95% CI = 0.70, 0.95, respectively). When the pregnancy complications were separated, this protective effect of low plasma copper was seen in all but GH however, these were not statistically significant. Women with lower plasma zinc and selenium (1st tertile) also had decreased risk of any pregnancy

complication when compared to high plasma zinc and plasma selenium (aRR = 0.86; 95% CI = 0.74, 1.00 and aRR: 0.84; 95% CI = 0.73, 0.96, respectively). Given that the strongest effect was observed with plasma copper, we sought to assess whether an association between plasma copper status at 15±1 weeks' gestation was still observed after adjust for plasma zinc and plasma selenium (Supplementary Table S4.1). A significant decreased risk for any complication was observed in women within the first and second tertile of plasma copper when compared to women in the third tertile indicating a relationship independent of plasma zinc or selenium status (aRR = 0.81; 95% CI = 0.69, 0.61 and aRR = 0.84; 95% CI = 0.72, 0.98, respectively). There was no significant effect of maternal iron status in early pregnancy on developing any pregnancy complication. However, women with plasma iron in the 1st and 2nd tertile were at increased risk of GH when compared to women in the 3rd tertile (aRR = 1.78; 95% CI = 1.05, 3.02 and aRR = 1.87; 95% CI = 1.12, 3.11, respectively).

A sensitivity analysis was performed on the relationship between plasma copper and CRP in order to determine the relationship between copper, inflammation and pregnancy outcome. Serum CRP measured at 15±1 weeks' gestation was used as an indirect marker of inflammation. Women with CRP ≥20.3 ng/mL were excluded as normal circulating CRP ranges between 0.4 to 20.3 ng/mL [26] and levels higher than 20 ng/mL are often associated with infection. Supplementary Table 2 shows the effects of plasma copper status in all (n = 1065) women as well as those with CRP <20.3 ng/mL (n = 717). The point estimates for the effects for lower plasma copper in the women whose CRP was <20.3 ng/mL was similar to those for all women (1st tertile: all women = 0.77 vs. women with CRP <20.3 ng/mL = 0.74 & 2nd tertile: all women = 0.81 vs. women with CRP <20.3 ng/mL = 0.78) indicating the possibility that the effects of plasma copper status in this study were likely to be independent of inflammation.

Given that copper and zinc share similar electro-chemical properties and biological pathways [18], the interactions between these two trace minerals was

analysed in relation to pregnancy complications (Table 4.3). Combined low zinc status (1st tertile) and low copper status (1st tertile) was associated with a reduced risk of any pregnancy complications compared with high zinc status and high copper status (2nd and 3rd tertile combined for both trace minerals)(aRR = 0.72; 95% CI = 0.62, 0.85). Similarly, combined low copper and high zinc was also associated with reduced risk of any pregnancy complication when compared to combined high zinc and high copper (aRR = 0.76; 95% CI = 0.62, 0.94) suggesting copper status is a more important determinant of pregnancy outcome. When each pregnancy complication was analysed separately, a similar protective effect of combined low copper and low zinc was seen in PE, GDM, sPTB and SGA. For SGA, this was statistically significant (aRR = 0.62; 95% CI = 0.41, 0.94). For GDM, combined low copper and high zinc, as well as high copper and low zinc, also appeared to increase the risk when compared to combined high copper and high zinc although, these were not statistically significant (aRR = 1.46; 95% CI = 0.57, 3.69 and RR = 1.78; 95% CI = 0.77, 4.10, respectively).

4.4 Discussion

In this study, we have comprehensively assessed plasma trace minerals in early pregnancy and their association with multiple pregnancy complications. Contrary to previous observations of the negative impacts of poor trace element status on pregnancy outcome, we observed a protective effect of lower levels of plasma copper, zinc and selenium at the beginning of pregnancy on the risk of any pregnancy complication. However, this relationship is highly likely to be driven by copper status rather than zinc or selenium as plasma copper was found to be associated with adverse pregnancy outcome after adjusting for plasma zinc and selenium. This provides justification for further work into elucidating the role of copper in pregnancy, and the interaction with other trace elements, particularly plasma zinc, in early pregnancy.

TABLE 4.2. Adjusted relative risks of pregnancy complications from all complications, preeclampsia (PE), gestational hypertension (GH), gestational diabetes mellitus (GDM), spontaneous preterm birth (sPTB) and small-for-gestational age (SGA) according to plasma levels of trace minerals categorised based on population tertiles

	All women	Pregnancy Complication											
		Any Complication		PE		GH		GDM		sPTB		SGA	
	n	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)
Copper			**0.77**		0.71		1.11		0.69		0.62		**0.66**
<27.9 µmol/L	360	150 (42)	**(0.66, 0.91)**	21 (6)	(0.42, 1.18)	33 (9)	(0.71, 1.75)	13 (4)	(0.35, 1.37)	20 (6)	(0.34, 1.15)	34 (9)	**(0.44, 1.00)**
27.9 -32.5 µmol/L	349	156 (45)	**0.81 (0.70, 0.95)**	27 (8)	0.88 (0.55, 1.42)	36 (10)	1.16 (0.75, 1.80)	13 (4)	0.66 (0.33, 1.32)	16 (5)	**0.52 (0.28, 0.98)**	49 (14)	0.95 (0.66, 1.38)
>32.5 µmol/L	356	201 (56)	1.0†	37 (10)	1.0†	39 (11)	1.0†	25 (7)	1.0†	29 (8)	1.0†	51 (14)	1.0†
Zinc													
<8.3 µmol/L	357	160 (45)	**0.86 (0.74, 1.00)**	26 (7)	0.80 (0.48, 1.32)	42 (12)	1.01 (0.66, 1.56)	15 (4)	0.82 (0.43, 1.55)	15 (4)	0.85 (0.47, 1.52)	42 (12)	0.75 (0.52, 1.08)
8.3-9.9 µmol/L	351	165 (47)	0.91 (0.78, 1.06)	29 (8)	0.91 (0.56, 1.47)	32 (9)	0.91 (0.58, 1.43)	14 (4)	0.97 (0.50, 1.88)	27 (8)	0.91 (0.51, 1.60)	36 (10)	**0.65 (0.44, 0.96)**
>9.9 µmol/L	356	182 (51)	1.0†	30 (8)	1.0†	34 (10)	1.0†	22 (6)	1.0†	23 (6)	1.0†	56 (16)	1.0†
Selenium			**0.84**		0.72		0.94		1.08		0.76		0.81
<0.9 µmol/L	449	203 (45)	**(0.73, 0.96)**	33 (7)	(0.45, 1.18)	49 (11)	(0.62, 1.42)	19 (4)	(0.55, 2.15)	25 (6)	(0.44, 1.31)	49 (11)	(0.55, 1.19)
0.9-1.0 µmol/L	199	83 (42)	**0.80 (0.66, 0.96)**	15 (8)	0.82 (0.45, 1.48)	16 (8)	0.77 (0.44, 1.35)	11 (6)	1.71 (0.78, 3.77)	9 (5)	0.62 (0.29, 1.33)	25 (13)	0.96 (0.61, 1.50)
>1.0 µmol/L	417	221 (53)	1.0†	37 (9)	1.0†	43 (10)	1.0†	21 (5)	1.0†	31 (7)	1.0†	60 (14)	1.0†
Iron			1.08		0.81		**1.78**		1.16		1.11		1.22
<15.6 µmol/L	356	180 (51)	(0.92, 1.27)	31 (9)	(0.49, 1.35)	47 (13)	**(1.05, 3.02)**	19 (5)	(0.53, 2.56)	24 (7)	(0.60, 2.03)	45 (13)	(0.81, 1.83)
15.6-21.2 µmol/L	353	171 (48)	1.08 (0.92, 1.26)	26 (7)	0.87 (0.52, 1.46)	40 (11)	**1.87 (1.12, 3.11)**	20 (6)	1.46 (0.74, 2.89)	18 (5)	0.78 (0.43, 1.43)	52 (15)	1.36 (0.92, 2.01)
>21.2 µmol/L	355	155 (44)	1.0†	28 (8)	1.0†	20 (6)	1.0†	12 (3)	1.0†	23 (6)	1.0†	37 (10)	1.0†

*Adjusted relative risks were adjusted for age, maternal body mass index and smoking status at 15±1 weeks' gestation (no versus yes).
†Reference category

TABLE 2.3. Adjusted relative risks (aRR) of pregnancy complications from all complications, preeclampsia (PE), gestational hypertension (GH), gestational diabetes mellitus (GDM), spontaneous preterm birth (sPTB) and small-for-gestational age (SGA) based on stratification by combined plasma copper and plasma zinc concentrations

	All women	Pregnancy complication											
		Any Complications		PE		GH		GDM		sPTB		SGA	
	n	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)
Low Cu Low Zn	519	222 (43)	**0.72 (0.62, 0.85)**	36 (7)	0.67 (0.39, 1.16)	55 (11)	0.94 (0.57, 1.55)	17 (3)	0.71 (0.31, 1.61)	27 (5)	0.55 (0.30, 1.01)	57 (11)	**0.62 (0.41, 0.94)**
Low Cu High Zn	192	85 (44)	**0.76 (0.62, 0.94)**	13 (7)	0.75 (0.37, 1.51)	14 (7)	0.67 (0.34, 1.34)	9 (5)	1.46 (0.57, 3.69)	9 (5)	0.49 (0.21, 1.12)	27 (14)	0.81 (0.49, 1.34)
High Cu Low Zn	197	105 (53)	0.86 (0.72, 1.03)	19 (10)	0.87 (0.47, 1.61)	20 (10)	0.86 (0.47, 1.56)	17 (9)	1.78 (0.77, 4.10)	14 (7)	0.75 (0.37, 1.50)	22 (11)	0.64 (0.38, 1.07)
High Cu High Zn	156	95 (61)	1.0†	17 (11)	1.0†	19 (12)	1.0†	8 (5)	1.0†	15 (10)	1.0†	28 (18)	1.0†

Low Copper (Cu) ≤32.5 μmol/L (1st and 2nd tertile combined), High Copper >32.5 μmol/L (3rd tertile) , Low Zinc (Zn) ≤9.91 μmol/L (1st and 2nd tertile combined), High Zinc >9.91 μmol/L (3rd tertile)

*Adjusted relative risks were adjusted for age, maternal body mass index and smoking status at 15±1 weeks' gestation (no versus yes).

†Reference category

Trace elements like copper, zinc and selenium are integral to supporting cellular and tissue physiology [27]. Indeed, animal models of copper, zinc and selenium deficiency in pregnancy consistently show negative effects on fertility, fetal growth and offspring health and wellbeing [28-32]. Copper status in pregnancy has been infrequently reported in the literature and has focused predominantly on copper deficiency as adequate supply of copper during pregnancy is necessary for early embryonic development [33]. Circulating levels of copper increase during pregnancy due to the increase in copper-carrying proteins in the blood mediated by estrogen [34, 35]. Furthermore, in this population, circulating plasma copper was similar to pregnancy reference ranges (10-90th centile: 26.4 to 33.8 μmol/L) and to those reported in other populations of pregnant women [26, 34, 36-38]. Thus, our findings of higher copper in early pregnancy in women who subsequently develop a pregnancy complication, which are similar to other studies that have measured copper concentrations in late pregnancy [15-17], suggest higher levels of circulating copper may contribute to adverse pregnancy outcomes.

Of the three trace minerals, zinc in pregnancy has been most widely studied. In human populations, zinc deficiency in pregnancy may be associated with delivery of a low birthweight infant as well as with hypertensive disorders of pregnancy, particularly in populations of women vulnerable to inadequate zinc intakes in pregnancy [39]. However, inconsistencies between the studies on the effect of zinc deficiency in pregnancy reflects real uncertainty within the literature. Furthermore, the evidence with respect to the relationship between selenium status and adverse pregnancy outcomes has not clearly been established [40]. Never-the-less, our findings that lower levels of zinc and selenium at 15±1 weeks' gestation is associated with a protective effect on pregnancy outcome is in conflict with current dogma that deficiencies in these nutrients are detrimental to pregnancy health. However, given that both plasma zinc and selenium positively correlated with plasma copper, and the protective

effect of lower plasma copper persisted once plasma zinc and selenium were adjusted for, implicates plasma copper status as the main contributor to adverse pregnancy outcomes in this study. This is further supported by the finding on combined copper and zinc status as whilst combined low copper and low zinc was associated with reducing the risk of all pregnancy complications, equivalently, low copper and high zinc also appeared to reduce the risk.

The greatest effect observed in this study was in the association between lower plasma copper in early pregnancy and the development of any pregnancy complication. This suggests a role for this trace mineral either within the causal pathway of, or as an early pregnancy biomarker for, adverse pregnancy outcomes. PE, GDM, sPTB and SGA are all often associated with placental dysfunction [41]. Pregnancies complicated by placental dysfunction can be characterised by impaired trophoblast invasion and transformation of the uterine spiral arteries resulting in inadequate uteroplacental blood flow or compromised fetal placental vasculature development [42]. It is still unclear as to what causes the initial failure of the placenta to form correctly. However, it is likely to be a combination of both genetic and environmental factors with increased oxidative and cellular stress, as well as inflammatory mediators, thought to be key [43]. Elevated free copper can be a source of oxidative stress [44] and thus, it is possible that elevated copper may be a potential contributor to increased oxidative stress in the placenta, particularly in early pregnancy.

Another potential hypothesis is that early pregnancy copper status may be a biomarker for an increased inflammatory response which itself predisposes women to developing a pregnancy complication. Pregnancy itself is considered a pro-inflammatory state, particularly during the peri-implantation period [45]. However, even in pregnancy, the inflammation response is tightly regulated and slight deviations can result in compromised tissue function. Indeed, inflammation, as well as obesity which induces persistent low-grade inflammation, are associated with increasing the risk of a number of pregnancy

complications like PE and GDM [46-49]. However, we observed a protective effect of lower copper concentrations on the risk of pregnancy complications after adjusting for BMI. Furthermore, in the sensitivity analysis, in which all women with a possible infection or exacerbated inflammatory response were excluded, very little change to the point estimates for risk of a pregnancy complication with copper levels in the first and second tertiles compared to the third tertile was found suggesting plasma copper is not necessarily just a biomarker for inflammation.

The overall aim of this study was to determine whether early pregnancy plasma trace mineral status was associated with pregnancy complications. Despite the tight biological regulation of these minerals in the circulation [50], lower levels of plasma copper, zinc and selenium may be protective against a number of the major pregnancy complications assessed. However, there are a number of limitations, most notably lack of high quality dietary intake data, which may have implications for the results of this study. Plasma and serum measures are not generally considered accurate determinants of nutrient status [51] and non-fasting blood samples were collected which is also likely to have an effect on short-term mineral status. Although, it is possible that higher circulating levels of these trace elements represent reduced bioavailability within cells, where the minerals are required. Hence, it is conceivable that lower circulating levels of copper, zinc and selenium may be associated with a reduced risk of pregnancy complications as more of the trace elements are being utilised with tissues where required. Furthermore, given the lack of suitable alternatives in which to determine women's nutritional status in this pregnancy cohort, measuring plasma minerals may still be informative in understanding the physiology of pregnancy and pregnancy complications.

In conclusion, we observed a very clear association between plasma copper concentrations in early pregnancy and the risk of pregnancy complications that warrants further investigation particularly within the context

of other trace element status. It is conceivable that elevated plasma copper status at the beginning of pregnancy may simply be a biomarker of inflammation which may underlie the development of a particular pregnancy complication. However, our data suggest it is more likely to have a mechanistic role, potentially in the placenta through oxidative stress or inflammatory pathways that warrants further investigation. Much could be gained if we expand our understanding of maternal nutrition in pregnancy to incorporate simultaneous mechanistic and epidemiological studies, particularly on multiple micronutrients and their interactions in order to fully elucidate the importance of these factors in pregnancy success.

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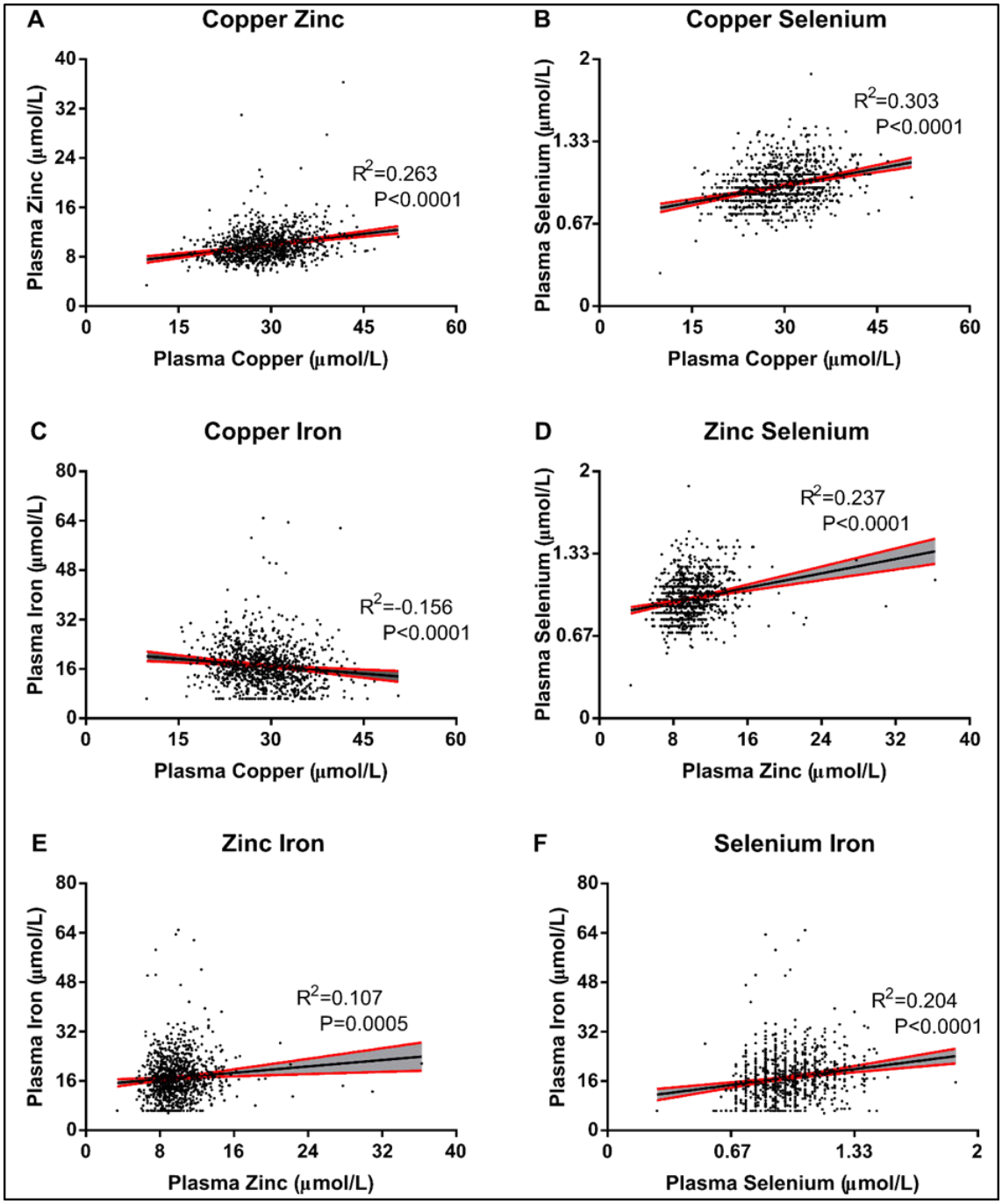
4.5 Supplementary Information



SUPPLEMENTARY FIGURE S4.1. Correlations between each of the plasma trace minerals at 15±1 weeks' gestation. Positive correlations were found between copper, zinc and selenium (A,B and D). Iron on the other hand was negatively correlated with copper (C) whilst positively correlated with zinc (E) and selenium (F).

SUPPLEMENTARY TABLE S4.1. Adjusted relative risks of pregnancy complications from all complications, preeclampsia (PE), gestational hypertension (GH), gestational diabetes mellitus (GDM), spontaeous preterm birth (sPTB) and small-for-gestational age (SGA) according to plasma levels of copper, zinc and selenium categorised based on population tertiles.

	All women	Pregnancy Complications											
		Any Complication		PE		GH		GDM		sPTB		SGA	
	n (%)	*n* (%)	aRR (95% CI)	*n* (%)	aRR (95% CI)	*n* (%)	aRR (95% CI)	*n* (%)	aRR (95% CI)	*n* (%)	aRR (95% CI)	*n* (%)	aRR (95% CI)
Copper*			**0.81**		0.72		1.10		0.69		0.72		0.67
<27.9 µmol/L	360	150 (42)	**(0.69, 0.96)**	21 (6)	(0.43, 1.23)	33 (9)	(0.69, 1.77)	13 (4)	(0.32, 1.48)	20 (6)	(0.37, 1.42)	34 (9)	(0.44, 1.02)
27.9 -32.5 µmol/L	349	156 (45)	**0.84 (0.72, 0.98)**	27 (8)	0.90 (0.56, 1.45)	36 (10)	1.16 (0.75, 1.79)	13 (4)	0.65 (0.32, 1.34)	16 (5)	0.57 (0.30, 1.09)	49 (14)	0.96 (0.65, 1.41)
>32.5 µmol/L	356	201 (56)	1.0†	37 (10)	1.0†	39 (11)	1.0†	25 (7)	1.0†	29 (8)	1.0†	51 (14)	1.0†
Zinc**			0.95		0.85		1.02		0.98		1.00		0.80
<8.3 µmol/L	357	160 (45)	(0.80, 1.11)	26 (7)	(0.48, 1.50)	42 (12)	(0.66, 1.60)	15 (4)	(0.48, 2.00)	15 (4)	(0.57, 1.78)	42 (12)	(0.55, 1.18)
8.3-9.9 µmol/L	351	165 (47)	0.95 (0.82, 1.11)	29 (8)	0.95 (0.58, 1.55)	32 (9)	0.92 (0.59, 1.43)	14 (4)	1.06 (0.53, 2.12)	27 (8)	0.98 (0.55, 1.73)	36 (10)	0.68 (0.46, 1.00)
>9.9 µmol/L	356	182 (51)	1.0†	30 (8)	1.0†	34 (10)	1.0†	22 (6)	1.0†	23 (6)	1.0†	56 (16)	1.0†
Selenium***			0.94		0.74		0.91		1.45		0.90		0.87
<0.9 µmol/L	449	203 (45)	(0.80, 1.10)	33 (7)	(0.44, 1.24)	49 (11)	(0.59, 1.39)	19 (4)	(0.68, 3.10)	25 (6)	(0.51, 1.60)	49 (11)	(0.58, 1.28)
0.9-1.0 µmol/L	199	83 (42)	0.86 (0.71, 1.04)	15 (8)	0.82 (0.45, 1.50)	16 (8)	0.75 (0.43, 1.32)	11 (6)	2.05 (0.89, 4.71)	9 (5)	0.71 (0.32, 1.53)	25 (13)	0.99 (0.63, 1.56)
>1.0 µmol/L	417	221 (53)	1.0†	37 (9)	1.0†	43 (10)	1.0†	21 (5)	1.0†	31 (7)	1.0†	60 (14)	1.0†

*Adjusted relative risks compared to all women were adjusted for age, maternal body mass index, smoking status at 15±1 weeks' gestation (no versus yes) and plasma zinc and selenium at 15±1 weeks' gestation.

** Adjusted relative risks compared to all women were adjusted for age, maternal body mass index, smoking status at 15±1 weeks' gestation (no versus yes) and plasma copper and selenium at 15±1 weeks' gestation.

***Adjusted relative risks compared to all women were adjusted for age, maternal body mass index, smoking status at 15±1 weeks' gestation (no versus yes) and plasma copper and zinc at 15±1 weeks' gestation.

†Reference category

SUPPLEMENTARY TABLE S4.2. Sensitivity analysis to determine the relationship between plasma copper and inflammation where by women with serum C-reactive protein (CRP) levels ≥20 mg/L were excluded and then relative risks of pregnancy complications from all complications, preeclampsia (PE), gestational hypertension (GH), gestational diabetes mellitus (GDM), spontaeous preterm birth (sPTB) and small-for-gestational age (SGA).

		Pregnancy Complications											
	All women	All Complications		PE		GH		GDM		sPTB		SGA	
	n (%)	*n* (%)	RR* (95% CI)	*n* (%)	RR* (95% CI)	*n* (%)	RR* (95% CI)	*n* (%)	RR* (95% CI)	*n* (%)	RR* (95% CI)	*n* (%)	RR* (95% CI)
All Women													
Copper													
<27.9 µmol/L	360	150 (42)	**0.77 (0.66, 0.91)**	21 (6)	0.71 (0.42, 1.18)	33 (9)	1.11 (0.71, 1.75)	13 (4)	0.69 (0.35, 1.37)	20 (6)	0.62 (0.34, 1.15)	34 (9)	**0.66 (0.44, 1.00)**
27.9 -32.5 µmol/L	349	156 (45)	**0.81 (0.70, 0.95)**	27 (8)	0.88 (0.55, 1.42)	36 (10)	1.16 (0.75, 1.80)	13 (4)	0.66 (0.33, 1.32)	16 (5)	**0.52 (0.28, 0.98)**	49 (14)	0.95 (0.66, 1.38)
>32.5 µmol/L	356	201 (56)	1.0†	37 (10)	1.0†	39 (11)	1.0†	25 (7)	1.0†	29 (8)	1.0†	51 (14)	1.0†
CRP <20.3 ng/mL													
Copper													
<27.9 µmol/L	297	118 (40)	**0.74 (0.61, 0.90)**	17 (6)	0.87 (0.42, 1.81)	21 (7)	0.87 (0.48, 1.58)	8 (3)	0.65 (0.27, 1.57)	18 (6)	0.63 (0.32, 1.26)	27 (9)	0.68 (0.40, 1.14)
27.9 -32.5 µmol/L	246	104 (42)	**0.78 (0.64, 0.95)**	18 (7)	1.12 (0.55, 2.29)	25 (10)	1.14 (0.65, 1.99)	9 (4)	0.75 (0.32, 1.78)	8 (3)	**0.34 (0.15, 0.78)**	30 (12)	0.89 (0.54, 1.48)
>32.5 µmol/L	174	95 (55)	1.0†	12 (7)	1.0†	18 (10)	1.0†	10 (6)	1.0†	16 (9)	1.0†	23 (13)	1.0†

*Adjusted relative risks compared to all women were adjusted for age, maternal body mass index and smoking status at 15±1 weeks' gestation (no versus yes).
†Reference category

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Chapter 5

Vitamin D levels in an Australian and New Zealand cohort and the association with pregnancy outcome

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ABSTRACT

Background: Pregnant women are at increased susceptibility to vitamin D deficiency. Hence, there is continuing interest in determining how vitamin D influences pregnancy health. We aimed to compare vitamin D status in two distinct populations of pregnant women in Australia and New Zealand and to investigate the relationship between vitamin D status and pregnancy outcome. This included evaluating possible effect measure modifications according to fetal sex.

Methods: Serum 25-hydroxy vitamin D (25(OH)D) was measured at 15±1 weeks' gestation in 2800 women from Adelaide and Auckland who participated in the multi-centre, prospective cohort SCreening fOr Pregnancy Endpoints (SCOPE) study.

Results: Mean serum 25(OH)D in all women was 68.1 ± 27.1 nmol/L and 28% (n = 772) were considered vitamin D deficient (<50 nmol/L). Serum 25(OH)D was lower in the women recruited in Adelaide when compared to the women recruited in Auckland and remained lower after adjusting for covariates including maternal body mass index and socioeconomic index (Adelaide: 58.4 ± 50.3 vs. Auckland: 70.2 ± 54.5 nmol/L, P<0.001). A 53% decreased risk for gestational diabetes mellitus (GDM) was observed with high (>81 nmol/L) "standardised" vitamin D status when compared to moderate-high (63-81 nmol/L, aRR: 0.47; 95% CI: 0.23, 0.96). Marginal sex-specific differences occurred between vitamin D status and GD: women carrying a female fetus had a 56% decreased risk for GDM in those with low-moderate levels of standardised vitamin D (44-63 nmol/L) compared to moderate-high levels (aRR: 0.44; 95% CI: 0.20, 0.97), whilst in women carrying a male fetus, a 55% decreased risk of GDM with found with high standardised vitamin D when compared to moderately-high vitamin D, but this was not statistically significant (aRR: 0.45; 95% CI: 0.15, 1.38).

Conclusions: High serum 25(OH)D at 15±1 weeks' gestation was shown to be protective against the development of GDM. A possible association between fetal sex, vitamin D status and GDM provides further questions and encourages continual research and discussion into the role of vitamin D in pregnancy, particularly in vitamin D replete populations.

5.1 Introduction

With an increasing prevalence of vitamin D deficiency and insufficiency reported both in Australia and New Zealand, as well as worldwide [1], there is continuing interest in determining how vitamin D deficiency may influence health in

pregnancy. Evidence suggests that vitamin D deficiency is associated with a number of pregnancy complications including preeclampsia (PE), gestational diabetes mellitus (GDM) and spontaneous preterm birth (sPTB) [2, 3]. However, inconsistencies between studies reflect uncertainty about the true effect of vitamin D deficiency on pregnancy outcome [4, 5]. This may be explained, in part, by inadequate control of related risk factors and confounders in statistical analyses, variations between assays that measure vitamin D and significant heterogeneity between studied populations [5].

Vitamin D status is determined by measuring circulating serum levels of 25-hydroxy vitamin D_{2+3} (25(OH)D). In Australia and New Zealand, the deficiency cut-offs are based on the role of vitamin D in bone health where serum 25(OH)D ≥50 nmol/L at the end of winter is required for optimal musculoskeletal health [1]. Furthermore, it has been established that serum 25(OH)D ≥50 nmol/L is recommended during pregnancy and lactation [6]. The incidence of vitamin D deficiency (<50 nmol/L) is frequent among pregnant women even in areas such as Australia and the North Island of New Zealand where sunlight exposure is high. Studies focused on high-risk populations, for example, veiled, dark-skinned or obese women in Australia and New Zealand, report between 50-94% of women to be vitamin D deficient [7-9]. Reports from lower-risk groups have indicated that vitamin D deficiency occurs in 25-55% of pregnant women [10-12].

There are numerous studies which have shown that vitamin D deficiency is associated with adverse pregnancy outcomes, particularly in populations that reside at higher latitudes [4]. However, studies in women from Australia and New Zealand are less consistent. Previous studies on pregnant Australian and New Zealand women have reported that while circulating 25(OH)D was significantly lower in women with PE, sPTB, GDM and those who delivered a small-for gestational age (SGA) infant, no association between vitamin D deficiency and these pregnancy complications was found after adjusting for covariates [13-15]. Differences in ethnicity [16], solar exposure and geographical

location, as well as genetics [17], are known to affect 25(OH)D status and therefore influence study outcomes. Furthermore, the gestation at which vitamin D was measured is also important. Measuring circulating 25(OH)D in early pregnancy is common and potentially clinically useful as this is prior to when many of the pregnancy complications that affect late gestation manifest.

Using a robust, validated chemiluminescent-based assay to measure serum 25(OH)D [18], we aimed to investigate the differences between vitamin D status in early pregnancy in two distinct populations of nulliparous women from Australia and New Zealand. We also aimed to examine the relationship between serum 25(OH)D at 15±1 weeks' gestation and the risk of an adverse pregnancy outcome and included determining the effect modification of fetal sex on the association between maternal vitamin D status and pregnancy outcome.

5.2 Methods

5.2.1 Study Participants

This study utilised data collected from the multi-centre, prospective cohort Screening for Pregnancy Endpoints (SCOPE) study [19]. Nulliparous women carrying a singleton pregnancy were recruited between November 2004 and September 2008 in Adelaide (Australia) and Auckland (New Zealand). At 15±1 weeks' gestation, women were interviewed by a research midwife and asked questions on maternal demographics and lifestyle and had physical measurements taken, including height and weight. Records included ethnicity, age, body mass index (BMI), socioeconomic index (SEI) and multivitamin use [20]. BMI was calculated as weight (kg)/height^2 (m^2). Obesity was defined as BMI ≥30 kg/m^2, overweight as BMI ≥25 and <30 kg/m^2, normal weight as BMI >20 and <25 kg/m^2 and underweight as BMI ≤20 kg/m^2. SEI was calculated using the New Zealand SEI of occupational status, deriving a number between 10 and 90 based on the woman's occupation; a higher number indicates higher socioeconomic status [21]. This population of women was predominantly Caucasian and thus

maternal ethnicity was categorised into 2 main groups; Caucasian and non-Caucasian. Uncomplicated pregnancies were defined as those without any pregnancy disorder who delivered an appropriate weight for gestational age infant at term (≥37 weeks gestation) [22]. Pregnancy complications studied included PE, gestational hypertension (GH), GDM, sPTB and SGA and have been previously defined [19, 20, 22, 23].

5.2.2 Measurement of serum 25(OH)D

Non-fasting whole peripheral blood samples were collected into non-heparinised tubes at 15±1 weeks' gestation. Serum was processed within 4 hours of collection and stored at -80°C until required. Unlike the previously published data on vitamin D in the Auckland cohort [15], serum 25(OH)D was measured using the IDS-iSYS chemiluminescent-based assay (Abacus, ALS) as per the manufacturer's instructions. Both serum 25(OH)D3 and 25(OH)D2 were measured independently and combined to provide a total 25(OH)D in nmol/L [18].

5.2.3 Statistical Analysis

Statistical analyses were performed in R (v3.1.1) [24]. Data were checked for normality using a Shapiro-Wilks test and differences between women recruited in Adelaide compared to Auckland were tabulated and compared using a Welch's t-test (continuous variables) or Fisher's exact test (categorical variables).

Given that the month in which the serum was sampled heavily influences vitamin D status, "standardised" serum 25(OH)D concentrations were calculated as previously described [25] in order to normalise against seasonal variation. Quartiles within the standardised concentrations were then used to create cut-points and designated 'low' (<44 nmol/L), 'low-moderate' (44-63 nmol/L), 'moderate-high' (63-81 nmol/L) and 'high' (>81 nmol/L) categories of serum 25(OH)D.

Generalised linear models (Poisson with log link and robust variance estimates) were used to calculate the risk ratios for pregnancy complications by standardised serum 25(OH)D concentrations calculated among the women who had an uncomplicated pregnancy. Potential confounders on vitamin D status was assessed using linear modelling. Maternal age, BMI, SEI, alcohol consumption at 15±1 weeks' gestation (never/former vs. current), recreational walking (1-3 times/week and ≥4 times/week vs. never), ethnicity (Caucasian vs. non-Caucasian) and recruitment site (Adelaide vs. Auckland) were significantly associated with serum 25(OH)D and along with smoking status at 15±1 weeks' gestation (never/former vs. current) included as main effects within the generalised linear models. These analyses were also repeated but using the current definitions of vitamin D deficiency: <25, 25-50, 50-75 and >75 nmol/L in the non-standardised data [6]. We also stratified based on fetal sex to evaluate possible effect measure modifications according to whether the mother was carry a male or female fetus.

5.3 Results

5.3.1 Population Characteristics

Of 3229 women recruited as part of SCOPE at the Adelaide and Auckland centres, serum samples at 15±1 weeks' gestation to measure 25(OH)D were available for 2800 (87%) women of whom, 1156 (41%) were recruited in Adelaide and 1644 (59%) were recruited in Auckland. Maternal characteristics are shown in Table 5.1. Compared to women who were recruited at the Adelaide site, women recruited in Auckland were older, had a lower BMI, less likely to smoke or drink alcohol during pregnancy and more likely to eat fruit and undertake recreational walks. Mean ± SD SEI of the Auckland women was also higher compared to that in women recruited in Adelaide (Auckland: 48.0 ± 14.8 vs. Adelaide: 27.7 ± 10.5, P<0.001). Given that all these factors significantly influenced serum 25(OH)D in the linear regression model, it was unsurprising

that vitamin D status of the women was significantly different between the two recruitment sites. However, after adjusting for maternal age, BMI, SEI, smoking status and alcohol consumption at 15±1 weeks' gestation, ethnicity, recreational walking and season, the women recruited in Adelaide still had significantly lower serum 25(OH)D when compared with those in Auckland (Adelaide: 58.4 ± 50.3 vs. Auckland: 70.2 ± 54.5 nmol/L, P<0.001) indicating the influence of other confounders not measured as part of the study on vitamin D status.

TABLE 5.1. Participant characteristics and comparison of characteristics between women recruited at the Adelaide SCOPE site versus Auckland

	All women (n = 2800)	Adelaide (n = 1156)	Auckland (n = 1644)	*P* value*
Age *yrs*, mean (SD)	28 (6)	23.73 (5.11)	30.44 (4.82)	**<0.0001**
BMI kg/m^2, mean (SD)	25.8 (5.44)	27.04 (6.56)	24.88 (4.26)	**<0.0001**
Ethnicity				**<0.0001**
Caucasian	2449 (87)	1060 (92)	1389 (84)	
Non Caucasian	351 (13)	96 (8)	255 (16)	
Smoking status, n (%)				**<0.0001**
No	2152 (77)	704 (61)	1448 (88)	
Quit during pregnancy	306 (11)	175 (15)	131 (8)	
Smoking	342 (12)	277 (24)	65 (4)	
Alcohol Consumption, n (%)				**<0.0001**
No	1480 (53)	708 (61)	772 (47)	
Stopped during pregnancy	1185 (42)	397 (34)	788 (48)	
Consuming alcohol	135 (5)	51 (4)	84 (5)	
Fruit Intake, n (%)				**<0.0001**
≥1x per day	2032 (73)	586 (51)	1446 (88)	
3-6x per week	405 (14)	272 (24)	133 (8)	
1-2x per week	223 (8)	181 (16)	42 (3)	
1-3x per month or less	140 (5)	117 (10)	23 (1)	
Recreational Walking				**<0.0001**
Never	428 (15)	265 (23)	163 (10)	
1-3 times/week	1773 (63)	668 (58)	1105 (68)	
≥4 times/week	590 (22)	221 (19)	369 (23)	
Time watching TV				**<0.0001**
<5 hours per day	2404 (86)	901 (78)	1503 (92)	
≥5 hours per day	387 (14)	253 (22)	134 (8)	
Season serum was sampled				0.3213
Summer	636 (23)	278 (24)	358 (22)	
Autumn	705 (25)	273 (24)	432 (26)	

Winter	727 (26)	300 (26)	427 (26)	
Spring	732 (26)	305 (26)	427 (26)	
Serum 25(OH)D *nmol/L*, mean (SD)	68.09 (27.14)	60.06 (23.68)	73.74 (27.99)	**<0.0001**
Vitamin D Status				**<0.0001**
<25 nmol/L	99 (4)	48 (4)	51 (3)	
25-50 nmol/L	673 (24)	375 (32)	298 (18)	
50-75 nmol/L	928 (33)	422 (37)	506 (31)	
>75 nmol/L	1098 (39)	311 (27)	787 (48)	

*P values for continuous variables were determined using a Welch's t-test and categorical variables a Fisher's exact test comparing Adelaide and Auckland women.

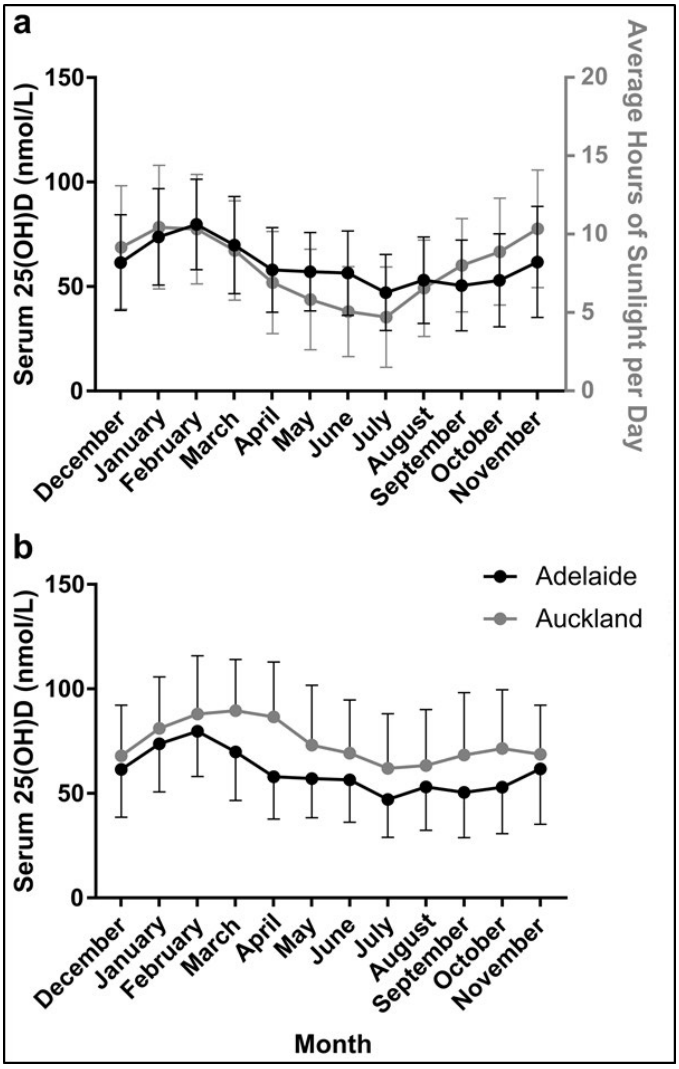

FIGURE 5.6. Seasonal variation in serum 25(OH)D. **a**. Comparison of serum 25(OH)D levels based on month of sampling in the women recruited in Adelaide (black line & left axis) and average hours of sunlight per day in Adelaide (grey line & right axis). Seasonal variation in vitamin D followed a similar pattern to hours of sunlight although was slightly shifted. **b**. Seasonal variation of serum 25(OH)D based on month of sampling between women recruited in Adelaide compared to Auckland. Data are mean ± SD

5.3.2 Standardising serum 25(OH)D based on seasonal variation

As expected, there was a seasonal influence on serum 25(OH)D and thus, "standardised" vitamin D concentrations were calculated to account for the month of serum collection. Average hours of sunlight per day in Adelaide from September 2005 to September 2008 were obtained and compared against serum vitamin D measured in the women recruited in Adelaide (Figure 1a; black line/left axis serum 25(OH)D and grey line/right axis average hours of sunlight). The means for serum 25(OH)D by month of sampling were also separated for women recruited in Adelaide and women recruited in Auckland (Fig. 5.1b).

5.3.3 Serum 25(OH)D and pregnancy outcome

Of the 2800 women present in this study, 1217 (43%) women went on to develop a pregnancy complication and included 13% (n = 161) who developed PE, 18% (n = 213) who developed GH, 8% (n = 92) who developed GDM, 11% (n = 139) who delivered preterm and 24% (n = 298) who delivered an SGA infant.

The association between "standardised" 25(OH)D with pregnancy complications is presented in Table 5.2. Using moderate-high as a reference (standardised vitamin D 63-81 nmol/L), there was no appreciable effect of having a low, low-moderate or high vitamin D with the risk of developing any pregnancy complication after adjusting for confounders. However, when each pregnancy complication was analysed separately, a 53% decreased risk for GDM was observed with high vitamin D status when compared to moderate-high status (aRR: 0.47; 95% CI: 0.23, 0.96). When women were categorised based on clinical definitions of vitamin D deficiency and serum 25(OH)D between 50-75 nmol/L used as the reference, no significant relationship between vitamin D status and adverse pregnancy outcome was found (Table 5.3). Although, the point estimates indicated a marginal increased risk for developing any pregnancy complication with severe (<25 nmol/L) vitamin D deficiency compared to those who had levels between 50-75 nmol/L (aRR: 1.10; 95% CI: 0.89, 1.36).

TABLE 5.2. Adjusted relative risks (aRR) of pregnancy complications from any complication, preeclampsia (PE), gestational hypertension (GH), gestational diabetes mellitus (GDM), spontaneous preterm birth (sPTB) and small-for-gestational age (SGA) according to vitamin D status categorised based on standardised quartiles and clinical definitions of vitamin D status

		Pregnancy Complications											
	All women	Any Complication		PE		GH		GDM		sPTB		SGA	
	n (%)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)
Standardised Quartiles**													
Low	711 (25)	328 (46)	0.95 (0.85, 1.07)	46 (6)	1.02 (0.66, 1.57))	57 (8)	0.97 (0.67, 1.42)	28 (4)	0.79 (0.47, 1.30)	36 (5)	0.83 (0.54, 1.29)	84 (12)	1.02 (0.75, 1.39)
Low-Moderate	755 (27)	318 (42)	1.00 (0.90, 1.12)	42 (6)	1.07 (0.69, 1.64)	65 (9)	1.06 (0.74, 1.52)	25 (3)	0.67 (0.39, 1.16)	33 (4)	0.87 (0.56, 1.35	72 (10)	0.98 (0.73, 1.32)
Moderate-High	702 (25)	309 (44)	1.0†	37 (5)	1.0†	50 (7)	1.0†	28 (4)	1.0†	39 (6)	1.0†	81 (12)	1.0†
High	630 (23)	258 (41)	0.93 (0.82, 1.06)†	35 (6)	1.24 (0.78, 1.96)	40 (6)	0.87 (0.58, 1.30)	10 (2)	**0.47 (0.23, 0.96)**	30 (5)	0.77 (0.48, 1.25)	60 (10)	0.80 (0.57, 1.11)
Vitamin D Status													
<25 nmol/L	99 (4)	51 (52)	1.10 (0.89, 1.36)	8 (8)	1.44 (0.68, 3.03))	6 (6)	0.70 (0.32, 1.52)	5 (5)	0.98 (0.38, 2.52)	7 (7)	1.44 (0.68, 3.04)	13 (13)	1.15 (0.66, 2.01)
25-50 nmol/L	673 (24)	301 (45)	0.93 (0.83, 1.03)	45 (7)	1.18 (0.80, 1.75)	59 (9)	0.97 (0.71, 1.34)	26 (4)	0.89 (0.54, 1.47)	29 (4)	0.75 (0.48, 1.16)	74 (11)	0.93 (0.69, 1.24)
>50-75 nmol/L	928 (33)	428 (46)	1.0†	48 (5)	1.0†	80 (9)	1.0†	35 (4)	1.0†	52 (6)	1.0†	107 (12)	1.0†
>75 nmol/L	1098 (39)	435 (40)	0.95 (0.86, 1.04)	60 (5)	1.28 (0.87, 1.87)	67 (6)	0.82 (0.59, 1.12)†	26 (2)	0.78 (0.48, 1.28)	51 (5)	0.86 (0.59, 1.26)	104 (9)	0.86 (0.67, 1.12)

*Relative risks compared to all women were adjusted for age, maternal body mass index, ethnicity (non Caucasian vs. Caucasian), smoking status at 15±1 weeks' gestation (no vs. yes), alcohol consumption at 15±1 weeks' (no vs yes), recreational walking (1-3x/week and ≥4x/week vs. never) and recruitment site (Auckland vs. Adelaide).

** Serum 25(OH)D was standardised for month that serum was sampled based as previously described [25].

†Reference category

TABLE 5.3. Adjusted relative risks (aRR) of pregnancy complications from any complication, preeclampsia (PE), gestational hypertension (GH), gestational diabetes mellitus (GDM), spontaeous preterm birth (sPTB) and small-for-gestational age (SGA) according to vitamin D status and stratified by fetal sex

	All women	Pregnancy Complications											
		Any Complication		PE		GH		GDM		sPTB		SGA	
	n (%)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)
Standardised Quartiles**													
Males													
Low	367 (26)	173 (47)	1.00 (0.85, 1.18)	24 (7)	1.24 (0.65, 2.34)	31 (8)	1.11 (0.64, 1.94)	18 (5)	1.25 (0.58, 2.68)	22 (6)	1.09 (0.60, 1.98)	43 (12)	1.14 (0.73, 1.79)
Low-Moderate	364 (26)	163 (45)	1.01 (0.86, 1.19))	20 (5)	1.11 (0.58, 2.12)	32 (9)	1.25 (0.73, 2.13)	13 (4)	1.06 (0.48, 2.38)	18 (5)	0.93 (0.49, 1.76)	45 (12)	1.23 (0.80, 1.88)
Moderate-High	359 (25)	152 (42)	1.0†	16 (4)	1.0†	23 (6)	1.0†	11 (3)	1.0†	18 (5)	1.0†	35 (10)	1.0†
High	325 (23)	111 (34)	0.86 (0.71, 1.04)	15 (5)	1.18 (0.59, 2.36)	17 (5)	0.90 (0.50, 1.62)	4 (1)	0.45 (0.15, 1.38)	15 (5)	0.97 (0.50, 1.88)	26 (8)	0.77 (0.47, 1.26)
Females													
Low	380 (28)	169 (44)	0.91 (0.77, 1.06)	23 (6)	0.85 (0.47, 1.55)	30 (8)	0.87 (0.52, 1.45)	13 (3)	0.51 (0.25, 1.04)	14 (4)	0.61 (0.31, 1.18)	45 (12)	0.88 (0.58, 1.35)
Low-Moderate	353 (26)	168 (48)	0.99 (0.85, 1.15)	24 (7)	1.03 (0.57, 1.85)	31 (9)	0.92 (0.57, 1.50)	9 (3)	**0.44 (0.20, 0.97)**	18 (5)	0.79 (0.43, 1.45)	33 (9)	0.74 (0.48, 1.13)
Moderate-High	334 (24)	149 (45)	1.0†	19 (6)	1.0†	28 (8)	1.0†	17 (5)	1.0†	21 (6)	1.0†	31 (9)	1.0†
High	314 (23)	128 (41)	1.00 (0.84, 1.19)	19 (6)	1.29 (0.70, 2.35)	20 (6)	0.85 (0.49, 1.46)	6 (2)	0.48 (0.19, 1.23)	12 (4)	0.60 (0.30, 1.21)	29 (9)	0.82 (0.53, 1.28)

*Relative risks compared to all women were adjusted for age, maternal body mass index, ethnicity (non Caucasian vs. Caucasian), smoking status at 15±1 weeks' gestation (no vs. yes), alcohol consumption at 15±1 weeks' (no vs yes), recreational walking (1-3x/week and ≥4x/week vs. never) and recruitment site (Auckland vs. Adelaide).

** Serum 25(OH)D was standardised for month that serum was sampled based as previously described [25].

†Reference category

5.3.4 Serum 25(OH)D, pregnancy outcome and fetal sex

As there is evidence to suggest that vitamin D metabolism within the placenta may differ with respect to fetal sex [26], we assessed the effect of "standardised" vitamin D status on pregnancy outcome stratified by fetal sex (Table 5.3). Although not statistically significant, point estimates for women carrying a male fetus indicated decreased risk of having any pregnancy complication with high serum 25(OH)D compared to moderately-high (aRR: 0.86; 9%% CI: 0.71, 1.04) . This was largely driven by a 55% decreased risk of GDM in women with a male fetus with high standardised serum 25(OH)D when compared to those carrying a male fetus with moderate-high serum 25(OH)D (aRR: 0.45; 95% CI: 0.15, 1.38). Conversely, if a woman was carrying a female fetus, there was generally no effect of having low, low-moderate or high standardised serum 25(OH)D on developing any pregnancy complication compared to moderate-high levels (Table 5.3). Although, a 56% decreased risk for GDM was observed in those with low-moderate levels of standardised vitamin D when compared to moderate-high levels for women carrying a female fetus (aRR: 0.44; 95% CI: 0.20, 0.97).

5.4 Discussion

This study adds to the current body of literature on vitamin D status in a population of pregnant Australian and New Zealand women and provides insight into normal circulating levels of 25(OH)D in early pregnancy. It is the largest prospective cohort study to assess vitamin D status within the international SCOPE cohort, comparing and combining two distinct populations of pregnant women living at similar latitude and provides a greater understanding of vitamin D deficiency and the risk of adverse pregnancy outcomes. We found, despite being at similar latitudes, circulating 25(OH)D was different between women recruited in Adelaide compared to women recruited in Auckland. This was independent of diet and lifestyles factors including BMI

and SEI and highlights the difficulty in understanding the role of vitamin D in pregnancy in human cohort studies. However, there was a protective association of having high vitamin D at 15±1 weeks' gestation and GDM once standardised based on month serum was sampled. Furthermore, there may be possible fetal sex specific differences in vitamin D status worth considering in future studies.

Despite the similar latitudes of Adelaide and Auckland (Adelaide: 34.93°S and Auckland 36.85°S), serum 25(OH)D was lower in the women recruited in Adelaide. Given the significant number of characteristic and lifestyle differences between the two populations, this is not overly surprising. Previous studies have shown a positive association between socioeconomic status and vitamin D in both pregnant and non-pregnant women [27, 28]. The lower SEI of the Adelaide women could therefore make them more susceptible to lower circulating 25(OH)D because of factors relating to disadvantage. Furthermore, increased BMI is known to be associated with reduced serum 25(OH)D [29-31] as adipose tissue is thought to sequester 25(OH)D [32]. However, after adjusting for factors shown to be associated with vitamin D status including BMI and recreational walking, serum 25(OH)D remained significantly lower in the women recruited in Adelaide suggesting the influence of other factors not measured as part of SCOPE for example, hours spent outside in the sun. As indicated by the seasonal variation in serum 25(OH)D, amongst women recruited in Adelaide, vitamin D status declined significantly from February to April whilst in the women recruited in Auckland, serum 25(OH)D in March and April remained elevated before declining.

Vitamin D deficiency has previously been associated with insulin resistance and type 2 diabetes given its role in supporting insulin secretion and pancreatic β-cell function [33]. Furthermore, inverse relationships between serum 25(OH)D and both fasting glucose and fasting insulin have been shown during pregnancy indicating poorer glycaemic control [14, 34]. Therefore, the protective role of high 25(OH)D 15±1 weeks' gestation against GDM, as seen with

an decreased risk of GDM in women within the 'high' quartile of standardised vitamin D compared to moderate-high, is consistent with knowledge about vitamin D and diabetes. This offers a potential physiological connection between vitamin D status and the progression of insulin resistance in pregnant women. Furthermore, studies have shown that vitamin D supports early placental development [35, 36] in which, abnormal placentation can be a characteristic of a number of pregnancy complications including GDM, PE, sPTB and SGA [37].

The placenta expresses all the necessary components to convert 25(OH)D to the active form and thus utilise active vitamin D either locally or in a paracrine manner [36, 38]. Vitamin D metabolism in the placenta has been shown to be influenced by testosterone production and thus varies by fetal sex [26]. Furthermore, sex specific differences in pregnancy outcome have also been reported whereby the risk of sPTB, PE and GDM are all higher in pregnancies with a male fetus [39-41]. In this study, we observed marginal sex-specific differences between early pregnancy vitamin D status and pregnancy outcome where-by high vitamin D status and carrying a male fetus was moderately associated with decreasing the risk of having any pregnancy complication. Conversely, high vitamin D status and carrying a female fetus was not associated with changing the risk of having any pregnancy complication. Indeed, for the risk of GDM an opposite effect of vitamin D status in early pregnancy was observed depending on fetal sex. This is similar to what has previously been shown in the relationship between vitamin D status at ≤26 weeks' gestation and placental pathology in pregnancy [42] suggesting that male and female fetuses respond differently to maternal vitamin D status.

Lack of statistically significant associations with other pregnancy complications may reflect the fact that this was a largely vitamin D-replete population (>72% with serum 25(OH)D >50 nmol/L) likely due to their residence latitude as low serum 25(OH)D was found in the SCOPE Ireland cohort [43]. Indeed many of the studies that have assessed the association between vitamin

D status and adverse pregnancy outcome that have reported statistically significant differences have been in populations with higher rates of vitamin D deficiency [2]. Inconsistencies in the literature may also reflect other causative factors in which vitamin D is a mediator. For example, active vitamin D (1,25-dihydroxyvitamin D_3) is the principal hormone that regulates calcium absorption within the intestine [44] and is integral to maintaining calcium homeostasis. During gestation, fetal demand for calcium increases and it is imperative that maternal vitamin D status remain adequate to support increased calcium absorption from the gut [45]. Therefore, vitamin D status may be important in populations where dietary calcium intake is low which is unlikely in the population of women studied here.

In conclusion, once standardised against month of sampling, we demonstrate a protective effect of high vitamin D with GDM. However, differences in vitamin D status between the women recruited in Adelaide and those recruit in Auckland reflect obvious difficulties in studying how vitamin D may support healthy pregnancies. A possible connection between fetal sex, vitamin D status and pregnancy complications provides further questions and encourages continual research and discussion into the role of vitamin D in pregnancy, particularly in vitamin D replete populations.

Ethics

This study was approved by the relevant human ethics committees and all participants provided written informed consent (Adelaide: Central Northern Adelaide Health Service Ethics of Human Research Committee on 2 September 2005, ethics number REC 1714/5/2008 and Auckland: Northern Region Ethics Committee on 23 April 2003, ethics number AKX/02/00/364).

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By signing the Statement of Authorship, each author certifies that:

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Chapter 6

Maternal calcium homeostasis is associated with uteroplacental dysfunction in a large prospective cohort of pregnant South Australian women

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ABSTRACT

Background: Adequate calcium intake and vitamin D status during pregnancy are integral to maintaining appropriate calcium supply to the fetus for fetal growth and development. Fetal growth restriction is associated with pregnancies complicated by preeclampsia (PE) and delivery of a small-for-gestational age (SGA) infant as well as with placental dysfunction. Vitamin D deficiency in pregnancy has been associated with PE and SGA but reports are inconsistent, possibly due to maternal calcium status.

Objectives: This study investigated the relationship between maternal vitamin D and calcium status at 15±1 weeks' gestation with the

development of PE and SGA, as well as mid-gestation measures of uteroplacental circulation.

Design: We used the SCOPE (Screening for Pregnancy Endpoints) Adelaide prospective pregnancy cohort that included 1065 pregnant nulliparous women. Total plasma calcium, serum 25-hydroxyvitamin D (25(OH)D), urinary calcium and urinary deoxypyridinoline (DPD) were measured at 15±1 weeks' gestation, uterine and umbilical artery resistance index (RI) was measured at 19-21 weeks' gestation.

Results: Total maternal plasma calcium (mean ± SD) was 2.06 ± 0.141 mmol/L and serum 25(OH)D was 59.41 ± 23.13 nmol/L in the whole cohort. Lower urinary calcium excretion was associated with greater risk of having an abnormal umbilical artery RI (aRR: 1.57; 95% CI: 1.11, 2.22) and delivering an SGA infant (aRR: 2.13; 95% CI: 1.29, 3.51) compared to excretion of higher levels of calcium. When combined, having both higher serum 25(OH)D and lower urinary calcium was associated with increased risk of SGA and an abnormal uterine artery RI (aRR: 1.55; 95% CI: 1.07, 2.24 and aRR: 1.79; 95% CI: 1.19, 2.68, respectively) compared to having higher levels of both suggesting maternal calcium status to be a more important determinant of placental development and fetal growth.

Conclusions: This is one of the first studies to assess both early pregnancy calcium and vitamin D status and their association with PE, SGA and measures of placental function in a large pregnancy cohort. Altered calcium homeostasis at 15±1 week's gestation was associated with an increased risk of uteroplacental dysfunction which can have subsequent consequences on fetal growth and development.

6.1 Introduction

Maternal calcium homeostasis during pregnancy is integral to supporting fetal bone growth and development. It is estimated that the average calcium demand of a human fetus is 30 g until birth, with the majority required in the third trimester when most bone mineralisation occurs [1]. Vitamin D and its metabolites, parathyroid hormone (PTH) and dietary calcium intake function together to regulate calcium metabolism [2]. Low circulating calcium stimulates the release of PTH which then acts to increase resorption of bone, reabsorption of calcium in the kidneys and the production of 1,25(OH)D_3, the active vitamin D metabolite, which functions to increase intestinal calcium absorption [3]. Once circulating calcium levels are restored, a negative feedback system signals to cease production of PTH. During pregnancy, calcium absorption approximately doubles, largely due to the significantly increased expression of 1,25(OH)D_3 as well as other factors like increased prolactin and placental lactogen production [4]. Placental expression of vitamin D metabolites and pathway enzymes also increases [5-7]. Hence, the system is designed to maintain the transfer of calcium from mother to fetus without any detriment to maternal bone health. However, in women with very low calcium intakes (<500 mg/day) or those who are vitamin D deficient/insufficient, this can result in a net calcium loss [8, 9] and have downstream consequences for fetal growth and maternal health.

Fetal growth restriction can be an outcome of pregnancies complicated by preeclampsia (PE) or accompany the delivery of a small-for-gestational age (SGA) infant [10] with the pathogenesis of these complications including placental dysfunction and insufficiency. Placental dysfunction can result from improper transformation of the maternal spiral arteries by invading placental trophoblast cells, as well as defective fetal villus formation and function (reviewed in [11]). The consequences are poor blood flow and nutrient exchange resulting in restricted fetal growth. Doppler ultrasound in mid-pregnancy

enables the assessment of the uteroplacental circulation *in vivo* and determine whether there is inadequate perfusion that may result in problems for the mother and/or fetus later in gestation [12]. Improper trophoblast invasion of the spiral arteries can be detected through the assessment of uterine artery blood flow [13] whilst Doppler assessment of the umbilical artery blood flow reflects the arborisation of the fetal chorionic villous tree [14]. Studies have shown that high resistance flow demonstrated by the waveforms from both uterine and umbilical arteries are associated with subsequent stillbirth, fetal growth restriction and PE [15-19] and thus provide a method by which to monitor placental function.

The link between vitamin D deficiency in pregnancy and adverse pregnancy outcomes has been studied extensively [20, 21]. In particular, there have been numerous reports on the association between vitamin D deficiency and PE, as well as with delivery of an SGA infant [22, 23]. However, these reports are inconsistent with other studies finding no association between vitamin D deficiency and the risk of pregnancy complications [20, 21]. Furthermore, less is known about the association between maternal calcium status and PE or SGA. Indeed, calcium supplementation is recommended as a preventative measure for PE based on studies which have shown an association between higher calcium intake and a lower risk of PE [24-28]. This is supported by a decreased urinary calcium-creatinine ratio both in early and late pregnancy in women who develop PE [29, 30] suggesting disrupted calcium homeostasis as a possible mechanism in the pathogenesis of PE. However, whether this is a consequence of low vitamin D, low maternal calcium intake or both has not clearly been defined.

The association between vitamin D status and adverse pregnancy outcomes in women from Australia and New Zealand remains uncertain and highly sample population dependant [31-33]. This is because vitamin D status is highly dependent upon variables such as ethnicity and body mass index (BMI) [34], geographic location and solar exposure, as well as genetics [35]. Furthermore, very few studies have assessed the association between vitamin D

status and calcium status with the risk of pregnancy complications. Recently, we reported that higher vitamin D levels in early pregnancy may be protective against the risk of developing gestational diabetes mellitus in a cohort of pregnant women recruited in Adelaide (Australia) and Auckland (New Zealand) [Chapter 5, Wilson *et al.,* Revisions pending BMC Pregnancy and Childbirth]. However, no association was found between vitamin D status and the risk of PE or SGA. The purpose of the present study was to further investigate maternal calcium status in addition to circulating 25(OH)D in early pregnancy with the development of PE and SGA, as well as placental function as both PE and SGA are associated with placental pathology. We hypothesised that, within this largely vitamin D replete population, maternal calcium status may be an important determinant of pregnancy outcome, placental development and fetal growth.

6.2 Methods

6.2.1 Design and Study Population

This study used data collected as part of the Screening for Pregnancy Endpoints (SCOPE) Study, a multi-centre, prospective cohort study of nulliparous pregnant women and was confined to those women attending the Lyell McEwin Hospital in Adelaide, Australia. Women were recruited at 15±1 weeks' gestation between November 2004 and September 2008. The study was approved by the Queen Elizabeth Hospital and Lyell McEwin Hospital Human Research Ethics committee (approval no: REC 1712/5/2008) and all women provided written consent.

6.2.2 Birth Outcomes

Uncomplicated pregnancies were defined as those without any pregnancy disorder and included women who delivered an appropriate weight for gestational age infant at term (≥37 weeks gestation) [36]. PE was diagnosed as

having high blood pressure (systolic blood press ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg) on at least two occasions after 20 weeks gestation in conjunction with proteinuria (24 h urinary protein ≥300 mg, a spot urine protein:creatinine ratio ≥30 mg/mmol, or urine dipstick protein ≥2) or any multisystem complication of PE or uteroplacental dysfunction [37]. SGA was defined as delivering an infant whose birthweight was <10th customised centile [38]. Ultrasound examination was performed at 19-21 weeks' gestation on both the umbilical and uterine arteries [39, 40]. Both the left and right uterine artery resistance index (RI) was measured and used to calculate mean uterine artery RI. Women who had a uterine or umbilical RI >90th centile were considered abnormal.

6.2.3 Maternal blood and urinary measures of calcium, 25(OH)D and deoxypridinoline

Maternal venous blood was collected at 15±1 weeks' gestation for both heparin plasma and serum samples, as well as a urine sample. Measurements of total plasma calcium and serum 25(OH)D have been described previously [Chapter 5, Wilson *et al.*, Revisions pending BMC Pregnancy and Childbirth, 41]. Urinary deoxy-pyridinoline (DPD), a marker of bone turnover, was measured using the Microvue Total DPD EIA Kit (*Quidel Corporation*) following manufacturer's protocol while the Cobas Integra 400+ Analyser (*Roche Diagnostics*) was used to measure urinary calcium and creatinine. Each assay was run on the Triad Multi Mode Microplate Reader (*Dynex Technologies*) and concentrations determined from standard curves. Values for urinary calcium and DPD were expressed as ratios to creatinine in order to correct for variations in urine volume and dilution.

6.2.4 Statistics

All statistical analyses were performed in R (v3.1.1) [42]. Spearman's correlational analyses were used to assess the relationships between

"standardised" serum 25(OH)D, plasma calcium, urinary calcium-creatinine (Ca:Cr) and urinary DPD-creatinine (DPD:Cr) as well as with uterine and umbilical artery RI as measures of placental function. Generalised linear models (Poisson with log link and robust variance estimates) were used to calculate the adjusted risk ratios (aRR) for PE, SGA and uteroplacental dysfunction. Population tertiles were used to create cut-points for each biochemical measure. For "standardised" serum 25(OH)D: lower <41 nmol/L, medium 41-61 nmol/L, higher >61 nmol/L. For plasma calcium: lower <2.0 mmol/L, medium 2-2.1 mmol/L, higher >2.1 mmol/L. For urinary Ca:Cr: lower <0.46, medium 0.46-0.83, higher >0.83. For urinary DPD:Cr: lower <7.2, medium 7.2-10.8, higher >10.8. Maternal age, body mass index (BMI), smoking status at 15±1 weeks' gestation (no vs. yes), alcohol consumption at 15±1 weeks' gestation (no vs. yes) and fruit intake at 15±1 weeks' gestation (≥3x per month vs. <3x per month) were included as main effects within the generalised linear models. These analyses were repeated for combinations of vitamin D and calcium status: serum 25(OH)D and plasma calcium, serum 25(OH)D and urinary calcium, plasma and urinary calcium and urinary calcium and DPD. Each participant was assigned to one of four categories based on whether they had "lower" levels of both (1st tertile), "higher" levels of both (2nd and 3rd tertile combined) or a combination of "lower and higher". Finally, multinomial logistic regression analysis was performed using the *nnet* package in R to assess the effect of vitamin D and calcium status on PE and/or SGA with or without an abnormal uterine or umbilical artery RI. Women who did not have PE and/or SGA nor an abnormal uterine or umbilical artery RI were selected as the reference category. Significance was determined with a $P<0.05$.

6.3 Results

6.3.1 Study Participants

Of the 1164 women recruited in Adelaide, plasma samples were available for 1065 (92%). Maternal characteristics of the study participants are summarised in Table 6.1. The mean ± SD maternal age and BMI was 24 ± 5.1 y, and 27 ± 6.52 kg/m^2, respectively. One quarter of the women were smoking cigarettes at 15±1 week's gestation but very few (4%) were regularly drinking alcohol. Low fruit consumption (<3 times/month) was recorded for 112 women (11%) indicating a proportion of the population were likely to have poorer dietary habits. Data from Doppler ultrasound assessment at 19-21 weeks' gestation of uterine and umbilical artery RI were available for 1043 (98%) women, for whom, 12% recorded an abnormal uterine artery RI and 18% recorded an abnormal umbilical RI (Table 6.1).

6.3.2 Calcium and Vitamin D Status

Total maternal plasma calcium and 25(OH)D in serum (mean ± SD) for all women studied were 2.06 ± 0.141 mmol/L and 59.41 ± 23.13 nmol/L, respectively (Table 6.1). The interquartile range for plasma calcium was from 2.00 to 2.15 mmol/L and similar to normal reference ranges for women in the second trimester of pregnancy [43]. Mean ± SD urinary Ca:Cr and DPD:Cr ratios were 0.69 ± 0.42 and 10.28 ± 18.01, respectively (Table 6.1).

Spearman's correlational analyses showed a positive correlation between plasma calcium and serum 25(OH)D and between urinary Ca:Cr and urinary DPD:Cr (Fig. 6.1). Serum 25(OH)D was negatively correlated with urinary DPD:Cr. Neither serum 25(OH)D nor urinary DPD:Cr were correlated with urinary Ca:Cr.

TABLE 6.3. Baseline characteristics of the women recruited to the SCOPE[1] Adelaide pregnancy cohort at 15±1 week's gestation

	Participants (n = 1065)
Age *y*, mean (SD)	24 (5.1)
BMI kg/m^2, mean (SD)	27.0 (6.52)
Smoking status, n (%)	
No	651 (61)
Quit during pregnancy	152 (14)
Smoking	262 (25)
Alcohol Consumption, n (%)	
No	658 (62)
Stopped during pregnancy	362 (34)
Consuming alcohol	45 (4)
Fruit Intake, n (%)	
≥3x per month	953 (89)
<3x per month	112 (11)
Abnormal uterine artery resistance index, n (%)	
No	923 (88)
Yes	122 (12)
Abnormal umbilical artery resistance index, n (%)	
No	854 (82)
Yes	189 (18)
Plasma Ca *mmol/L*, mean (SD)	2.06 (0.14)
Serum 25(OH)D *nmol/L*, mean (SD)	59.41 (23.13)
Urinary Ca:Cr, mean (SD)	0.69 (0.42)
Urinary DPD:Cr, mean (SD)	10.28 (18.01)

[1]SCOPE: Screening for Pregnancy Endpoints

6.3.3 Pregnancy Outcome

There were 85 women who developed PE (8%) and the incidence of SGA was 13% (134 pregnancies). Table 6.2 shows the association between calcium and vitamin D status with PE and SGA. Using higher levels (>0.83) as the reference category, we found that women categorised as having lower urinary Ca:Cr ratio (<0.46) were 2.13 times more likely to deliver an SGA infant (aRR: 2.13; 95% CI: 1.29, 3.51). Moderate protective effects of lower plasma calcium (<2.0 mmol/L) with PE and SGA were also found, although these were not statistically significant. Maternal vitamin D status and urinary DPD excretion were not associated with PE or SGA. However, an increasing trend in the risk for PE with decreasing DPD:Cr was observed (P = 0.045).

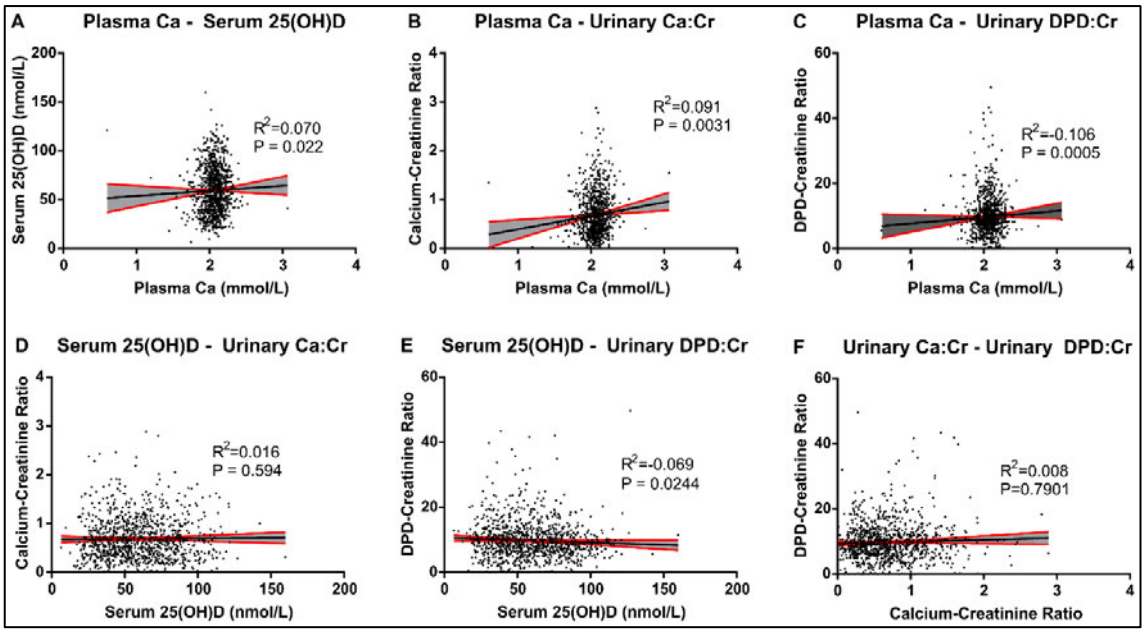

FIGURE 6.7. Spearmans's correlations between plasma calcium, serum 25(OH)D, urinary calcium-creatinine ratio (Ca:Cr) and urinary deoxypyridinoline-creatinine ratio (DPD:Cr) at 15±1 week's gestation. Significant positive correlations were found between plasma calcium and serum 25(OH)D (A), urinary Ca:Cr (B) and urinary DPD:Cr (C). No significant correlations were found between serum 25(OH)D and urinary Ca:Cr (D) but there was a negative correlation with urinary DPD:Cr (E). No significant correlation was found between urinary Ca:Cr and urinary DPD:Cr (F).

We further sought to determine if there was any relationship between combinations of vitamin D, plasma calcium and urinary calcium or DPD status and PE or SGA (Table 6.3). The medium and higher categories were combined for serum 25(OH)D, plasma calcium and urinary calcium or DPD. There was a 55% increased risk for SGA in women with higher standardised 25(OH)D and lower urinary calcium excretion when compared to women who had higher standardised 25(OH)D and higher urinary calcium excretion (aRR: 1.55; 95% CI: 1.07, 2.24). Similarly, women who had both lower urinary calcium and urinary DPD excretion had a more than 2-fold increased risk for SGA compared to those who had higher urinary calcium and DPD excretion (aRR: 2.05; 95% CI: 1.31, 3.20). There was no association between any of the combinations of vitamin D or calcium status with PE.

TABLE 6.4. Adjusted relative risks (aRR) of preeclampsia (PE), small-for-gestational age (SGA) infant and indictors of uteroplacental dysfunction with maternal vitamin D and calcium status categorised based on population tertiles at 15±1 weeks' gestation

	All women (n = 1065)	PE (n = 85)		SGA (n = 134)		Abnormal Uterine Artery RI (n = 122)		Abnormal Umbilical Artery RI (n = 189)	
	n	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)
Standardised serum 25(OH)D** (nnmol/L)									
<41	336	29 (9)	0.96 (0.60, 1.54)	40 (12)	0.78 (0.53, 1.14)	53 (16)	0.90 (0.60, 1.35)	96 (29)	1.17 (0.86, 1.59)
41-61	343	24 (7)	0.80 (0.48, 1.33)	38 (11)	0.78 (0.53, 1.14)	47 (14)	0.92 (0.61, 1.37)	57 (17)	1.01 (0.73, 1.39)
>61	385	32 (8)	1.00†	56 (15)	1.00†	22 (6)	1.00†	36 (9)	1.00†
		P for trend	0.852		0.191		0.744		0.419
Plasma Calcium (mmol/L)									
<2.0	307	20 (7)	0.64 (0.35, 1.15)	30 (10)	0.78 (0.47, 1.30)	32 (10)	0.78 (0.51, 1.18)	50 (16)	0.84 (0.61, 1.16)
2.0-2.1	378	25 (7)	0.79 (0.46, 1.36)	50 (13)	1.13 (0.74, 1.73)	38 (10)	0.74 (0.50, 1.10)	65 (17)	0.88 (0.65, 1.18)
>2.1	380	40 (11)	1.00†	54 (14)	1.00†	52 (14)	1.00†	74 (19)	1.00†
		P for trend	0.069		0.094		0.210		0.281
Urinary Ca:Cr									
<0.46	359	32 (9)	1.18 (0.65, 2.16)	59 (16)	**2.13 (1.29, 3.51)**	53 (15)	1.51 (0.98, 2.32)	82 (23)	**1.57 (1.11, 2.22)**
0.46-0.83	366	31 (8)	1.33 (0.74, 2.39)	38 (10)	1.38 (0.81, 2.37)	38 (10)	1.23 (0.77, 1.98)	65 (18)	**1.42 (1.00, 2.04)**
>0.83	328	22 (7)	1.00†	36 (11)	1.00†	28 (8)	1.00†	42 (13)	1.00†
		P for trend	0.387		**0.033**		0.055		**0.010**
Urinary DPD:Cr									
<7.2	341	32 (9)	1.71 (1.00, 2.91)	54 (16)	1.39 (0.94, 2.06)	34 (10)	**1.55 (1.02, 2.37)**	40 (12)	0.78 (0.54, 1.11)
7.2-10.8	358	31 (9)	1.50 (0.88, 2.55)	40 (11)	1.05 (0.69, 1.60)	40 (11)	1.32 (0.86, 2.05)	63 (18)	1.24 (0.92, 1.66)
>10.8	354	22 (6)	1.00†	39 (11)	1.00†	45 (13)	1.00†	86 (24)	1.00†
		P for trend	**0.045**		0.096		0.102		0.346

*aRR are compared to all women who were not diagnosed with the outcome of interest and were adjusted for maternal age, maternal body mass index and smoking status at 15±1 weeks' gestation (no versus yes), alcohol consumption at 15±1 weeks' gestation (no vs. yes) and fruit intake at 15±1 weeks' gestation (≥3x per month vs. <3x per month)

**Serum 25(OH)D was standardised against month serum sample was taken

†Reference category

Ca:Cr = calcium-creatinine ratio; DPD:Cr = deoxypyridinoline-creatinine ratio

TABLE 6.5. Adjusted relative risks (aRR) of preeclampsia (PE), small-for-gestational age (SGA) infant and PE and/or SGA as an indictor of uteroplacental dysfunction with combinations of maternal vitamin D and calcium status at 15±1 weeks' gestation

	All women (n = 1065)	PE (n = 85)		SGA (n = 134)		Abnormal Uterine Artery RI (n = 122)		Abnormal Umbilical Artery RI (n = 189)	
	n	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)
Standardised Serum 25(OH)D (nmol/L) and Plasma Calcium (mmol/L)**									
<41 & <2.0	112	11 (10)	1.06 (0.57, 1.97)	13 (12)	0.75 (0.43, 1.30)	14 (13)	0.82 (0.45, 1.50)	27 (24)	1.21 (0.82, 1.79)
<41 & >2.0	224	18 (8)	0.86 (0.52, 1.43)	27 (12)	0.80 (0.53, 1.20)	39 (17)	0.97 (0.63, 1.49)	69 (31)	1.00 (0.72, 1.39)
>41 & <2.0	196	9 (5)	0.52 (0.26, 1.05)	17 (9)	0.61 (0.37, 1.01)	18 (9)	0.92 (0.58, 1.46)	23 (23)	0.71 (0.47, 1.06)
>41 & >2.0	533	47 (9)	1.00†	77 (14)	1.00†	51 (10)	1.00†	70 (13)	1.00†
Standardised Serum 25(OH)D (nmol/L) and Urinary Ca:Cr**									
<41 & <0.46	121	12 (10)	1.21 (0.66, 2.20)	17 (14)	1.19 (0.71, 2.01)	17 (14)	0.97 (0.51, 1.83)	44 (36)	1.43 (0.96, 2.13)
<41 & >0.46	214	17 (8)	0.90 (0.52, 1.56)	23 (11)	0.96 (0.60, 1.54)	36 (17)	1.41 (0.89, 2.25)	52 (24)	1.21 (0.84, 1.73)
>41 & <0.46	238	20 (8)	0.93 (0.54, 1.58)	42 (18)	**1.55 (1.07, 2.24)**	36 (15)	**1.79 (1.19, 2.68)**	38 (16)	1.34 (0.97, 1.85)
>41 & >0.46	479	36 (8)	1.00†	51 (11)	1.00†	30 (6)	1.00†	55 (11)	1.00†
Plasma Calcium (mmol/L) and Urinary Ca:Cr									
<2.0 & <0.46	109	7 (6)	0.65 (0.26, 1.63)	12 (11)	1.46 (0.84, 2.54)	16 (15)	1.28 (0.76, 2.17)	25 (23)	1.24 (0.83, 1.84)
<2.0 & >0.46	194	13 (7)	0.78 (0.40, 1.50)	41 (21)	0.53 (0.27, 1.07)	15 (8)	0.78 (0.45, 1.35)	25 (13)	0.80 (0.53, 1.20)
>2.0 & <0.46	250	25 (10)	1.07 (0.62, 1.86)	45 (18)	**1.58 (1.04, 2.38)**	37 (15)	1.26 (0.85, 1.86)	57 (23)	1.21 (0.89, 1.64)
>2.0 & >0.46	500	40 (8)	1.00†	18 (4)	1.00†	51 (10)	1.00†	82 (16)	1.00†
Urinary DPD:Cr and Urinary Ca:Cr									
<0.46 & <7.2	95	11 (12)	1.36 (0.70, 2.63)	23 (24)	**2.05 (1.31, 3.20)**	16 (17)	**1.90 (1.20, 3.02)**	15 (16)	0.80 (0.49, 1.29)
<0.46 & >7.2	181	18 (10)	1.49 (0.88, 2.53)	21 (12)	1.25 (0.80, 1.94)	18 (10)	1.16 (0.73, 1.86)	25 (14)	0.80 (0.53, 1.18)
>0.46 & <7.2	292	22 (8)	1.15 (0.68, 1.96)	39 (13)	1.35 (0.90, 2.03)	37 (13)	1.20 (0.77, 1.85)	67 (23)	**1.39 (1.04, 1.87)**
>0.46 & >7.2	485	34 (7)	1.00†	50 (10)	1.00†	48 (10)	1.00†	82 (17)	1.00†

*aRR are compared to all women who were not diagnosed with the outcome of interest and were adjusted for maternal age, maternal body mass index and smoking status at 15±1 weeks' gestation (no versus yes), alcohol consumption at 15±1 weeks' gestation (no vs. yes) and fruit intake at 15±1 weeks' gestation (≥3x per month vs. <3x per month)

**Serum 25(OH)D was standardised against month serum sample was taken

†Reference category

6.3.4 Uteroplacental Dysfunction

The strongest association was observed between maternal calcium status and SGA and indicated the greatest effect on fetal growth. Thus, we tested the association between maternal vitamin D and calcium status on mid-gestation abnormal uterine and umbilical artery RI as indicators of potential uteroplacental dysfunction. Whilst there was no association between serum 25(OH)D, plasma calcium, urinary Ca:Cr with abnormal uterine artery RI at 19-21 weeks' gestation, women in the 1st tertile (<7.2) urinary DPD:Cr, were at an increased risk of having an abnormal RI when compared to those in the 3rd tertile (>10.8)(Table 6.2: aRR: 1.55; 95% CI: 1.02, 2.37). Furthermore, decreasing urinary calcium excretion at 15±1 weeks' gestation was associated with increased risk of an abnormal umbilical artery RI (Table 6.2, Lower: aRR: 1.57; 95% CI: 1.11, 2.22 and Medium: 1.42; 95% CI: 1.00, 2.04).

For combinations of vitamin D and calcium levels, similar to the results found with SGA, there was a 1.79-fold increased risk of having an abnormal uterine artery RI in women with both higher serum 25(OH)D and lower calcium excretion when compared to those with higher levels of both (Table 6.3, aRR: 1.79; 95% CI: 1.19,). Furthermore, women with lower urinary DPD and calcium were also at an increased risk of having an abnormal uterine artery RI at 19-21 weeks' gestation (Table 6.3, aRR: 1.90; 95% CI: 1.20, 3.02). On the other hand, women with higher urinary DPD excretion, indicating more bone reabsorption, and lower calcium excretion were at an increased risk of having an abnormal umbilical artery RI when compared to women with higher urinary excretion of both (Table 6.3, aRR 1.39; 95% CI: 1.04, 1.87).

Finally, we further classified women based on whether there was evidence of placental dysfunction based on Doppler ultrasound who also went on to develop a pregnancy complication given that not all women who had an abnormal uterine or umbilical artery RI in mid-pregnancy developed PE or SGA (Table 6.4 and Table 6.5). A 3- to 4-fold increased risk of having an abnormal

uterine artery RI and having PE and/or SGA in women was found with decreasing urinary DPD (Lower: aRR: 4.30; 95% CI: 1.55, 11.92 and Medium: aRR: 3.20; 95% CI: 1.13, 9.02). There was also an increased risk of having both an abnormal uterine artery RI and having PE and/or SGA with lower and medium urinary calcium excretion (Lower: aRR: 1.57; 95% CI: 1.11, 2.22 and Medium: aRR: 1.42; 95% CI: 1.00, 2.04). Women with plasma calcium levels in the 2nd tertile (2-2.1 mmol/L) were protected against having both an abnormal uterine artery RI and developing PE and/or SGA (aRR: 0.32; 95% CI: 0.13-0.76).

6.4 Discussion

To our knowledge, this is one of the first studies to assess the relationship between maternal calcium and vitamin D status and PE, SGA, and measures of placental function in mid-gestation that provide an indication of impaired uteroplacental blood flow. We have demonstrated, in this large prospective study, a protective association between higher urinary calcium excretion and outcomes associated with abnormal fetal growth and placental dysfunction. Urinary calcium excretion can be used as a representation of maternal calcium intake [44, 45] and provides further evidence for the importance of calcium and vitamin D in pregnancy that may have implications for placental development and function.

Incomplete trophoblast invasion into the maternal uterine spiral arteries has been described in pregnancies complicated by fetal growth restriction, as well as PE [11]. However, not all women who develop PE or deliver a SGA infant demonstrate this defect. Doppler ultrasound of the uterine arteries provides a surrogate of trophoblast invasion whereby a high RI indicates reduced perfusion on the maternal side most likely due to inadequate spiral artery remodelling [12].

TABLE 6.4. Adjusted relative risks (aRR) of preeclampsia (PE) and/or small-for-gestational age (SGA) infant with and without an abnormal UTERINE artery RI with maternal vitamin D and calcium status categorised based on population tertiles at 15±1 weeks' gestation

	No PE, SGA or abnormal uterine artery RI (n = 768)	No PE, SGA but an abnormal uterine artery RI (n = 83)		PE and/or SGA, no abnormal uterine artery RI (n = 155)		PE and/or SGA and abnormal uterine artery RI (n = 39)	
	n (%)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)
Standardised serum 25(OH)D** (nmol/L)							
<41	244 (73)	24 (7)	0.80 (0.46-1.40)	49 (15)	0.81 (0.54-1.24)	13 (4)	0.96 (0.43-2.10)
41-61	253 (74)	26 (8)	0.83 (0.48-1.43)	44 (13)	0.75 (0.49-1.15)	12 (3)	0.93 (0.42-2.06)
>61	270 (70)	33 (9)	1.00†	62 (16)	1.00†	14 (4)	1.00†
Plasma Calcium (mmol/L)							
<2.0	238 (78)	22 (7)	0.85 (0.47-1.52)	34 (11)	**0.63 (0.40-1.00)**	10 (3)	0.49 (0.23-1.08)
2.0-2.1	273 (72)	31 (8)	0.99 (0.58-1.70)	61 (16)	1.01 (0.68-1.52)	7 (2)	**0.32 (0.13-0.76)**
>2.1	257 (68)	30 (8)	1.00†	60 (16)	1.00†	22 (6)	1.00†
Urinary Ca:Cr							
<0.46	252 (70)	41 (11)	**1.82 (1.04-3.19)**	75 (21)	**1.60 (1.05-2.42)**	12 (3)	**1.57 (1.11, 2.22)**
0.46-0.83	266 (73)	18 (5)	0.74 (0.38-1.43)	35 (10)	0.75 (0.46-1.21)	20 (5)	**1.42 (1.00, 2.04)**
>0.83	242 (74)	21 (6)	1.00†	44 (13)	1.00†	7 (2)	1.00†
Urinary DPD:Cr							
<7.2	233 (68)	26 (8)	1.27 (0.71-2.26)	54 (16)	1.28 (0.83-1.97)	19 (6)	**4.30 (1.55-11.92)**
7.2-10.8	265 (74)	26 (7)	1.07 (0.60-1.89)	49 (14)	1.02 (0.66-1.58)	15 (4)	**3.20 (1.13-9.02)**
>10.8	262 (74)	28 (8)	1.00†	51 (14)	1.00†	5 (1)	1.00†

*aRR are compared to women who were not diagnosed with PE or SGA and did not have an abnormal uterine artery resistance index (RI) and were adjusted for maternal age, maternal body mass index and smoking status at 15±1 weeks' gestation (no versus yes), alcohol consumption at 15±1 weeks' gestation (no vs. yes) and fruit intake at 15±1 weeks' gestation (≥3x per month vs. <3x per month)

**Serum 25(OH)D was standardised against month serum sample was taken

†Reference category

Ca:Cr = calcium-creatinine ratio; DPD:Cr = deoxypyridinoline-creatinine ratio

TABLE 5. Adjusted relative risks (aRR) of preeclampsia (PE) and/or small-for-gestational age (SGA) infant with and without an abnormal UMBILICAL artery RI with maternal vitamin D and calcium status categorised based on population tertiles at 15±1 weeks' gestation

	No PE, SGA or abnormal umbilical artery RI (n = 713)	No PE, SGA but an abnormal umbilical artery RI (n = 136)		PE and/or SGA, no abnormal umbilical artery RI (n = 141)		PE and/or SGA and abnormal umbilical artery RI (n = 53)	
	n (%)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)	*n* (%)	aRR* (95% CI)
Standardised serum 25(OH)D** (nmol/L)							
<41	222 (66)	46 (14)	1.14 (0.73-1.79)	42 (13)	0.78 (0.50-1.21)	20 (6)	1.17 (0.60-2.28)
41-61	235 (69)	44 (13)	1.03 (0.65-1.62)	42 (12)	0.78 (0.50-1.22)	14 (4)	0.82 (0.40-1.69)
>61	255 (66)	46 (12)	1.00†	57 (15)	1.00†	14 (4)	1.00†
Plasma Calcium (mmol/L)							
<2.0	228 (74)	32 (10)	0.67 (0.41-1.08)	26 (8)	**0.47 (0.29-0.78)**	18 (6)	0.83 (0.43-1.59)
2.0-2.1	250 (66)	53 (14)	0.99 (0.64-1.52)	56 (15)	0.95 (0.63-1.44)	12 (3)	0.51 (0.25-1.06)
>2.1	235 (62)	51 (13)	1.00†	59 (16)	1.00†	23 (6)	1.00†
Urinary Ca:Cr							
<0.46	241 (67)	52 (14)	1.46 (0.91-2.35)	57 (16)	1.26 (0.81-1.96)	30 (8)	**3.12 (1.44-6.77)**
0.46-0.83	231 (63)	51 (14)	1.49 (0.92-2.41)	41 (11)	1.01 (0.63-1.62)	14 (4)	1.63 (0.69-3.89)
>0.83	230 (70)	33 (10)	1.00†	42 (13)	1.00†	9 (3)	1.00†
Urinary DPD:Cr							
<7.2	232 (68)	27 (8)	0.62 (0.37-1.04)	54 (16)	1.44 (0.91-2.27)	19 (6)	1.36 (0.65-2.84)
7.2-10.8	232 (65)	58 (16)	1.29 (0.84-1.97)	44 (12)	1.16 (0.72-1.85)	20 (6)	1.58 (0.77-3.25)
>10.8	238 (67)	51 (14)	1.00†	44 (12)	1.00†	20 (6)	1.00†

*aRR are compared to women who were not diagnosed with PE or SGA and did not have an abnormal umbilical artery resistance index (RI) and were adjusted for maternal age, maternal body mass index and smoking status at 15±1 weeks' gestation (no versus yes), alcohol consumption at 15±1 weeks' gestation (no vs. yes) and fruit intake at 15±1 weeks' gestation (≥3x per month vs. <3x per month)

**Serum 25(OH)D was standardised against month serum sample was taken

†Reference category

Ca:Cr = calcium-creatinine ratio; DPD:Cr = deoxypyridinoline-creatinine ratio

Our finding that reduced urinary calcium excretion is associated with increased risk of having an abnormal uterine artery RI and pregnancy complicated by PE and/or SGA indicates a strong association between maternal calcium status, early placental development and fetal growth. The relationship between urinary calcium excretion, calcium intake and intestinal calcium absorption is complex and not fully understood but higher calcium intakes do result in higher calcium excretion [44, 45]. Adequate calcium intake during pregnancy is essential to support fetal growth, particularly bone growth and development. In cases where maternal calcium intakes are low or insufficient, this may result in increased maternal bone resorption in order to mobilise calcium for the fetus [7]. Indeed, the risk for having an abnormal uterine artery RI and pregnancy complicated by PE and/or SGA was highest in the women with lower urinary DPD. This may indicate problems whereby the mother does not supply an adequate amount of calcium to support fetal growth and placental development and is worthy of further investigation.

In pregnancies in which fetal growth restriction has occurred, the placentas may lack the functional or structural capabilities to support optimal fetal growth [46]. The fetal chorionic villi are composed of several different cell types of which, the syncytiotrophoblasts, the cells which line the intervillous space and cover the fetal villi, are extremely important in coordinating the exchange of oxygen, nutrients and wastes between maternal and fetal circulations [47]. Indeed, particular dysfunction of syncytiotrophoblast cells is thought to underlie the pathophysiology of PE and contribute to fetal growth restriction [48]. Once again, lower urinary calcium excretion was associated with increasing the risk of having an abnormal umbilical artery RI and pregnancy complicated by PE and/or SGA and highlights the importance of calcium to the development of the feto-placental circulation. Calcium is a universal intracellular second messenger involved in many cellular processes [49] and likely to be extremely important in maintaining syncytiotrophoblast function. Furthermore,

a protective effect of having lower plasma calcium was observed in women who developed PE and/or SGA but no abnormal uterine or umbilical RI at 19-21 weeks' gestation. One explanation for this may be that more calcium is being utilised where required in cells and tissues such as the placenta, lowering levels in the circulation to allow proper establishment of the uteroplacental circulation.

Hypocalciuria has been previously associated with PE and was thought to result from decreased dietary calcium intakes, decreased absorption of calcium in the digestive system, increased fetal and/or placental calcium demands or renal dysfunction [50-52]. Indeed, it has been proposed that the early pregnancy urinary Ca:Cr ratio may be a predictor of the development of PE [30] as reduced calcium excretion has been shown to be a characteristic of the disease. In the current study however, we did not find any association between the urinary Ca:Cr ratio nor vitamin D status at 15±1 weeks' gestation and PE. This may reflect the fact that surveys of pregnant Australian women indicate that the majority meet the recommended daily intakes of calcium [53, 54]. Although dietary intake of calcium was not measured directly in this population, circulating plasma levels of total calcium fell between the reference range of 2.05-2.25 mmol/L for women in the second trimester of pregnancy [43] indicating that the women included in the present study were not calcium deficient. Furthermore, calcium supplementation has been shown to reduce the risk of PE and is more effective in women with low dietary calcium intakes [28, 55, 56].

As previously mentioned, the literature suggesting associations between maternal vitamin D deficiency and pregnancy complications is equivocal [20, 21]. In this study, maternal vitamin D status was not associated with PE, SGA or uteroplacental dysfunction. However, significant relationships were found when combined with maternal calcium status. Women with both lower vitamin D and lower urinary calcium excretion at 15±1 weeks' gestation were at increased risk of having an abnormal umbilical artery RI at 19-21 weeks' gestation compared to those who had higher levels of both. This is consistent with mechanistic

understanding of vitamin D metabolism in pregnancy as higher levels of vitamin D are required to increase intestinal calcium absorption and renal reabsorption to meet fetal and placental calcium demands [3]. However, having both higher serum 25(OH)D and lower urinary calcium was associated with increasing the risk of delivering an SGA infant or having an abnormal uterine artery RI at 19-21 weeks' gestation. This suggests that, at least within this population, calcium status is likely a more important determinant of appropriate placental development and fetal growth.

The current study is strengthened by the use of a well-characterised cohort of pregnant women. However, there are a number of limitations which need to be considered when interpreting the data. Plasma calcium for example, is subject to a number of inter- and intra-patient variability [57] that cannot be controlled for in a real-world study design. This study assumes calcium intake was adequate in this population of women as this information is not available. However, evidence from other surveys in pregnant Australian women show adequate calcium consumption [53, 54]. Therefore, it is highly likely that the majority of the studied population were meeting their recommended daily intakes of calcium. Furthermore, urinary calcium excretion was measured. This best reflects chronic calcium status and thus incorporates dietary calcium intake over time, offering a surrogate from limited dietary data and supports the findings between plasma calcium and pregnancy outcome. Finally, given how tightly regulated plasma calcium is, and the difference of just 0.1 mmol/L calcium between the lower, medium and higher calcium groups, renders the biological importance of the findings on plasma calcium in the present study uncertain. As this is an observational study, the mechanisms by which disrupted calcium homeostasis may contribute to uteroplacental dysfunction cannot be determined. However, the associations in this study would suggest a strong effect of calcium in mediating appropriate placental development worth further research.

In conclusion, the current study shows altered calcium homeostasis in early pregnancy is associated with increased risk of uteroplacental dysfunction with consequences for fetal growth. Continued research is required to determine whether these associations are causal but highlights the importance of adequate calcium intakes in pregnancy and the potential use of urinary and plasma calcium measurements in early pregnancy to assist stratifying women at risk for a placental dysfunction. Furthermore, calcium status may explain the disparate published findings on the association of vitamin D deficiency and pregnancy complications and this should be assessed in future studies.

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Chapter 7

Characterisation of 5-methylcytosine and 5-hydroxymethylcytosine in human placenta cell types across gestation

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ABSTRACT

The placenta is one of the most important organs to human reproduction. However, very little is understood about placental development at a molecular level. Placental dysfunction is often associated with pregnancy complications including preeclampsia (PE) as well as with maternal micronutrient deficiencies. Whilst the exactly how micronutrient deficiencies cause placental dysfunction remain unclear, disruption of epigenetic mechanisms, such as DNA methylation have been implicated in the mechanistic link. However, studying placental DNA methylation is complicated by the presence of multiple different cell types each of which is likely to have its own unique methylome. We used immunohistochemistry (IHC) and

immunofluorescence (IF) to localise 5-methylcytosine (5-mC) and 5-hydroxy-methylcytosine (5-hmC) in placenta tissue from first/second trimester and term. IHC analysis of tissue sections showed levels of 5-mC increased across gestation. When specific trophoblast cell subtypes: cytotrophoblasts (CTB) and syncytiotrophoblasts (STB), were isolated and labelled using IF, levels of both 5-mC and 5-hmC were increased in term CTBs compared to first/second trimester. Staining intensity of 5-hmC was also increased in first trimester STBs compared to CTBs. Finally, comparison of IHC staining in term tissue from PE and uncomplicated pregnancies revealed levels of 5-mC to be higher in placentas from PE pregnancies. Our analysis confirmed that 5-mC and 5-hmC staining intensity increased across gestation and was different between CTB and STB and provides a solid foundation for future research focused on single cell populations. Very little is known about the role of 5-hmC in placental development but our data suggest it is not merely an intermediate step during demethylation but also a marker of cellular identity likely to influence gene expression and placental function. Finally, differences in DNA methylation profiles between different cell types of the placenta may be indicative of different functions and requires further study in order to elucidate what changes accompany placental pathologies as well as how perturbed micronutrient status affects DNA methylation profiles.

7.1 Introduction

The placenta is arguably the most important organ for human reproduction. It is shared by both mother and fetus, conducts oxygen, nutrient and waste transfer to the growing fetus and is responsible for the secretion of a variety of hormones and steroids imperative to correct maternal adaptation to pregnancy [1]. Thus, it is unsurprising that defective placentation and consequently placental

dysfunction underlies many of the major obstetric diseases including preeclampsia (PE) and fetal growth restriction (FGR) [2]. However, whilst a significant amount is understood about the down-stream effects of placental dysfunction, the cause of inadequate placental development and function are poorly understood.

Placental development is highly organised and results in numerous cell types with specialised functions. The most important cell types of the placenta are the trophoblasts which derive from the trophectoderm [3]. Trophoblast differentiation can follow one of two pathways: the villous pathway to form cytotrophoblasts (CTBs) and syncytiotrophoblasts (STBs) or CTB cell column pathway to form extravillous CTBs [4]. In terms of mediating oxygen, nutrient and waste exchange between maternal and fetal circulation, the CTBs and STBs are imperative to this process. STBs form a continuous epithelial layer on the surface of the villous tree and is maintained throughout pregnancy by constant fusion of the underlying CTBs [5]. Furthermore, STB stress is hypothesised to be a major factor associated with placental dysfunction in pregnancies complicated by PE [6]. Yet despite an intense amount of research in recent years, comprehensive analysis of key molecular processes which govern human trophoblast differentiation as well as how disruption to these processes may contribute to STB stress are poorly understood.

Epigenetic regulation of the placental transcriptome is emerging as an important mediator of placental cell differentiation. Environmental factors such as smoking, obesity, poor nutrition and micronutrient deficiencies, which are associated with placental dysfunction, are also known to impact the epigenome [7, 8]. The most widely studied epigenetic modification in the placenta is DNA methylation: the addition of a methyl group to primarily the cytosine DNA base forming 5-methylcytosine (5mC) [9]. DNA methylation is crucial to many cellular processes including the regulation of gene expression and genomic imprinting. The genome of the placenta is hypomethylated compared to other organs [10, 11].

However, how or why this is the case is unclear. To add complexity, data is emerging on the oxidised derivative of 5-mC, 5-hydroxymethylcytosine (5-hmC) [12], and the roles this modification plays in the regulation of cellular processes. Indeed, there is increasing evidence to suggest 5-hmC is not merely a passive intermediate in the DNA demethylation process but contributes its own epigenetic functions [13].

Recently, we reported that DNA methylation in the placenta could accurately predict gestational age and was accelerated in placentas from pregnancies complicated by early onset PE [14]. However, studying DNA methylation in the placenta is complicated by the presence of numerous different cell types which carry their own unique methylomes [9]. Furthermore, before a detailed understanding of what changes occur to the epigenome of the placenta in pregnancy complications, we first need to develop a greater understanding of how the placental transcriptome is epigenetically regulated in normal placental development. In this study, we sought to use visualisation techniques, immunohistochemistry (IHC) and immunofluorescence (IF), to characterise the localisation of 5-mC and 5-hmC in placenta tissue across gestation, as well as in tissues from pregnancies complicated by PE. Furthermore, we aimed to determine whether localisation of both markers differed between isolated CTB and STB cells with the hypothesis that the different trophoblast populations would display different staining intensities based on previous published literature [15]. Our analysis confirmed that 5-mC and 5-hmC staining intensity increased across gestation and is different between CTB and STB cells. Moreover, the staining intensity of 5-mC appeared to be greater in placenta tissue from pregnancies complicated by PE and reinforces the need to use more sensitive methodologies on extracts from individual cell populations rather than whole tissue.

7.2 Materials and Methods

7.2.1 Tissue samples

First and second trimester placenta tissue was obtained with written informed consent from the Adelaide Pregnancy Advisory Centre (PAC) with ethics approved by the University of Adelaide Human Ethics committee (ethics number: HREC/16/TQEH/33). Term placental tissue was collected from women recruited as part of the Screening Tests to Predict Poor Outcomes of Pregnancy (STOP) study. The study was also approved by the University of Adelaide Human Ethics committee (ethics number: HREC/14/WCHN/90) and all women provided written informed consent.

7.2.2 Immunofluorescent labelling of 5-mC, 5-hmC, PEG-10 and PSG-1 in placenta tissue

Paraffin-embedded paraformaldehyde (first and second trimester) or formalin (term) fixed sections, cut at 3 μm thickness, were used for double-label IF or IHC. Tissue sections were dewaxed and rehydrated according to standard protocols. Antigen retrieval was performed using boiling citrate buffer (10 mM, pH 6.0) for 15 minutes, followed by a hydrochloric acid digestion (1.5 M) step for 30 minutes at room temperature. Sections were then blocked in 10% goat serum with 0.3% Triton-X in PBS for 30 minutes to reduce non-specific binding. Antibodies were diluted in 10% bovine serum albumin (BSA) in PBS with 0.3% Triton-X as outlined in Table 7.1 and applied to the sections in a humidified environment overnight at 4°C. For IF, antigens were visualised by applying fluorophore-conjugated anti-mouse (5-mC and PSG-1) or anti-rabbit (5-hmC and PEG-10) secondary antibodies (Table 1) for 3 hours at room temperature. Nuclei were counterstained using 4',6-diamidino-2-phenylindole (DAPI). For IHC, biotin conjugated anti-mouse or anti-rabbit secondaries (Table 1) were applied for 1 hour at room temperature followed by another hour incubation with streptavidin horseradish peroxidase (strep-HRP; *Dako*). Sections were washed

and staining visualised using 3,3′-diaminobenzidine (DAB; *Sigma*). Nuclei were counter-stained with haematoxylin.

TABLE 7.1. Antibodies and dilutions used for immunohistochemistry and immunofluorescence

Antigen	Supplier	Species	Clone	Dilution	Antigen Retrieval
5-methylcytosine	GeneTex	Monoclonal Mouse	33D3	Cells: 1/400 Tissue: 1/200	Cells: Yes, HCl Tissue: Yes, Citrate & HCl
5-hydroxy-methycytosine	Active Motif	Polyclonal Rabbit		Cells: 1/5000 Tissue: 1/10,000	Cells: Yes, HCl Tissue: Yes, Citrate & HCl
Paternally-expressed gene 10 (PEG-10)	Abcam	Monoclonal Rabbit	EPR20051	Cells: 1/100 Tissue: 1/200	Cells: No Tissue: Yes Citrate
Pregnancy specific beta-1-glycoprotein 1 (PSG-1)	Abcam	Monoclonal Mouse	BAP3	Cells: 1/100 Tissue: 1/250	Cells: No Tissue: Yes Citrate
Alexa Fluor 488 Anti-Mouse	Life Technologies	Goat		1/750	
Alexa Fluor 555 Anti-Rabbit	Life Technologies	Goat		1/750	

7.2.3 Isolation of first and second trimester cytotrophoblast and syncytiotrophoblast cells

First trimester cytotrophoblast and syncytiotrophoblast cells were isolated from tissue following a modified protocol outlined in [30]. Initially, villous tissue was washed in PBS in order to remove maternal blood before being incubated for 10 minutes at 37°C with 10 mL 0.25% trypsin (*Life Technologies*) with 200 μg/mL DNAse (*Roche*) in PBS per gram of tissue. The supernatant, containing largely syncytiotrophoblasts and extravillous cytotrophoblasts, was collected and the villous tissue washed eight times with 20 mL PBS. Each wash, together with the initial digest supernatant, was passed through a 100 μM filter into a 50 mL tube containing 5 mL fetal calf serum (FCS; *Sigma*) to remove debris before being passed through a 70 μM filter to remove smaller

contaminating cells. The filtrate was discarded and the syncytiotrophoblast cells remaining on the filter collected for immunofluorescent labelling.

Continuing with the cytotrophoblast isolations, the villous tissue was digested for a second time in 10 mL 0.25% trypsin with 200 μg/mL DNAse in PBS per gram of tissue on gentle agitation for 7 minutes at 4°C before being left stationary at 4°C overnight. The following morning, villi were washed ten times in PBS with the supernatant collected into 50 mL tubes containing 5 mL FCS and centrifuged at 450 g^{-1} for 8 minutes. Supernatant was aspirated, cell pellets combined into 15 mL per gram of tissue DMEM media (*Life Technologies*) containing 10% FCS and 1% Antibiotic-Antimycotic (*Life Technologies*) and then incubated for 10 minutes at 37°C in 5% CO2 on a 10 cm petri dish to allow contaminating fibroblasts to adhere to the plastic surface. The cell suspension was then collected and the petri dishes washed twice with 5 mL DMEM media in order to maximise recovery of cytotrophoblasts which were then used for immunofluorescent staining.

For isolation of cytotrophoblasts and syncytiotrophoblasts from second trimester tissues, the protocol follows that for isolation of first trimester with slight modifications on day two of the procedure. Following the second, overnight digest, villous tissue was washed ten times in PBS and the supernatant collected into 50 mL tubes containing 5 mL FCS. Tubes were centrifuged at 450 g-1 for 8 minutes and the cells resuspended in 6 mL of 1x Ca/Mg-free Hank's buffered saline solution (HBSS) to yield approximately 8 mL of cell suspension. The cell suspension was gently layered onto two 50 mL falcon tubes containing a 70-5% Percoll® gradient (*Sigma*) and centrifuged for 20 minutes at 1200 g^{-1} without brake. Following centrifugation, the cytotrophoblasts, situated within the layer ranging from 27-12 mL was collected into a clean falcon tube, diluted with DMEM media and centrifuged at 1000 g^{-1} for 10 minutes. The supernatant was aspirated, cells resuspended in 1x HBSS for cell counts and then centrifuged for another 5 minutes at 1000 g^{-1}. Following this, the cells were resuspended in DMEM media to a concentration of 107 cells/mL and transferred to 1.5 mL microcentrifuge tubes for negative selection and the removal of contaminating

lymphocytes and fibroblasts. As per Dynabead® protocol (*Life Technologies*), mouse anti-human CD9 antibody (*RnD Systems*) was added to each microcentrifuge tube at a concentration of 1 μg/106 cells and incubated on a rotator for 10 minutes at 4°C. Following incubation, anti-mouse Dynabeads® were washed as suspended as per manufacturer's protocol, added to the cells and incubated for a further 30 minutes on a rotator at 4°C to allow binding. After the incubation, microcentrifuge tubes were placed onto a magnetic separator and the supernatant containing the isolated cytotrophoblast cells collected to be used for immunofluorescent staining.

7.2.4 Isolations of term cytotrophoblast cells

Cytotrophoblasts and syncytiotrophoblasts were isolated from term placenta tissue following the protocol outlined in [31]. Placenta tissue was collected from elective caesarean sections, washed in PBS and heavily dissected to dissociate placenta villi fragments from vessels and connective tissue. Approximately 50 g of tissue was then digested three times for 20 mins in 150 mL of enzyme digestion buffer containing 0.25% trypsin and 1% DNAse at 37°C in a shaking water bath. Between each digest, supernatant was collected into 50 mL tubes containing 5 mL FCS and cell pellets centrifuged at 1000 g^{-1} for 15 minutes. Cell pellets from all three digests were then combined in 1x HBSS solution and the remaining isolation protocol followed that outlined for second trimester placenta cytotrophoblast isolations.

7.2.5 Immunofluorescent labelling of 5-mC, 5-hmC, PEG-10 and PSG-1 in isolated placenta cells

Isolated cytotrophoblast and syncytiotrophoblast cells were spun onto slides using cytocentrifugation (6-8x105 cells/dot, 1000 g^{-1}, 5 minutes; CytospinTM, *Thermo Fisher Scientific*). Cells were then fixed with 1:1 acetone-methanol for 10 minutes on ice and immunostaining proceeded as previously described.

7.2.6 Microscopic analysis and image quantification

IHC staining of 5-mC and 5-hmC in tissue sections were visualised using the Nanozoomer 2.0-HT C9600-13 (Hamamatsu). Random systematic sampling of the tissue was used to select 10 representative fields of each section at 40x magnification using the NDP View2 software (*Hamamatsu*). DAB staining intensity was then quantified using the VIA software (*Leading Edge Software*). Double-label IF was visualised using the Nikon A1+ Confocal microscope system and images created using the NIS-Element Advanced Research microscope imaging software (*Nikon*). Analysis of 5-mC and 5-hmC in isolated cells was visualised using the Nikon Eclipse Ni-U microscope and fluorescent intensity measured using ImageJ Version 1.48 software (*National Institutes of Health*) [32].

7.2.7 Statistics

All statistical analysis was performed in R (v3.1.1) [33]. Analysis of Variance (ANOVA) with a Tukey post-hoc comparison or Mann-Whitney test was used to calculate exact P-values and results are reported as median and interquartile range. Statistical significance was deemed at $P < 0.05$.

7.3 Results

7.3.1 Identification and isolation of CTB and STB cells

Double-labelled IF was used to identify CTB and STB cells in tissue sections using antibodies against two proteins: Paternally-expressed gene 10 (PEG-10) and Pregnancy specific beta-1-glycoprotein 1 (PSG-1). These proteins are expressed by CTBs (PEG-10) [16] and STBs (PSG-1) [17], and allowed the two cell types to be clearly distinguished (Fig. 7.1A) and confirmed the purity of >95% CTBs with the occasional clump of contaminating STB in trophoblast isolations (Fig. 7.1B). Furthermore, double-labelled IF revealed punctate staining of both 5-mC and 5-hmC, confirmed co-localisation of both marker (Fig. 7.2A) and allowed assessment of 5-mC and 5-hmC between different cell types in tissue sections staining with PEG-10 and PSG-1, respectively (Fig. 7.2B & 7.2C).

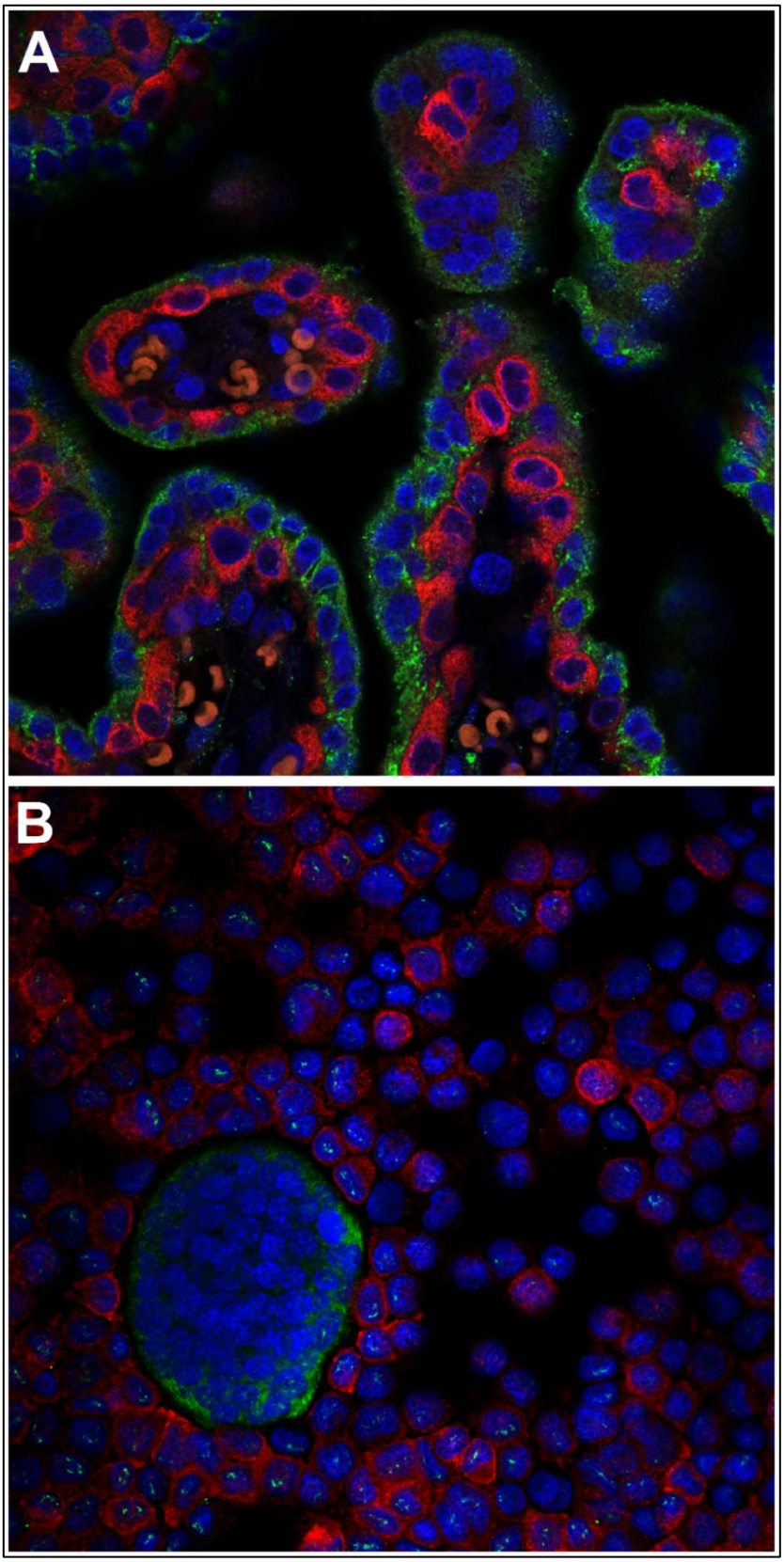

FIGURE 7.1. Immunofluorescent double-labelling of Paternally-expressed gene 10 (PEG-10; cytotrophoblasts RED) and Pregnancy specific beta-1-glycoprotein 1 (PSG-1; syncytiotrophoblasts GREEN). Representative images are from an 8 weeks' gestation tissue section (**A**) and trophoblast cell isolated from 15 weeks' gestation tissue (**B**).

7.3.2 Comparison of 5-mC and 5-hmC expression in placenta tissue across gestation

DNA methylation in the placenta increases across gestation until term [10, 11]. IHC staining of 5-mC and 5-hmC revealed localisation within the nuclei the CTBs, STBs and stromal cells (Fig. 7.3). Staining intensity, quantified using Video Image Analysis (VIA), of 5-mC increased in placenta tissue across gestation (Fig. 7.3E, P = 0.031). Conversely, 5-hmC intensity did not differ between tissues from first trimester compared to term (Fig. 7.3F).

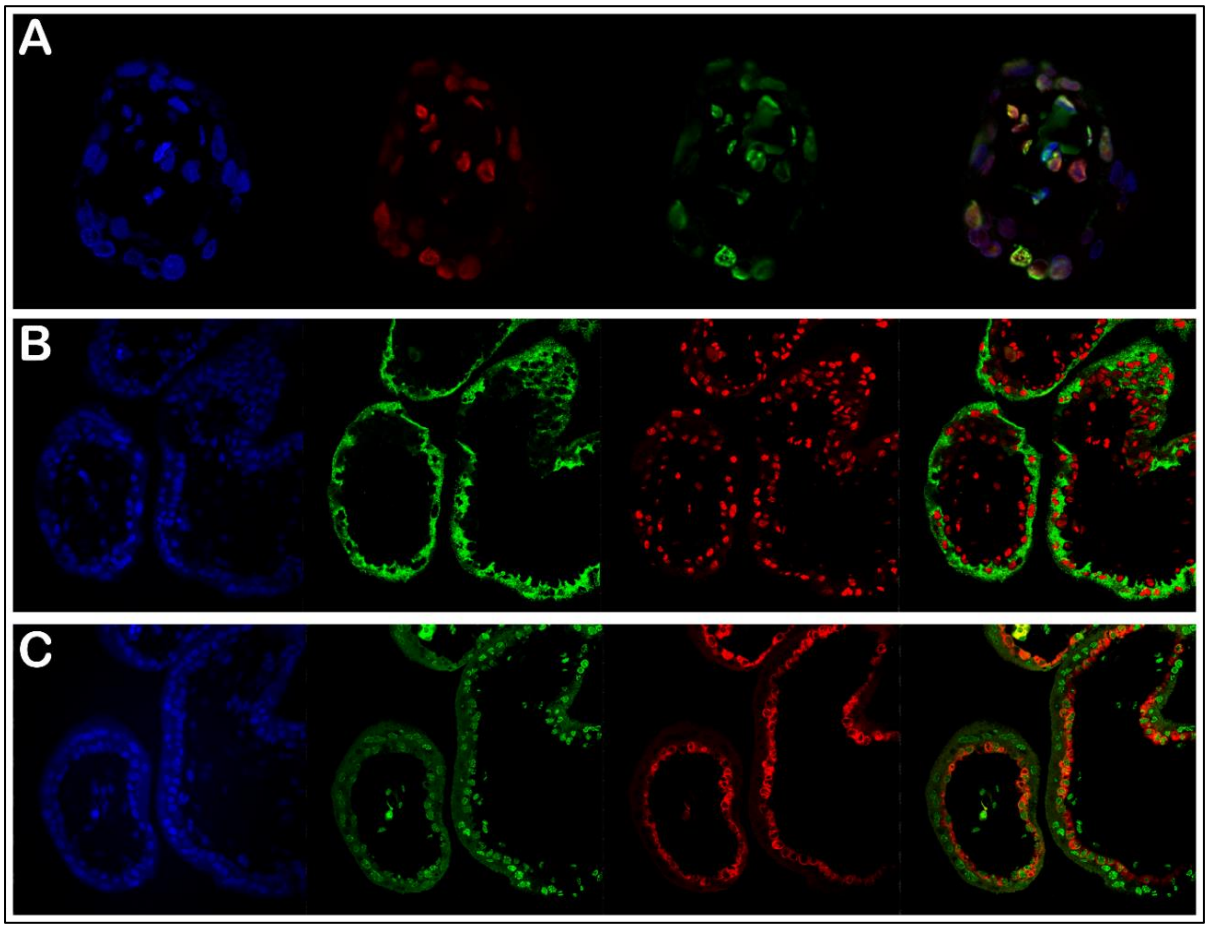

FIGURE 7.2. Immunofluorescent double-labelling in first trimester (7 weeks' gestation) tissue sections. Panel **A:** Placental villous tissue stained with 4',6-Diamidine-2'-phenylindole (DAPI; Blue, Nuclei), 5-methylcytosine (5-mC; Green), 5-hydroxymethylcytosine (5-hmC; Red) and all three images merged. Panel **B**: Placenta tissue section stained with Dapi (Blue, Nuclei), Pregnancy specific beta-1-glycoprotein 1 (PSG-1; Green, syncytiotrophoblasts), 5-hmC (Red) and PSG-1 and 5-hmC images merged. Panel **C:** First trimester placenta tissue section stained with DAPI (Blue, Nuclei), 5-mC (Green), Paternally-expressed gene 10 (PEG-10; Red, cytotrophoblasts) and 5-mC and PEG-10 images merged.

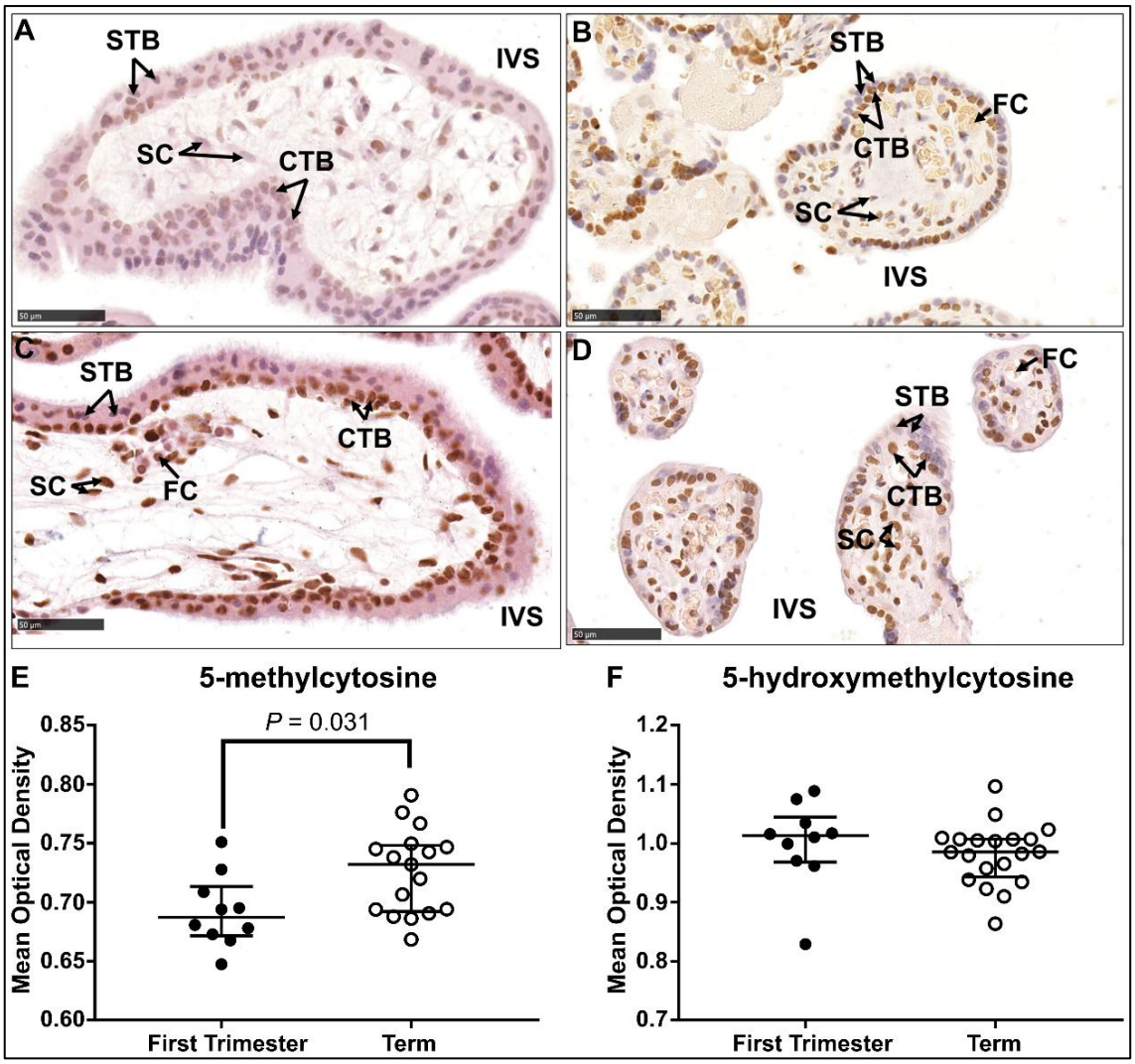

FIGURE 7.3. Immunohistochemical labelling of 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) in first trimester and term tissue sections. Representative images of 5-mC labelling in a first trimester section (**A**) and term tissue section (**B**). Representative images of 5-hmC labelling in a first trimester section (**C**) and term tissue section (**D**). Video image analysis (VIA) quantification of staining intensity revealed an increase in levels of 5-mC in tissue sections from term placenta compared to first/second trimester (**E**). There was no difference in the staining intensity of 5-hmC between first/second trimester and term tissue sections (**F**). Data are median and interquartile range. Significance was determined using a Mann-Whitney test. CTB: cytotrophoblast, FC: fetal capillary, IVS: intervillous space, SC: stromal cell, STB: syncytiotrophoblast.

7.3.3 5-mC and 5-hmC expression in isolated CTB and STB cells

CTB and STB cells were isolated from first and second trimester tissue, as well as term tissue, and stained for 5-mC and 5-hmC to determine whether staining intensity was similar in the different trophoblast subpopulations (Fig. 7.4). In the CTBs, mean fluorescence intensity (MFI) of 5-mC was significantly higher in cells isolated from term tissue when compared to first and second trimester CTBs (Fig. 7.4E, $P = 0.0021$ and $P = 0.0006$, respectively). This was similar for 5-hmC staining intensity (Fig. 7.4F, $P = 0.0005$ and $P = 0.0027$, respectively). STBs were not readily obtained from term isolations and therefore could not be compared with STB cells collected in early gestation. However, in first and second trimester isolated cells, MFI of 5-hmC was significantly higher in STBs compared to CTBs (Fig. 7.4H, $P = 0.0011$).

7.3.4 Assessment of 5-mC and 5-hmC in placenta tissue from pregnancies complicated by PE

DNA methylation profiles are different in placenta tissue from pregnancies complicated by PE compared to uncomplicated pregnancies [18-21]. Thus, we sought to determine whether there was a difference in 5-mC and 5-hmC staining between term placenta tissue collected from uncomplicated and PE pregnancies (Fig. 7.5). Staining intensity of 5-mC was significantly higher (Fig. 7.5A, $P = 0.028$) in placenta tissue from PE pregnancies compared to uncomplicated but 5-hmC staining intensity did not differ (Fig. 7.5B).

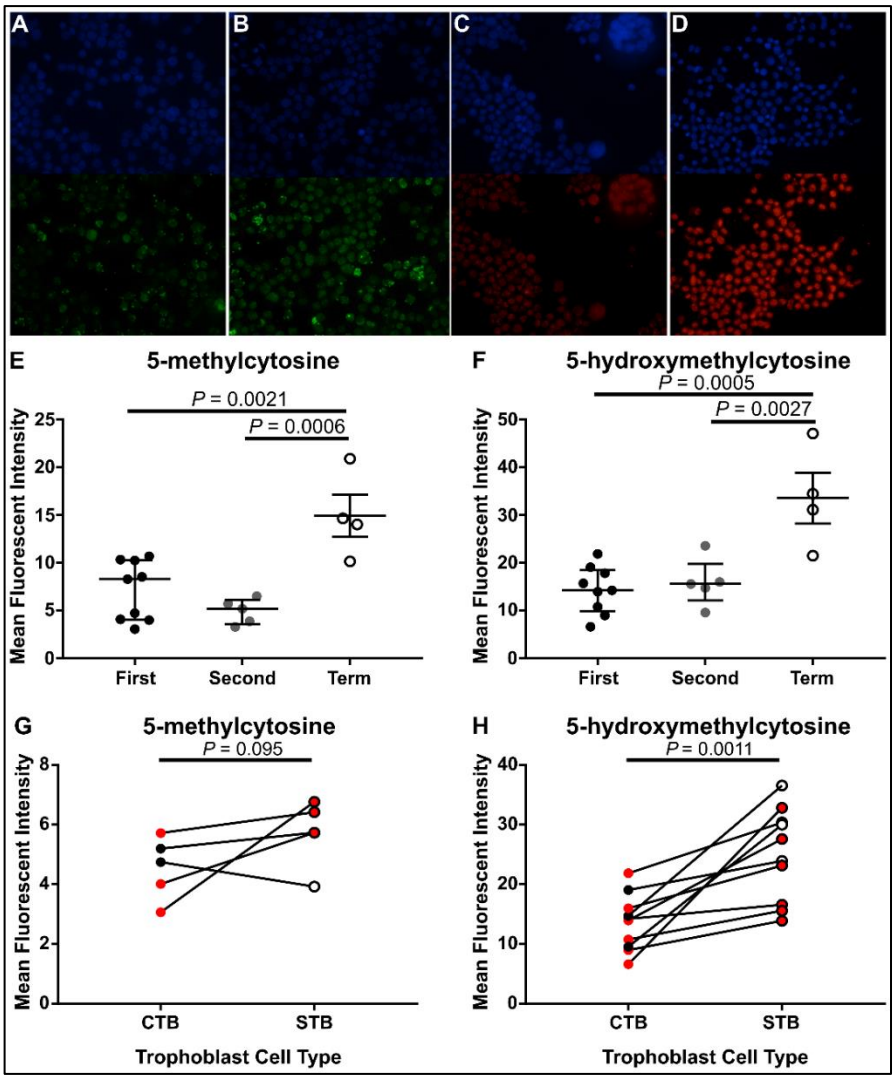

FIGURE 7.4. Immunofluorescent labelling of 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) in cytotrophoblast (CTB) and syncytiotrophoblast (STB) cells isolated from first (6-12 weeks' gestation) and second trimester (13-22 weeks' gestation) and term placenta tissue. Representative images of 4',6-Diamidine-2'-phenylindole (DAPI; Blue, Nuclei) and 5-mC (Green) staining in cells from second trimester (15 weeks' gestation) (A) and term (B). Representative images of DAPI (Blue) and 5-hmC (Red) staining in cells from first trimester (7 weeks' gestation) (C) and term (D). Quantification of staining intensity using ImageJ image analysis software revealed an increase in levels of both 5-mC (E) and 5-hmC (F) in CTB cells isolated from term tissue compared to both first and second trimester. There was no difference in staining intensity of neither 5-mC nor 5-hmC between first and second trimester CTBs. A moderate increase in staining intensity of 5-mC in isolated STB cells compared to CTB cells collected from first/second trimester placenta (G) and a significant increase in levels of 5-hmC in STBs compared to CTBs (H) was found. Red dots indicate cells collected from first trimester placenta tissue (<13 weeks' gestation). Data are median and interquartile range. Significance was determined using an ANOVA with Tukey post-hoc comparison (E & F) or Mann-Whitney test (G & H).

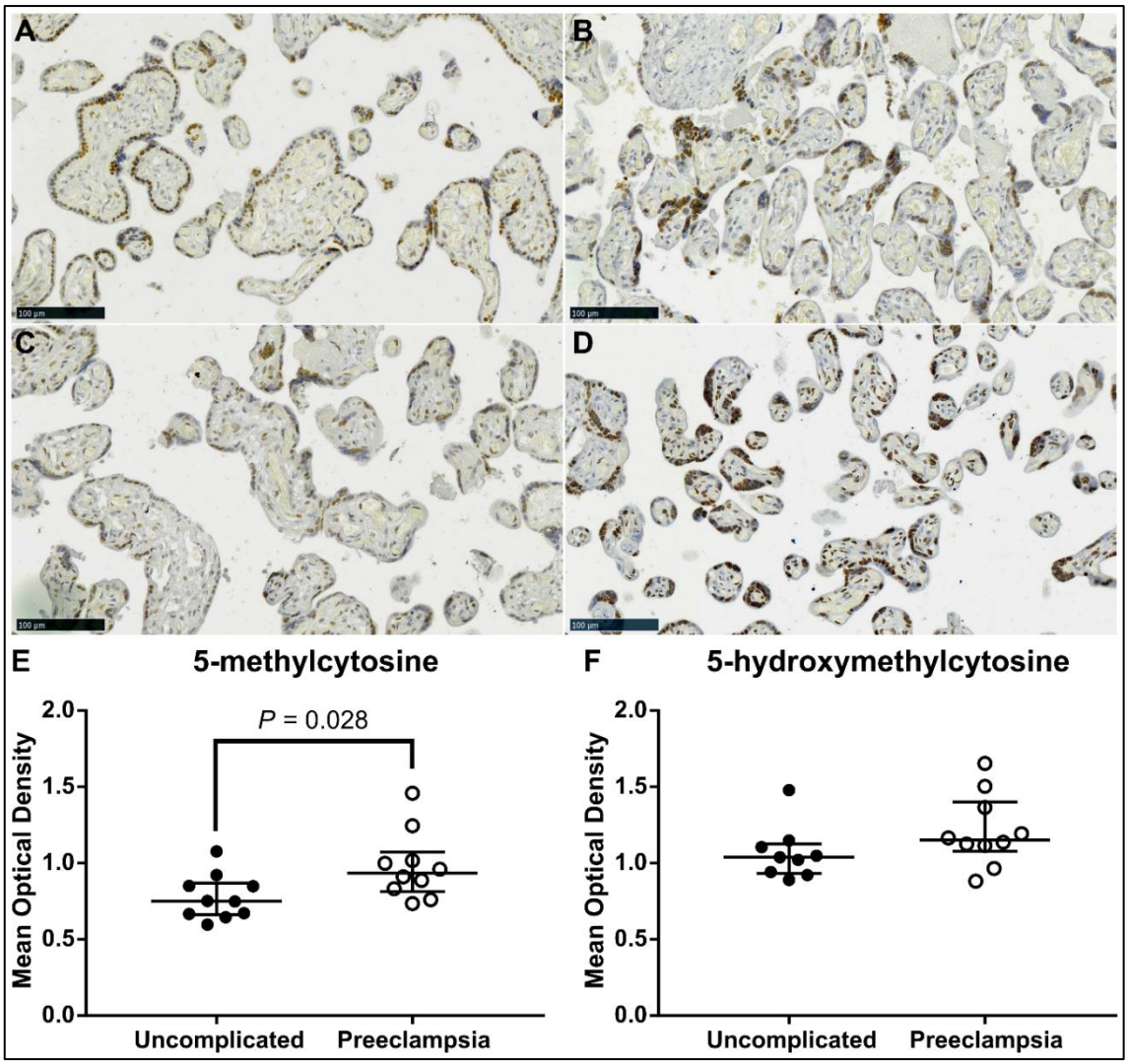

FIGURE 7.85. Video image analysis (VIA) of immunohistochemical staining of 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) in term placenta sections from pregnancies complicated by preeclampsia (PE) and uncomplicated. Representative images of 5-mC staining in placenta samples from uncomplicated (**A**) and PE (**B**) pregnancies and 5-hmC staining in placenta in an uncomplicated (**C**) and PE (**D**) pregnancy. Intensity of 5-mC staining was greater in tissue sections from PE pregnancies compared to uncomplicated (**E**). However, staining intensity of 5-hmC did not differ (**F**). Data are median and interquartile range. Significance was determined using a Mann-Whitney test.

7.4 Discussion

Studying DNA methylation in the placenta is complicated by the presence of numerous different cell types, each with their own unique and specific function and hence molecular and epigenetic profile. We sought to overcome this by using visualisation techniques to localise and quantify DNA methylation markers 5-

mC and 5-hmC in placenta tissue and trophoblast cells across gestation. Our assessment revealed dynamic changes in levels of both markers across gestation, as well as between different trophoblast cell types at the same gestation. This preliminary data provides a platform for further work to elucidate how changes to epigenetic modifications, including how 5-mC and 5-hmC contribute to placental pathology and pregnancy complications.

To our knowledge, there has only been one other study that has used IHC to assess 5-mC and 5-hmC localisation in human placenta tissue [15]. Fogarty et al. observed differences in the levels of 5-mC and 5-hmC in CTBs compared to STBs in tissue sections across gestation. 5-mC was more abundant in CTBs whilst STBs showed greater staining for 5-hmC. Indeed, in the current study, STBs isolated from first and second trimester tissue had a higher staining intensity of 5-hmC compared to CTBs isolated from the same samples. However, 5-mC was also abundantly expressed within STB cells with a moderate increase in levels compared to CTBs. Differences between the two studies may be explained by different 5-mC antibodies, staining protocols and in this study, levels were measured in isolated cells not tissue sections. Furthermore, we used a more sensitive method of detection and quantification in IF. What both studies do highlight, however, is that the methylomes of the different trophoblast cell types are likely to vary substantially. Thus, further research into the biological importance of these differences is warranted.

Of relevance to placental dysfunction is the observation that staining intensity of 5-mC was higher in placenta tissue sections collected from PE pregnancies compared to uncomplicated pregnancies. DNA methylation patterns, determined using sequencing arrays, have been shown to be different in placentas from PE pregnancies [18-20], as well as in placentas from other pregnancy complications [26-28] compared to uncomplicated pregnancies. However, evidence on whether or not these changes in DNA methylation result in differences in gene expression is conflicting [20, 21]. This may be explained, in

part, by the fact that DNA was extracted from whole tissue homogenates. The etiology of PE that is characterised by placental dysfunction suggests STB stress to be a major factor [6]. Our laboratory is currently optimising protocols to isolate STB cells from tissue in order to study this particular cell type more specifically. Future experiments including analysis of both DNA methylation profiles and gene expression patterns within the STB cell type and to assess changes to these molecular profiles under adverse conditions, for example, increased oxidative stress. Such research is integral to understand what governs molecular and cellular changes in placenta cells providing a solid foundation to elucidate what occurs in pathological pregnancies.

One of the most interesting findings in this study is the increased staining intensity of 5-hmC in CTB cells across gestation. Oxidation of 5-mC to form 5-hmC is mediated by the Ten-eleven translocation (Tet) enzymes and knockdown models of these enzymes have shown 5-hmC to be central to maintain developmental pathways and early embryogenesis [22, 23]. Furthermore, there is a strong association between active transcription and genomic regions with elevated 5-hmC levels [13], 5-hmC is known to localise to areas of DNA damage and promote genome stability [24] and progressive loss of 5-hmC is a hallmark of tumorigenesis [25]. Thus, there is accumulating evidence suggesting that perturbations to this DNA methylation modification may contribute significantly to disease phenotypes and genomic instability. From this study, the relevance of changes in 5-hmC levels across gestation in CTB and STB cells to trophoblast differentiation or function cannot be determined. Furthermore, there was no difference in staining intensity of 5-hmC in tissue sections from placentas from PE pregnancies compared to uncomplicated pregnancies. One disadvantage to using VIA to quantify staining intensity, is the inability to easily differentiate staining in different cell types. Indeed, visually, staining intensity of 5-hmC appeared greater in the syncytial knots of the placental tissue sections from PE compared to uncomplicated pregnancies. Further analysis is required to

determine whether there is a difference in 5-hmC staining intensity in STB cells of PE placentas but the results presented here provide the first steps to better understanding how 5-hmC contributes to placental development and placental dysfunction.

One of the major advantages in using IHC and IF was the ability to visually assess which cell types expressed 5-mC and 5-hmC in placenta across gestation. In this study, we observed clear changes to the levels of both markers which were confirmed using quantification analyses. However, quantifying IHC and IF staining is inherently difficult due to a number of assumptions [29]. For example, quantification assumes a linear relationship between antigen-antibody binding; more antigen leads to more antibody binding and results in a greater visual signal either in the presence of darker staining or greater fluorescent intensity. Such assumptions cannot easily be validated and thus experiments using more sensitive techniques such as methylation sequencing should be undertaken to confirm our results here. Never-the-less, the current study provides solid evidence that epigenetic profiles can differ significantly between different cell types within a complex tissue and reinforces the need to focus sequencing experiments on individual cell populations rather than whole tissue extracts.

This study presents visual evidence of the dynamic localisation patterns of 5-mC and 5-hmC in placenta trophoblast cells across gestation. Importantly, it shows stark differences in the levels of these DNA methylation markers in different trophoblast subtypes, reinforcing the need to focus future sequencing experiments on individual cell populations. Furthermore, this is one of only a few studies to analyse 5-hmC in placenta, providing a solid rationale for further study on how this modification is important to placental development and function. Finally, key to fully understanding the importance of epigenetics in placental pathologies is to first determine how modifications such as DNA methylation influence normal placental development. Studies like the one

presented here pave the way to increasing knowledge of human placental development at a molecular level and will ultimately lead to novel therapeutic targets to prevent and treat placental dysfunction and improve pregnancy outcome.

Acknowledgments

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Chapter 8

General Discussion

The primary aims of this project were to further the understanding of how maternal micronutrient status during pregnancy influences placental development and function, as well as pregnancy outcome. Focusing on a number of key micronutrients: zinc, copper, vitamin D and calcium, the work presented in this thesis has provided novel insight into both the mechanisms behind how these micronutrient deficiencies influence pregnancy physiology, as well as how they are clinically relevant to human pregnancy. It is the first to report on how zinc deficiency in pregnancy affects maternal hemodynamic adaptations to pregnancy; one of the first to comprehensively assess both vitamin D and calcium status in pregnancy; and the first to use immunofluorescence to characterise and quantify changes in DNA methylation in placenta trophoblast cells across gestation.

8.1 Overall Significance

8.1.1 Zinc deficiency in pregnancy effects fetal growth and pregnancy physiology

Chapter 1 and 2 present my research on the effects of zinc deficiency during pregnancy on pregnancy outcome and highlights the importance of adequate maternal zinc status on fetal growth and placental morphogenesis [1, 2]. In pregnant women, the systematic review of the literature indicated that being zinc deficient during pregnancy may be associated with increasing the risk for

reduced fetal birthweight and hypertensive disorders of pregnancy. Indeed, this was confirmed in my mouse model in which, moderate dietary zinc deficiency resulted in fetal growth restriction, disrupted placental morphogenesis and substantial alterations to the maternal blood pressure profile over pregnancy. Thus, this study provided a mechanistic interpretation of what is observed in human cohort studies. Furthermore, this study was one of the first to utilise the highly sensitive radio-telemetry technique in un-restrained mice during pregnancy to continuously measure blood pressure. Both studies illustrate the importance for continual education of women on appropriate nutrition and lifestyle choices when contemplating pregnancy. They also reinforce the need to develop strategies that alleviate the burden of zinc deficiency in pregnant women and women of reproductive age that is particularly prevalent in the developing world.

8.1.2 Early pregnancy copper status is associated with increasing the risk of any pregnancy complication

Our analysis of plasma trace elements in early pregnancy in the Adelaide SCOPE cohort [3] revealed a clear association between plasma copper status and adverse pregnancy outcome. A protective effect of lower plasma copper at the 15±1 weeks' gestation was observed and is in contrast with the current dogma that lower levels of trace elements are detrimental to human health [4]. This is an important finding as many pregnancy supplements contain these minerals on the basis of very minimal evidence to suggest the efficacy of their inclusion [5]. Furthermore, whilst this study cannot determine cause-effect scenarios, the data presented highlights possible mechanistic pathways by which higher circulating copper concentrations may impact placental development that has been previously unappreciated. These hypotheses need to be confirmed using animal

and *in vitro* models but provide focus for moving the pregnancy nutrition field forward.

8.1.3 Evidence that both maternal vitamin D and calcium status are important for fetal growth and placental function

I used the comprehensive, well-characterised SCOPE database to assess the effects of vitamin D status on pregnancy outcome in 2800 women across two distinct pregnancy populations; Adelaide and Auckland. This was the largest study to assess vitamin D status in pregnancy from the international SCOPE consortium to date. Furthermore, it offered a unique perspective on the effects of social demographics on vitamin D status by comparing the two different populations independent of different sampling and analysis techniques. A recent meta-analysis of 20 observational studies which included 16,515 individuals [6] supported the findings that the risk of GDM was lower with higher vitamin D levels. However, disparate published findings, of which my study contributes to, on the association between vitamin D deficiency and other pregnancy complications still remain [7,8]. This may be explained by differences in maternal calcium status within different populations that are studied. Indeed, my follow-up study focused on calcium and vitamin D status in the Adelaide SCOPE women confirmed a clear relationship between markers of calcium status, placental function and fetal growth. To the best of our knowledge, this is one of the first detailed investigations to comprehensively assess both vitamin D and calcium status in pregnant women and their associations with adverse pregnancy outcomes. It also highlights the requirement for future endeavours to focus on multiple micronutrients and their interactions. This, despite increasing the complexity of the studies, will ultimately allow us to provide stronger nutritional recommendations to pregnant women.

8.1.4 Dynamic DNA methylation profiles are exhibited in the human placenta across gestation

The final research in this thesis presents the first steps in understanding epigenetic regulation of placental cell development at the level of the individual cell type. This was in order to focus future studies on elucidating how perturbations in micronutrient homeostasis impacts placental function at an epigenetic level. Focusing DNA methylation, this study presented visual evidence on the dynamic localisation patterns of 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) across gestation. Most importantly, it characterised potential difference in patterns of DNA methylation in the specific cytotrophoblast and syncytiotrophoblast subpopulations which is supported by recent single cell RNA-sequencing experiments [9, 10]. Thus, reinforces the requirement for future sequencing experiments to focus on single cell populations rather than whole tissue extracts. Finally, the study provides evidence to further explore potential epigenetic functions of 5-hmC in trophoblast differentiation. Whilst such a hypothesis would need to be confirmed using more sensitive methodologies, these results provide a new foundation for investigations into the functional significance of 5-hmC in regulating placental development.

8.2 Problems Encountered and Limitations

8.2.1 Animal models in studying the placenta

Comprehensive analysis of the effects of zinc deficiency in pregnancy showed the influence of zinc on pregnancy physiology with a number of limitations. The radio-telemetry data suggested reduced placental perfusion may be a contributing factor to fetal growth restriction in this model. However, neither placental perfusion nor direct measures of placental function such as glucose uptake were measured. Whilst double-labelled immunohistochemical analysis

was used to assess structural correlates of placental function, we can only assume that blood flow into the placental bed was perturbed. Although, this is highly likely given that fetal growth was restricted and does provide an avenue for future research.

A second limitation of this study was with the use of radio-telemetry to continuously measure blood pressure. Whilst providing a huge amount of accurate, highly specific data on maternal blood pressure in an un-restrained animal, there were problems that occurred which reduced the number of animals in which useable data was obtained. Most notably, the length of time in which sampling occurred (45 days) meant a number of probes malfunctioned towards the end of the experiment. Furthermore, given the surgery occurred before the animals were mated, those that did not become pregnant despite multiple mating attempts could not be used in the study. However, a testament to the potential of this technology is that we were able to observe clear differences in the blood pressure profiles between the diet groups with a sample size of 8 highlighting the sensitivity and specificity of this technique.

8.2.2 Circulating nutrients in human cohort studies

Unfortunately, detailed information on dietary intakes of nutrients such as food frequency questionnaires were not included as part of the original SCOPE study. Whilst information such as frequency of fruit, green leafy vegetables and oily fish consumption was collected, and provided some basic understanding of dietary patterns within the women studied, lack of high quality dietary information presented a real limitation for my human cohort studies. Diet is the main source of zinc, copper, selenium and calcium; vitamin D can also be obtained from the diet, as well as through UV exposure. A more comprehensive analysis on the associations between maternal micronutrient status and adverse pregnancy outcome may have been undertaken if more detailed dietary information was

available. Consideration needs to be given when interpreting micronutrient status is circulation as trace elements, calcium and vitamin D levels are heavily influenced by inter- and intra-patient factors. However, given the lack of more suitable alternatives that assess maternal micronutrient status, and food frequency questionnaires have their own limitations [11], measuring micronutrients in circulation can still be informative in advancing our understanding of how different micronutrients contribute to pregnancy success.

8.2.3 Immunofluorescence of DNA methylation markers

One of the greatest benefits of using immunofluorescence and immuno-histochemistry is the ability to visualise the location of specific proteins and other biomarkers within a complex tissue structure. However, to create visually stunning images whilst maintaining accuracy and specificity relies heavily on a number of factors including the primary antibody and staining protocol; both of which proved to be major limitations in studying 5-mC using immuno-fluorescence. DNA denaturation using hydrochloric acid was required in order to allow antigen-antibody binding of the 5-mC antibody. Specific antigen retrieval was also necessary and results varied depending on whether heat-mediated retrieval with citrate buffer or pronase digestion was used. Such steps within the protocol were particularly challenging for double-labelling with cell specific markers as these antigens were easily destroyed by such aggressive treatments. Never-the-less a functioning protocol was achieved in which studying DNA methylation using immunofluorescence in different cell types of the placenta was achieved and will be continually used in future experiments.

8.3 Future Directions

The importance of adequate nutrition in pregnancy has long been understood and unquestionable progress has been made in educating women on the importance of different micronutrients, such as folate and iodine, are for fetal

development and pregnancy success. However, despite decades of research, we have barely scratched the surface of all that we could possibly know. The research presented here continues to offer more detailed insights into how certain micronutrients support placental development and provides new avenues for future exploration.

The importance of considering micronutrient interactions was consistently explored through this thesis, however, a deeper understanding is still required. For example, my mouse model provided conclusive evidence that maternal zinc deficiency results in fetal growth restriction as a consequence of inappropriate placental morphogenesis. Yet zinc was conserved in the placenta and fetus at what appeared to be the expense of iron. This observation offers numerous questions about the importance of zinc-iron interactions. For example: what placental transport systems are affected by maternal zinc deficiency that causes a reduction in iron transport? And, is this transport system disrupted in the placentas from different species including humans? In our human cohort study, we did not observe an association between zinc deficiency at 15±1 weeks' gestation and an increased risk of hypertensive disorders of pregnancy or SGA. However, we could not study in detail the influence of iron in this cohort due to the unavailability of more precise determinants of iron status. Future work focused on the interaction between zinc and iron, particularly within the placenta, will continue to untangle the mechanisms by which trace elements support fetal growth *in utero*. Moreover, this information may be useful in the development of strategies to combat micronutrient deficiencies in the developing world.

Paradoxically, the placenta is one of the most important organs to human health yet one of the least understood at the cellular and molecular level. As indicated in the limitations section of this chapter, a significant proportion of time was utilised in optimising a functioning protocol to study DNA methylation using immunofluorescence. Now such a protocol is available, it is possible to

implement it in future study designs. Experiments assimilating knowledge gained on zinc, iron, copper and calcium in regulating placental development with epigenetic regulation of trophoblast cell function may provide information valuable to placenta researchers.

Finally, current methodologies for assessing and monitoring placental function are limited. The key to improving reproductive success is accurate, early diagnosis of disease including placental dysfunction. Thus, there is an inherent need to develop non-invasive methods that accurately determine the health of the placenta and pregnancy in real-time so that intervention strategies may be implemented in a time appropriate manner. Indeed, we have identified a number of potential micronutrients measured in circulation that may be useful in including in screening tools for predicting pregnancy outcome. In order for this idea to be pursued, assessment of micronutrient status should be undertaken in a much larger cohort of women and include high quality dietary data. Furthermore, it would be useful to include a more socioeconomically and ethnically diverse population. However, if proven useful, the inclusion of routine micronutrient screening in a clinical setting may be immensely valuable to decision-making and implementation of appropriate interventions for pregnant women who may be at risk of developing a pregnancy complication.

8.4 Conclusions

The work presented in this thesis has explored several key micronutrients, their interactions and their effects on pregnancy outcome. It offers a unique perspective into the role of maternal micronutrient status in pregnancy and demonstrates the complexities and multi-faceted nature of micronutrient interactions in pregnancy physiology. The work provides a basis for the advancement of research into how maternal nutrition might influence the epigenome of the placenta and the consequences this has to fetal growth *in utero.* Furthermore, the human cohort studies presented may be beneficial in the

development of early pregnancy screening tools that will allow a more accurate assessment of a woman's risk of a pregnancy complication and thus allow early intervention strategies to be employed if required.

Finally, to quote Jennifer Brea (TedSummit, June 2016) – *"I don't know" is a beautiful thing. "I don't know" is where discovery starts. And if we can [be willing to say "I don't know"] and approach the great vastness of all that we do not know, rather than fear uncertainty, maybe we can greet it with a sense of wonder."* What this thesis does not show directly but embodies in its entirety is the willingness to not only say "I don't know" but to also say "I want to know".

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Appendix A

Publication Formats

nutrients

MDPI

Review

Association between Maternal Zinc Status, Dietary Zinc Intake and Pregnancy Complications: A Systematic Review

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Abstract: Adequate zinc stores in the body are extremely important during periods of accelerated growth. However, zinc deficiency is common in developing countries and low maternal circulating zinc concentrations have previously been associated with pregnancy complications. We reviewed current literature assessing circulating zinc and dietary zinc intake during pregnancy and the associations with preeclampsia (PE); spontaneous preterm birth (sPTB); low birthweight (LBW); and gestational diabetes (GDM). Searches of MEDLINE; CINAHL and Scopus databases identified 639 articles and 64 studies were reviewed. In 10 out of 16 studies a difference was reported with respect to circulating zinc between women who gave birth to a LBW infant (≤2500 g) and those who gave birth to an infant of adequate weight (>2500 g), particularly in populations where inadequate zinc intake is prevalent. In 16 of our 33 studies an association was found between hypertensive disorders of pregnancy and circulating zinc; particularly in women with severe PE (blood pressure ≥160/110 mmHg). No association between maternal zinc status and sPTB or GDM was seen; however; direct comparisons between the studies was difficult. Furthermore; only a small number of studies were based on women from populations where there is a high risk of zinc deficiency. Therefore; the link between maternal zinc status and pregnancy success in these populations cannot be established. Future studies should focus on those vulnerable to zinc deficiency and include dietary zinc intake as a measure of zinc status.

Keywords: zinc; pregnancy; pregnancy complications; dietary zinc intake; circulating zinc

1. Introduction

Adequate maternal nutrition, particularly before and during pregnancy, is imperative to the health of both the mother and child [1,2]. Poor nutrition in pregnancy may lead to inappropriate nutrient partitioning between the mother and fetus, which can be deleterious to the health of both [3]. Each year, 3.5 million deaths in women and children are attributed to undernutrition [4]. Zinc deficiency is predicted to be responsible for 1% of all deaths globally and 4.4% of deaths in children aged 6 months to 5 years [5]. The World Health Organization (WHO) prioritized minimizing zinc deficiency in developing nations as part of the Millennium Development Goal 1: to eradicate extreme poverty and hunger [6]. Therefore, understanding the effects of zinc deficiency on pregnancy and fetal growth is very important.

Zinc is an essential component of over 1000 proteins including antioxidant enzymes, metalloenzymes, zinc-binding factors and zinc transporters. These are required for a variety of biological

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Zinc is a critical regulator of placental morphogenesis and maternal hemodynamics during pregnancy in mice

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Zinc is an essential micronutrient in pregnancy and zinc deficiency impairs fetal growth. We used a mouse model of moderate zinc deficiency to investigate the physiological mechanisms by which zinc is important to placental morphogenesis and the maternal blood pressure changes during pregnancy. A 26% reduction in circulating zinc ($P = 0.005$) was exhibited in mice fed a moderately zinc-deficient diet. Zinc deficiency in pregnancy resulted in an 8% reduction in both near term fetal and placental weights (both $P < 0.0001$) indicative of disrupted placental development and function. Detailed morphological analysis confirmed changes to the placental labyrinth microstructure. Continuous monitoring of maternal mean arterial pressure (MAP) revealed a late gestation decrease in the zinc-deficient dams. Differential expression of a number of regulatory genes within maternal kidneys supported observations on MAP changes in gestation. Increased MAP late in gestation is required to maintain perfusion of multiple placentas within rodent pregnancies. Decreased MAP within the zinc-deficient dams implies reduced blood flow and nutrient delivery to the placenta. These findings show that adequate zinc status is required for correct placental morphogenesis and appropriate maternal blood pressure adaptations to pregnancy. We conclude that insufficient maternal zinc intake from before and during pregnancy is likely to impact *in utero* programming of offspring growth and development largely through effects to the placenta and maternal cardiovascular system.

During pregnancy, the placental vasculature provides the interface between the fetus and the mother for exchange of nutrients and wastes. Adequate placental function underpins normal fetal development[1]. Defects in placental development and function are implicated in a number of clinical pregnancy complications. These include preeclampsia (PE)[2], a common hypertensive disorder of pregnancy[3] and fetal growth restriction (FGR)[4–7], defined as birth weight, adjusted for gestational age, of $\leq 5^{th}$ percentile[3]. Together these conditions pose a lifelong risk of morbidity and mortality for both the mother and infant. Increased placental oxidative stress is hypothesised to be

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Clinical studies

Early pregnancy maternal trace mineral status and the association with adverse pregnancy outcome in a cohort of Australian women

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ARTICLE INFO

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ABSTRACT

Maternal micronutrient deficiencies in pregnancy can have profound effects on fetal development and pregnancy outcome. Plasma trace minerals including copper, zinc, selenium and iron have been shown to be extremely important in supporting reproduction. We sought to determine whether there is an association between maternal trace mineral status in early pregnancy and pregnancy complications using a prospective cohort study of 1065 pregnant Australian women who were recruited as part of the Screening for Pregnancy Endpoints (SCOPE) study in Adelaide. Copper, zinc, selenium and iron present in the plasma were measured using mass spectrometry in samples collected at 15 ± 1 weeks' gestation. After adjusting for covariates, women with lower plasma copper (< 27.9 μmol/L and 27.9–32.5 μmol/L) had decreased risk for any pregnancy complication when compared with women with high plasma copper (> 32.5 μmol/L) (aRR = 0.87; 95% CI = 0.76, 0.99 and aRR = 0.88; 95% CI = 0.78, 1.00, respectively). This was also observed when adjusting for plasma zinc and selenium status (< 27.9 μmol/L: aRR = 0.81; 95% CI = 0.69, 0.96 and 27.9–32.5 μmol/L: aRR = 0.84; 95% CI = 0.72, 0.98). Combined low copper and zinc status was also associated with a reduced risk of any pregnancy complication as compared with high copper and zinc status (aRR = 0.80; 95% CI = 0.70, 0.93). These results provide justification for further work into elucidating the mechanistic role of trace elements in early pregnancy, as well as their interactions in supporting successful pregnancy outcomes.

Maternal nutrient stores and diet supply all the macro- and micronutrients to support optimal fetal growth essential for successful pregnancy [1]. Hence, it is not surprising that maternal deficiencies in key micronutrients can have profound effects on fetal development and pregnancy outcome [2]. Pregnancy complications including pre-eclampsia (PE), gestational diabetes mellitus (GDM), spontaneous preterm birth (sPTB) and fetal growth restriction (FGR) together affect 25% of first pregnancies and predict lifelong morbidity and mortality for both the mother and infant [3]. Furthermore, micronutrient deficiencies which tend to be associated with decreased consumption of foods rich in micronutrients, have also been associated with the development of PE, GDM, sPTB, FGR, as well as gestational hypertension (GH) [2].

Extensive investigations into micronutrient deficiencies have focused on those common within pregnant populations including folate and vitamin D [4]. However, evidence is emerging about the importance of trace minerals like iron, zinc and copper in supporting successful pregnancy [5]. It is known that trace minerals are crucial for the maintenance of cell proliferation and function with severe deficiencies in copper and zinc during pregnancy having been shown to have a teratogenic effect on the fetus [6]. This is likely driven by a reduction in the activity of key enzymes which require these metals structurally in order to function, as well as compromised oxidant defence systems [6]. It is also important to acknowledge the importance of micronutrients in mediating inflammation and the immune response. Animal models of iron, copper and zinc deficiencies have been shown to be associated with compromised immunity and increased susceptibility to infection [7]. Pregnancy complications including PE and FGR have been associated with increased oxidative stress and circulating markers of inflammation [8,9] and therefore there may be a causal connection

Abbreviations: FGR, fetal growth restriction; GDM, gestational diabetes mellitus; GH, gestational hypertension; PE, preeclampsia; sPTB, spontaneous preterm birth; SGA, small-for-gestational age

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Appendix B

Publications arising during PhD not included as part of the thesis

PLOS | ONE

RESEARCH ARTICLE

Vitamin D Receptor Gene Ablation in the Conceptus Has Limited Effects on Placental Morphology, Function and Pregnancy Outcome

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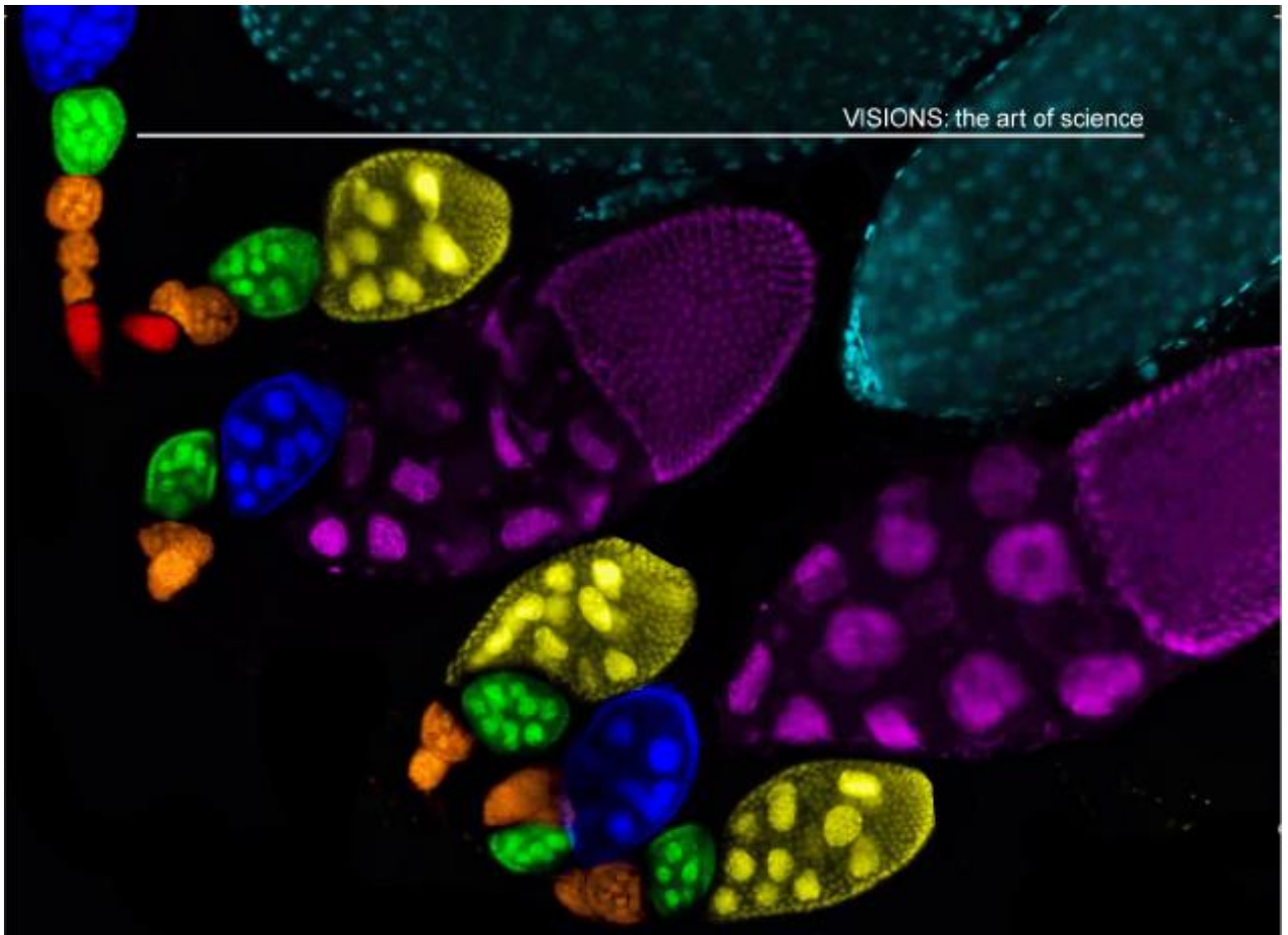
Abstract

Vitamin D deficiency has been implicated in the pathogenesis of several pregnancy complications attributed to impaired or abnormal placental function, but there are few clues indicating the mechanistic role of vitamin D in their pathogenesis. To further understand the role of vitamin D receptor (VDR)-mediated activity in placental function, we used heterozygous *Vdr* ablated C57Bl6 mice to assess fetal growth, morphological parameters and global gene expression in *Vdr* null placentae. Twelve $Vdr^{+/-}$ dams were mated at 10–12 weeks of age with $Vdr^{+/-}$ males. At day 18.5 of the 19.5 day gestation in our colony, females were euthanised and placental and fetal samples were collected, weighed and subsequently genotyped as either $Vdr^{+/+}$, $Vdr^{+/-}$ or $Vdr^{-/-}$. Morphological assessment of placentae using immunohistochemistry was performed and RNA was extracted and subject to microarray analysis. This revealed 25 genes that were significantly differentially expressed between $Vdr^{+/+}$ and $Vdr^{-/-}$ placentae. The greatest difference was a 6.47-fold change in expression of *Cyp24a1* which was significantly lower in the $Vdr^{-/-}$ placentae ($P<0.01$). Other differentially expressed genes in $Vdr^{-/-}$ placentae included those involved in RNA modification (*Snord123*), autophagy (*Atg4b*), cytoskeletal modification (*Shroom4*), cell signalling (*Plscr1*, *Pex5*) and mammalian target of rapamycin (mTOR) signalling (*Deptor* and *Prr5*). Interrogation of the upstream sequence of differentially expressed genes identified that many contain putative vitamin D receptor elements (VDREs). Despite the gene expression differences, this did not contribute to any differences in overall placental morphology, nor was function affected as there was no difference in fetal growth as determined by fetal weight near term. Given our dams still expressed a functional VDR gene, our results suggest that cross-talk between the maternal decidua and the placenta, as well as maternal



Ovarian follicle development is essential for the propagation of species. In *Drosophila melanogaster*, follicle development occurs through a spectacularly coordinated sequence within the highly polarized ovariole (pictured). A single ovary contains more than a dozen ovarioles that cluster together to form bud-like structures. Gentle mechanical disruption of the ovary allows for the separation and visualization of individual ovarioles and, thus, the progression of follicle development.

Here, *D. melanogaster* ovarioles were isolated and stained with 4',6-diamidino-2-phenylindole (DAPI) to identify the characteristic stages of follicular maturation. Follicle development occurs in a distinct anterior-to-posterior direction, beginning in the germarium (red) and progressing through a set of egg chambers of increasing numerical stage (orange, green, blue, yellow, and magenta), before terminating at a mature oocyte (cyan). Although not shown here, mature oocytes are connected to egg chambers within the same ovarioles via dorsal appendages.

Germ line stem cells, residing within the germarium, are a self-renewing population that divide to form a daughter stem cell, which remains in the germarium, and a cystoblast. The cystoblast undergoes sequential rounds of division to form an egg chamber housing 15 nurse cells that work in tandem to nourish a single developing oocyte. The egg chamber is additionally surrounded by numerous follicular cells. Ultimately, the developmentally competent egg will exit the assembly line several days later and enter the uterus where it may be fertilized. A fertilized egg activates during its deposition on an exterior surface allowing embryonic development to commence externally.

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