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Vitamin D supplementation to prevent Gestational Diabetes Mellitus

To improve offspring metabolic outcomes

Veronica Therese Boyle

Abstract

Background

Gestational diabetes mellitus (GDM) affects between 4 and 20% of pregnancies globally. Children of women with GDM are more likely to be obese and both child and mother are at increased life-long risk of developing type 2 diabetes mellitus. There is an association between lower 25-hydroxyvitamin D concentration and glycaemia in both pregnant and non-pregnant populations. Vitamin D deficiency is common in pregnant women. It is not known whether vitamin D supplementation in pregnancy could prevent GDM.

Aims

To investigate whether vitamin D supplementation in pregnancy improves glycaemia and whether maternal vitamin D status is associated with long term metabolic outcomes in children.

Methods

Samples were analysed from two large pregnancy studies.

The Screening for Pregnancy Endpoints (SCOPE) was an observational study with relevant samples available from 1710 participants. Serum samples collected at 14-16 weeks gestation were analysed 25-hydroxyvitamin D concentration by a LC-MS/MS method developed for this study. Associations were investigated between maternal vitamin D status at 15 weeks gestation and GDM later in pregnancy, offspring adiposity at 6 years of age as determined by percentage body fat and age-adjusted BMI z-score and maternal adiposity at 6 years follow-up.

The Maternal Vitamin D Osteoporosis (MAVIDOS) study was a randomised placebo controlled trial of vitamin D supplementation in pregnancy. Relevant samples were available from 910 women. Fructosamine, a measure of mean plasma glucose, and metabolome profiling using GC-MS, were performed on plasma samples collected at 34 weeks gestation after 19 weeks of vitamin D supplementation.
Results

Vitamin D status at 15 weeks gestation was not associated with the development of GDM in SCOPE participants. Maternal vitamin D status was negatively associated with percentage body fat in children at 6 years and negatively associated with BMI change in mothers 6 years post-partum.

Mean plasma fructosamine was not different between participants in the vitamin D supplementation and placebo groups of the MAVIDOS trial. 152 metabolites were detected by GC-MS. No individual metabolites were significantly different in relative abundance between the two groups; however fatty acids as a group were overall lower in relative abundance in the vitamin D intervention group.

Conclusion

Vitamin D status or supplementation in pregnancy was not associated with glycaemia later in pregnancy. Vitamin D status was negatively associated with measures of adiposity at 6 years follow-up in both mother and child and subtle changes in the plasma metabolome suggest vitamin D may have a small but real effect on metabolism.
Acknowledgements

I am grateful to everyone who helped me complete this work. There are some people in particular I want to mention. Firstly I would like to thank my supervisors, Professor Philip Baker, who presented me with this opportunity, and Associate Professor Timothy Kenealy. I am fortunate to have had supervisors with a wealth of knowledge and experience. I appreciate their guidance and advice. I have learnt so much from them.

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I thank the Investigators of the SCOPE and MAVIDOS studies, not only for providing samples but also their advice and feedback. I particularly thank Ms Rennae Taylor for her help, her encouragement and her willingness to listen. Also my special thanks to Dr Farhana Pinu, for her supervision and friendship.

I thank my parents for their patience and support. Finally, and most of all, I thank Suheelan Kulasegaran who has always supported and encouraged me.
Dedication

This thesis is dedicated to my grandmothers, Imelda Boyle (1st of October, 1925 – 29th September, 2004) and Joan Irwin (6th August, 1926 – 31st October, 2015).
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## Glossary

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<tr>
<td>GDM</td>
<td>Gestational diabetes mellitus</td>
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<tr>
<td>95% CI</td>
<td>95% confidence interval</td>
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<td>ADIPS</td>
<td>Australasian Diabetes in Pregnancy Society</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
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<tr>
<td>FPG</td>
<td>Fasting plasma glucose</td>
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<td>GCMS</td>
<td>Gas chromatography mass spectrometry</td>
</tr>
<tr>
<td>GLT</td>
<td>Glucose load test</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>Homeostatic Model Assessment for Insulin Resistance</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard Ratio</td>
</tr>
<tr>
<td>IADPSG</td>
<td>International Association of Diabetes and Pregnancy Study Group</td>
</tr>
<tr>
<td>IU</td>
<td>International Units</td>
</tr>
<tr>
<td>LCMS-MS</td>
<td>Liquid Chromatography Tandem Mass Spectrometry</td>
</tr>
<tr>
<td>MAVIDOS</td>
<td>Maternal Vitamin D Osteoporosis Study</td>
</tr>
<tr>
<td>MCF</td>
<td>Methylchloroformate</td>
</tr>
<tr>
<td>MSTFA</td>
<td>Methyl-N-(trimethylsilyl) trifluoroacetamide</td>
</tr>
<tr>
<td>NZ</td>
<td>New Zealand</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PBF</td>
<td>Percentage body fat</td>
</tr>
<tr>
<td>PTB</td>
<td>Preterm birth</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
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</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RR</td>
<td>Relative Risk</td>
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<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SCOPE</td>
<td>Screening for Pregnancy Endpoints</td>
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<tr>
<td>SGA</td>
<td>Small for gestational age</td>
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<tr>
<td>TMS</td>
<td>Trimethyl silyl</td>
</tr>
<tr>
<td>VDBP</td>
<td>Vitamin D binding protein</td>
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<tr>
<td>VDR</td>
<td>Vitamin D Nuclear Receptor</td>
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Chapter 1  Introduction

The aim of this thesis is to investigate whether vitamin D supplementation in pregnancy can improve glycaemia and thereby prevent the development of gestational diabetes mellitus and improve the metabolic health of offspring.

The concept for this investigation arose as a consequence of a larger clinical trial of nutrient supplementation in pregnancy to improve glucose tolerance in pregnancy therefore improving offspring outcomes: The Nutritional Intervention Preconception and during Pregnancy to maintain healthy glucose levels and offspring health (NiPPeR). The NiPPeR trial intervention supplement contains 200IU of vitamin D along with other micronutrients that have been proposed to improve glycaemic control. The supplement is taken twice daily providing 400IU of vitamin D daily.

The rationale for a multi-nutrient supplement is that it may have a greater likelihood of being effective than a single micronutrient. On the other hand, if the supplement is effective at improving glucose concentrations in pregnancy, it cannot be determined whether all, some or only one of the nutrients were efficacious.

The NiPPeR trial does not feature again in this thesis. It is mentioned here merely to provide the context in which this investigation was started.

The following introduction covers the background of gestational diabetes mellitus (GDM) and vitamin D and provides context as to what is already known in relation to vitamin D and glucose control.
Introduction

1.1 Section One: Gestational Diabetes Mellitus

1.1.1 Introduction and pathophysiology of GDM

Diabetes mellitus refers to a group of heterogenous conditions that all result in hyperglycaemia. Some forms of diabetes mellitus have been recognised since Classical times and the legend follows that the name is derived from the sweet taste observed by ancient Greek physicians on tasting the affected individuals urine (diabetes – to syphon water and mellitus – honey). Diabetes mellitus is broadly categorised based on underlying pathophysiology (American Diabetes Association, 2009) for example, type 1 diabetes mellitus is due to autoimmune destruction of the pancreas and type 2 diabetes mellitus in which profound insulin resistance cannot be overcome by insulin secretion.

Gestational diabetes mellitus (GDM) is so named because pregnancy is the precipitating factor and is defined as “any degree of glucose intolerance with onset or first recognition in pregnancy” (Metzger et al, 1998). GDM was recognised much more recently than type 1 and type 2 diabetes mellitus with the first description of glucose intolerance developing during pregnancy with resolution following delivery in 1882 (Duncan, 1882).

Pregnancy is a state of insulin resistance comparative to non-pregnancy (Catalano et al, 1999). This is believed to be an adaptive response to improve nutrient transfer to the fetus. Relative insulin resistance increases both glucose production in the liver and circulating amino acid and fatty acids in the fasting state thus facilitating greater nutrient availability to the placenta (Butte et al, 1999). Placental hormones, including placental growth hormone (Barbour et al, 2002) and progesterone (Sugaya et al, 2000), and a rise in cortisol (Carr et al, 1981, Robert A et al, 1982) have been directly implicated in causing insulin resistance. Other hormones such as human placental lactogen and prolactin induce leptin resistance (Rachael and David, 2008). Leptin resistance promotes adipose tissue deposition (Highman et al, 1998) and greater adipose tissue mass in turn increases insulin resistance (Kahn et al, 2006).

GDM occurs when the secretion of insulin from the pancreas cannot overcome insulin resistance. The effects of insulin resistance become more exaggerated and an even greater abundance of nutrients are available to the fetus (Chen et al, 2010, Scholtens et al, 2014). With a greater abundance of glucose and amino acids, the fetus produces more insulin. Insulin promotes growth of muscle, bone and adipose tissue (Fowden, 1993). In the presence of abundant nutrients, fetal growth is accelerated, leading to macrosomia (Simmons, 1995).
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Macrosomic infants are not just large for gestational age. They have asymmetric growth, with greater adiposity and abdominal organ growth compared to their head size (Naeye, 1965, Susa et al, 1984, McFarland et al, 1998). Assymetric growth can be difficult to assess and macrosomia is commonly defined as infants with a birth weight above the 90th centile or over 4000g or 4500g (Crowther et al, 2005, The HAPO Study Cooperative Research Group, 2008). A recent meta-analysis including data from 28,303 pregnancies found the odds of a hyperglycaemic women having a macrosomic infant was 2.06 (95% CI 1.86 to 2.28) compared to normoglycaemic women (Farrar et al, 2016). Due to large infant size, macrosomia increases the risk of obstructed labour and birth injury (Jolly et al, 2003). The hyperinsulinaemic fetus, with or without macrosomia, is at risk of developing neonatal hypoglycaemia. The immature liver cannot maintain gluconeogenesis at the rate delivered from the placenta and plasma glucose concentrations fall and in some cases hypoglycaemia can lead to brain injury. (Weiss et al, 1998, Ogunyemi et al, 2016).

There is abundant evidence that for the mother, GDM clearly signifies a propensity to develop metabolic disease. Ten years after diagnosis with GDM, up to two thirds of women will have developed type 2 DM (Kim et al, 2002). In a prospective cohort study comparing nulliparous women to women with and without GDM during their pregnancy, a past history of GDM was a stronger predictor than family history and BMI for developing type 2 DM in the following 20 years (Gunderson et al, 2007). A systematic review and meta-analysis including over 600,000 women found those who had GDM had a 7 fold increase in the risk of developing type 2 DM over a median follow-up of 5 years (Relative Risk (RR) 7.43 95% Confidence Interval (CI) 4.79-11.51) (Bellamy et al). After adjustment for pre-eclampsia and BMI, women who had GDM were more likely to suffer a cardiovascular event (adjusted Hazard Ratio (HR) 2.6 95% CI 2.3 – 3.0) (Kessous et al, 2013).

Evidence is gathering that GDM confers lifelong health risks to the offspring also. Given the strong genetic association between type 2 DM and GDM, it has been found, that the children of women with GDM are more likely to develop type 2 DM later in life (Huerta-Chagoya et al, 2015). However, children of women with type 1 DM also have an increased likelihood of developing type 2 diabetes/pre-diabetes in early adulthood compared to those not exposed to hyperglycaemia during pregnancy (OR 4.02 95% CI 1.31 – 12.33) (Clausen et al, 2008). This finding suggests that fetal programming contributes to at least some of the conferred risk.
Introduction

Animal models support this theory, for example, adult rat offspring of diabetic rats are more insulin resistant (Holemans et al, 1991).

An association has also been found between early exposure to GDM and adiposity. Studies have found that siblings born to a mother with GDM have a higher BMI and greater adiposity than their siblings not exposed to GDM (Pettitt et al, 1987, Lawlor et al, 2011). One of the largest of these studies is a Swedish prospective cohort study including 280 866 men from 248 293 families (Lawlor et al, 2011). Men born to mothers with GDM were 0.94 kg/m² heavier than their siblings born before a diagnosis of GDM at 18 years of age, after adjustment for maternal age, parity and BMI. A study of growth trajectories comparing children born to mothers with GDM to those who did not, found that differences in BMI growth trajectory occurred late in childhood at 10 to 13 years of age (difference in change of BMI 1.05 \( p = 0.005 \)) (Crume et al, 2011). The consequences of GDM have been briefly summarised in Table 1.1.

Table 1.1 Peripartum and long term consequences with GDM

<table>
<thead>
<tr>
<th>Peri-partum</th>
<th>Long Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal</td>
<td></td>
</tr>
<tr>
<td>Obstructed labour</td>
<td>Type 2 DM</td>
</tr>
<tr>
<td>Shoulder dystocia</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>Perineal tear</td>
<td></td>
</tr>
<tr>
<td>Pudendal nerve injury</td>
<td></td>
</tr>
<tr>
<td>Post-partum haemorrhage</td>
<td></td>
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<tr>
<td>Offspring</td>
<td></td>
</tr>
<tr>
<td>Birth injury</td>
<td>Obesity</td>
</tr>
<tr>
<td>Perinatal asphyxia</td>
<td>Type 2 DM</td>
</tr>
<tr>
<td>Neonatal hypoglycaemia</td>
<td></td>
</tr>
</tbody>
</table>
Introduction

1.1.2 Epidemiology of GDM

GDM affects 4%-25% of pregnant women depending on the background population demographics (Bener et al, 2011, Guariguata et al, 2014). GDM shares a common physiological mechanism with type 2 diabetes mellitus, insulin resistance, and the conditions share common risk factors, the most important being family history, ethnicity and adiposity. The prevalence of GDM in women with a family history of type 2 DM is double that of the background population (Di Cianni et al, 2003) and up to 35% in studied populations (Retnakaran et al, 2007). South East Asian and Indian women have the highest prevalence with between 1 in 5 and 1 in 4 women developing GDM while women of European descent have the lowest prevalence (Hunsberger et al, 2010).

The most important potentially modifiable risk factor for GDM is adiposity. In a meta-analysis including 70 studies, the likelihood of developing GDM increased significantly with each BMI category (overweight 25-30, obese 30-35 and morbidly obese >35) as shown in Table 1.2 (Torloni et al, 2009). In a composite of 56 studies included in the systematic review, the prevalence of GDM in women with a BMI >35 was 40%. Greater visceral fat, in particular, increases the likelihood of developing GDM. In a study of 485 women, being within the upper quartile of visceral adipose tissue at 11-14 weeks gestation was a predictor of developing GDM, while subcutaneous adipose tissue thickness was not (adjusted OR 4.2 95% CI 1.4 – 14.2 vs 1.5 95% CI 0.56 – 4.5) (De Souza et al, 2015).

<table>
<thead>
<tr>
<th>BMI category</th>
<th>Odds Ratios (95% CI) for developing GDM (Torloni et al, 2009)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 20</td>
<td>0.75 (0.69-0.82)</td>
</tr>
<tr>
<td>20 – 24.9</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>25 – 29.9</td>
<td>1.97 (1.77-2.19)</td>
</tr>
<tr>
<td>30 – 34.9</td>
<td>3.01 (2.68-3.24)</td>
</tr>
<tr>
<td>&gt;35</td>
<td>5.55 (4.27-7.21)</td>
</tr>
</tbody>
</table>

As expected, with increasing global obesity, epidemiological studies have shown an increased incidence of GDM (Dabelea et al, 2005, Hunt and Schuller, 2007, Seshiah et al, 2008). More women who developed GDM may be developing Type 2 DM later in life due to the increasing prevalence of obesity. A comparison of two Danish cohorts of women who had diet-treated GDM, from the same treatment centre between 1978-1985 and 1987-1996 showed a
Introduction

significantly higher prevalence of type 2 DM at a median follow-up of 9.8 years in the later cohort (18.3% in the early cohort vs 40.9% in the later cohort) (Lauenborg et al, 2004). The significant differences between the cohorts were in was both pre-pregnancy BMI and BMI at follow-up. The increase in GDM diagnoses in recent years may have resulted, in part, from changing thresholds in diagnostic criteria.

1.1.3 The diagnosis of GDM

The diagnosis of GDM is made by analysing glucose concentrations in a fasting state and/or in response to a glucose load. Table 1.3 summarises the recommendations of the guidelines discussed in this section (O'Sullivan and Mahan, 1964, National Diabetes Data Group, 1979, Carpenter and Coustan, 1982, International Association of Diabetes and Pregnancy Study Groups Consensus Panel, 2010, American College of Obstetricians and Gynecologists, 2011, Ministry of Health, 2014, NICE, 2015, American Diabetes Association, 2016). This table demonstrates the contention over the diagnostic criteria for GDM. A range of fasting, and post glucose challenge thresholds have been proposed. One problem is identifying an appropriate outcome measure. Early recommendations were based upon predictions of the likelihood for developing type 2 diabetes mellitus later in life (O'Sullivan, 1980). New guidelines, such as the International Association of Diabetes in Pregnancy Study Group (IADPSG) (Metzgar and IADPSG consensus panel, 2010), aim to establish a glucose concentration threshold more predictive of immediate adverse outcomes associated with GDM.

The IADPSG guidelines use a lower threshold of plasma glucose than those recommended in previous guidelines (Metzger et al, 1998). These new thresholds were based on the conclusions drawn from the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study (The HAPO Study Cooperative Research Group, 2008). In this study of over 25,000 participants, a small rise in fasting plasma glucose (FPG) (0.4 mmol/L), 1-hour post glucose load plasma glucose (1.7mmol/L) or 2 hour plasma glucose (1.3mmol/L), was associated with a greater likelihood of an infant birth weight above the 90th centile before the contemporaneous threshold for GDM was reached. This is supported by evidence from other studies showing a linear association between glycaemia and adverse pregnancy outcome risk with no obvious glucose threshold (Sermer et al, 1995, Jensen et al, 2008).
Introduction

Implementation of the IADPSG was predicted to significantly increase the proportion of women diagnosed with GDM (Cundy et al, 2014). Indeed, in Madrid, Spain, implementation of the IADPSG screening and diagnostic guideline resulted in an increase in the prevalence of GDM from 10% (American Diabetes Association criteria) to 35% (Duran et al, 2014). Due to a reduction in adverse outcomes, decreasing the number of admissions to the neonatal intensive care unit, the introduction of the IADPSG criteria was associated with a cost saving. In Beijing, the adoption of the IADPSG diagnostic criteria increased the prevalence of GDM from 7.98% (ADA) to 19.9%. An assessment of effect on outcomes or cost-effectiveness was not made (Shang and Lin, 2014).

These stricter glucose thresholds have not been adopted everywhere, due to concerns that the threshold is too low and will increase interventional harm, such as increased maternal hypoglycaemia and increased small for gestational age infants, without providing additional benefit (Ekeroma et al, 2015). There is no clear evidence for either substantial benefit or harm. For example, a study randomising 900 women with mild hyperglycaemia (FPG ≥ 5.3 nmol/L, 1 hour glucose ≥ 10.0 nmol/L, 2-hour ≥ 8.6 nmol/L, 3 hour ≥ 7.8 nmol/L) to either treatment (dietary advice and insulin if needed) or usual antenatal care showed no difference in the primary composite outcome of perinatal mortality, hypoglycaemia, hyperbilirubinaemia, neonatal hyperinsulinaemia, and birth trauma (Landon et al, 2009). On the other hand, infants in the treatment group had a lower birth weight and the percentage of large for gestational age infants was lower in the treatment group (7.1% vs 14.5% p <0.001) with no increase in small for gestational age infants (7.5% vs 6.4% p = 0.5).

Another debate concerns whether screening for GDM should be universal or for high risk women only. Screening takes the form of either a fasting oral glucose tolerance test (OGTT) or a non-fasting glucose load test (GLT) followed by an OGTT if a glucose threshold is met. With increasing prevalence of GDM and lowering of the glucose threshold, universal screening is becoming more commonly recommended. One exception is in the United Kingdom, where the National Institute for Clinical Excellence (NICE) recommends an OGTT on the basis of risk factors (NICE, 2015). NICE recommends a 75g 2 hour OGTT if at least one of the following is met: BMI above 30 kg/m², previous infant weighing 4.5kg or greater, previous gestational diabetes, first degree relative with diabetes mellitus, or high risk ethnic group.
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High risk screening is known to miss between 7 to 50% of women with GDM in comparison to universal screening, depending on the screening criteria and the population characteristics (Lavin, 1985, Naylor et al, 1997, Williams et al, 1999). However, whether universal screening improves outcomes at the population level is not known. A Cochrane systematic review identified only one study that directly compared outcomes with high risk compared to universal screening (Tieu et al, 2014). Fewer cases of GDM were identified in the high risk screening group, although prevalence was low in both groups (1.45% in the high risk group vs 2.7% in the universal screening group $p < 0.03$) (Griffin et al, 2000). The authors compared outcomes in those diagnosed with GDM, rather than comparing the outcomes of the whole high risk screening group to the whole universally screened group. Therefore few conclusions on the effect of high risk vs universal screening on the population can be drawn from this study.

1.1.4 Management of GDM

The management goal of GDM is to “normalise” plasma glucose (American Diabetes Association, 2016). Recommended glucose targets are given in Table 1.3. Blood glucose control has been demonstrated to reduce the risk of macrosomia and large gestational age infants, shoulder dystocia, pre-eclampsia and hypertensive disorders of pregnancy in women with GDM (Crowther et al, 2005).

Treatment consists initially of lifestyle changes. Diet modification has been shown to reduce maternal weight gain during pregnancy, lower the prevalence of large for gestational age infants and prevent shoulder dystocia, while a benefit of exercise on outcomes has not been established (Alwan et al, 2009). Which type of diet is most beneficial is more difficult to establish. Low glycaemic index diets, low carbohydrate diets, high monounsaturated fat acid diets and fibre enriched diets have been compared in a Cochrane meta-analysis with no diet showing clear benefit over the others (Han et al, 2013).

Insulin and/or metformin are introduced if plasma glucose concentrations cannot be controlled with diet alone. Metformin has been shown to have comparative safety to insulin for the management of GDM (Rowan et al, 2008). In a recent meta-analysis of 8 randomised trials, metformin (with or without addition of insulin) was associated with lower pregnancy weight gain, less neonatal hypoglycaemia and fewer admissions to neonatal intensive care.
Introduction

units compared to insulin without metformin (Zhu et al, 2016). No increase in adverse events was observed with metformin.

Whether management of GDM improves the long term metabolic outcomes of mother and offspring is not known (Falavigna et al, 2012).
### Table 1.3 Guidelines for the screening and diagnosis of GDM and recommended plasma glucose targets for GDM

<table>
<thead>
<tr>
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<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Target</td>
<td>Universal 50g non-fasting GLT</td>
<td>Universal Fasting 75g OGTT</td>
<td>Universal Fasting OGTT or non-fasting GLT</td>
<td>Risk factors OGTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Screening type</td>
<td></td>
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</tr>
</tbody>
</table>

### Diagnosis

<table>
<thead>
<tr>
<th>Test</th>
<th>Glucose load</th>
<th>2 hour OGTT 75g</th>
<th>2 hour OGTT 75g</th>
<th>3 hr OGTT² 100g</th>
<th>2 hr OGTT³ 75g</th>
<th>2 Hour OGTT 75g</th>
<th>3 Hour OGTT 100g</th>
<th>3 Hour OGTT 100g</th>
<th>3 hour OGTT 100g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 hour</td>
<td>≥ 5.5</td>
<td>≥ 5.1</td>
<td>5.3/5.8</td>
<td>≥ 5.1</td>
<td>≥ 5.6</td>
<td>≥ 5.8</td>
<td>≥ 5.3</td>
<td>≥ 5.8</td>
<td>≥ 5.8</td>
</tr>
<tr>
<td>2 hour</td>
<td></td>
<td>≥ 10.0</td>
<td>10.0/10.6</td>
<td>≥ 10.0</td>
<td>≥ 10.6</td>
<td>≥ 10.0</td>
<td>≥ 10.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 hour</td>
<td>≥ 9.0</td>
<td>≥ 8.5</td>
<td>8.6/9.2</td>
<td>≥ 8.5</td>
<td>≥ 7.8</td>
<td>≥ 9.2</td>
<td>≥ 8.6</td>
<td>≥ 9.2</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glucose targets</th>
<th>Fasting</th>
<th>1 hour postprandial</th>
<th>2 hour postprandial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting</td>
<td>≤ 5.0</td>
<td>≤ 5.3</td>
<td>≤ 5.3</td>
</tr>
<tr>
<td>1 hour postprandial</td>
<td>≤ 7.4</td>
<td>7.2 - 7.8</td>
<td>≤ 7.8</td>
</tr>
<tr>
<td>2 hour postprandial</td>
<td>≤ 6.7</td>
<td>≤ 6.7</td>
<td>≤ 6.4</td>
</tr>
</tbody>
</table>

**Abbreviations:** ACOG – American College of Obstetrics and Gynecology, ADA – American Diabetes association, FPG – fasting plasma glucose, GLT – glucose loading test, IADPSG – International Association of Diabetes in Pregnancy Study Group, NDDG – The National Diabetes Data Group, OGTT – oral glucose tolerance test

¹ If two or more thresholds meet or exceed threshold

² Plasma glucose concentrations given in mmol/L

³ Either one-step diagnostic pathway with a 2 hour 75g OGTT or a two-step pathway with a non-fasting 50g GLT followed by a 3 hour 100g glucose load OGTT if 1 hour plasma glucose ≥7.8. Diagnosis is confirmed when two or more plasma glucose concentrations meet or exceed the threshold.
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1.1.5 Interventions to prevent GDM

With the rise of obesity and the lowering of glucose thresholds to diagnosis GDM, the proportion of women diagnosed with GDM is rising exponentially. There is accumulating evidence that the children of women who had GDM, not only have increased health risks in the perinatal period but also in the long term. On the other hand, there are concerns that over treatment may put women and neonates at risk of treatment harm. In this context a population-wide intervention for the prevention of GDM is highly desirable.

Adiposity is an important contributor to the likelihood of developing GDM and is undoubtedly the major contributor to the rise in GDM prevalence. For this reason, interventions for the prevention of GDM have focused on lifestyle changes. However, there are at least three major challenges to this approach. First, lifestyle changes are hard to sustain. Even when people are highly motivated they require intensive support (MacLean et al, 2015). Second, it is not known whether weight loss and increasing exercise in pregnancy have adverse consequences (Cox Bauer et al, 2016). Third, while GDM is associated with adiposity and inactivity it also occurs in women who exercise and are not overweight (Efendić, et al, 1987).

A number of recent systematic reviews have assessed the evidence for interventions to prevent GDM. High fibre and low glycaemic index diets from early pregnancy have been trialled (Moses et al, 2006, Clapp III, 2007). A Cochrane systematic review concluded that the evidence was not yet sufficient to support these interventions (Tieu et al, 2008). A more recent meta-analysis, with the addition of one further trial, came to the same conclusion, however, the authors identified that any dietary advice reduced the risk of GDM (risk difference -0.05 95% CI -0.10 to -0.01) (Oostdam et al, 2011).

One randomised controlled trial of probiotics in pregnancy (Lactobacillus rhamnosus GG ATCC 53103 and Bifidobacterium lactis Bb-12) found significant reductions in the prevalence of GDM in the probiotic supplement group compared to the diet advice group and the control group (13% vs 36% vs 34%  p = 0.003) (Luoto et al, 2010). This is a very encouraging result but as yet it is the only trial of probiotics in pregnancy reporting on glycaemia, although there are a number of trials currently in process (Barrett et al, 2014).

Another Cochrane systematic review investigated “exercise only” interventions (Han et al, 2012). The authors did not find a reduction in the risk of developing GDM with an exercise
Introduction

intervention (RR 1.10 95% CI 0.66-1.84). A more recent meta-analysis that included 8 trials did find reduction in the risk of developing GDM with an exercise intervention compared to routine care (RR 0.69 95% CI 0.52-0.91 p = 0.009) (Sanabria-Martínez et al, 2015). Only one clinical trial was included in both reviews (Barakat et al, 2012). This was partly due to a number of new publications following the Cochrane review but also a difference in inclusion criteria; the more recent review did not include studies that gave any lifestyle or other advice to the control group.

Finally, a Cochrane systematic review and meta-analysis identified 13 randomised controlled trials comparing combined dietary and exercise intervention to no intervention for the prevention of GDM (Bain et al, 2015). The authors did not find a reduction in the risk of developing GDM with a combined intervention (RR 0.92, 95% CI 0.68-1.23). Of the six trials that reported birth weight, there was also no reduction in large for gestational age infants (RR 0.90, 95% CI 0.77-1.05).

Even if exercise is effective at preventing GDM, compliance is a major issue, especially in populations most likely to benefit. In a study investigating the effect of exercise in pregnancy in women with a BMI 25.5 ± 4.0, compliance with the exercise program over the period of the study was 75 ± 17% (Hopkins et al, 2010). When the same group investigated the effect of exercise in women with a mean BMI 31.6 ± 4.4, compliance was much lower (33% range 0 – 85%) (Seneviratne et al, 2016).

An increasing prevalence of GDM may, in turn, be contributing to increasing obesity and diabetes in the next generation. But there is not yet an effective preventative measure that can be implemented at a population level.
1.2 Section Two: Vitamin D

It was discovered almost simultaneously that supplementation with cod liver oil and exposure to sunlight could cure rickets. At the time when investigators were isolating the anti-ricketic factor in cod liver oil, other groups were demonstrating that exposure to adequate ultraviolet light without dietary supplementation was adequate to cure and prevention of rickets (Norman, 2011).

In 1921 the anti-ricketic factor was named vitamin D although its chemical structure was then unknown (McCollum et al, 1922). Fifteen years later, the chemical structure was characterised when it was shown that vitamin D₃ (cholecalciferol) was formed when 7-dehydrocholesterol was exposed to ultraviolet light and this was identical to the vitamin D in cod-liver oil (Windaus and Bock, 1937). The chemical structure of vitamin D₂ (ergocalciferol) had been characterised a few years earlier (Askew et al, 1931).

It is from this background that an endogenously synthesised steroid hormone has been named a vitamin. It is not, however, entirely inappropriate. Much of the world’s population does not synthesise adequate amounts of cholecalciferol (Wahl et al, 2012). Many factors of modern living result in lower sun exposure including migration to areas of high latitude and low sun hours, air pollution, indoor occupations and recreation, and sunscreen use (Kimlin et al, 2007, Golbahar et al, 2014). In a study of 320 adolescent girls in Korea, 63.8% were found to be vitamin D deficient as defined by a serum 25-hydroxyvitamin D <20ng/mL (Jang et al, 2013). Almost half of 1,873 pregnant women in Qatar by the same definition were deficient (Bener et al, 2013).

The most important and most well-known function of vitamin D is to increase the intestinal absorption of calcium, however, the vitamin D receptor (VDR) is found throughout the body in various tissue types (Stumpf et al, 1979, Clark et al, 1980). The earliest evidence of other functions was from the description of the syndrome known as hereditary vitamin D resistant rickets (HVDRR) (Federico et al, 1993). HVDRR is a rare autosomal recessive condition in which the VDR is either absent or dysfunctional. In the most severe cases, which account for about two thirds of reports, it is associated with alopecia. Hence the role of vitamin D in keratinocyte maturation was first recognised and it has since been found to have some role in differentiation of other cell types (Haussler et al, 1998). It was later discovered that hypercalcaemia in sarcoidosis was secondary to the activation of vitamin D in macrophages (Singer and Adams, 1986).
Introduction

1.2.1 Chemical structure and nomenclature

Vitamin D is a seco-steroid, meaning that it is based on a 4 carbon ring like all steroids, but with one ring broken. In the case of vitamin D it is the bond between carbons 9 and 10 carbon of the B ring (Pietraszek et al, 2013).

After the chemical structure was established, it was realised that vitamin D must undergo two steps of hydroxylation to become activated. This step-wise discovery has resulted in what can be a confusing nomenclature. For example, “vitamin D” can refer to vitamin D in all of its forms or more specifically vitamin D$_2$ and vitamin D$_3$ combined prior to hydroxylation.

Vitamin D$_3$ (cholecalciferol) and vitamin D$_2$ (ergocalciferol) differ from one another in that the D$_2$ form has a C-22/C23 double bond and a C-24 methyl group while D$_3$ has a single bond and hydrogen (Volmer et al, 2013). This reflects their different origin. Vitamin D$_2$ is derived from plants, when ergosterol is exposed to ultraviolet light. It can only be attained through diet. Vitamin D$_3$ is synthesised in the skin of vertebrates including humans. The structural difference has biological implications in the first step to activation, 25-hydroxylation (Zhu and DeLuca, 2012). Vitamin D$_3$ is the most studied, being the more plentiful form in mammals. Table 1.4 details vitamin D precursors and Table 1.5 details the various forms of vitamin D.
### Table 1.4 Vitamin D₃ precursors

<table>
<thead>
<tr>
<th>Pro-vitamin D₃</th>
<th>Pre-vitamin D₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-dehydroxycholesterol</td>
<td>Pre-cholecalciferol</td>
</tr>
<tr>
<td>7-DHC</td>
<td></td>
</tr>
</tbody>
</table>

![Chemical structures of Pro-vitamin D₃ and Pre-vitamin D₃](image-url)
### Table 1.5 Vitamin D<sub>2</sub> and D<sub>3</sub> in their various forms

<table>
<thead>
<tr>
<th>Vitamin D&lt;sub&gt;3&lt;/sub&gt;</th>
<th>Vitamin D&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholecalciferol</td>
<td>Ergocalciferol</td>
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Introduction

1.2.2 Vitamin D synthesis

Cholesterol is a vital component of cell membranes. Cholesterol is delivered to most cells via low density lipoproteins (LDL), however, mature keratinocytes lack LDL receptors. In order to maintain cell lipid structures, keratinocytes must synthesis cholesterol de novo. (Glossmann, 2010). 7-dehydrocholesterol (7-DHC) is the final precursor in the biosynthesis of cholesterol and large quantities are incorporated into the cell walls of the dermis and epidermis. On exposure to ultraviolet light the 9-10 carbon bond of 7-DHC is cleaved to form pre-vitamin D₃ (Holick et al, 1980).

Pre-vitamin D₃ is a labile molecule and at body temperatures it isomerises to vitamin D₃ through rotation around the carbon bond 5 and 6 (Zhu and Okamura, 1995). Vitamin D₃ binds with strong affinity to the vitamin D binding protein (VDBP) and is taken from the lipid layer into the circulation such that the reaction is driven in favour of the production of vitamin D₃ (Tian et al, 1994). This thermally-driven reaction continues after sun exposure for up to three days and peak levels of serum vitamin D are reached after 24 hours (Figure 1.1) (Holick et al, 1980).

Factors such as darker skin pigmentation and latitude further from the equator extend the time required for pre-vitamin D₃ to be produced, however, with adequate sunlight exposure the maximum amount produced remains the same for all skin types (Tsiaras and Weinstock, 2011). For example, a light skinned person at the equator at mid-day may require 10 minutes to convert the maximum available 7-DHC to vitamin D₃ while a person with darker skin may require 30 minutes. After half an hour of sunlight exposure, both have produced the same amount of vitamin D₃ (Holick et al, 1980).

Temperature and ultraviolet light have different effects on pre-vitamin D₃ and an equilibrium is reached so that about 10 to 15% of the 7-DHC in skin is converted to vitamin D₃. At body temperature, pre-vitamin D₃ becomes vitamin D₃, but on exposure to sunlight, pre-vitamin D₃ photoisomerises to tachysterol and lumisterol (Holick et al, 1981). Vitamin D remaining in the skin also undergoes photo isomerisation to suprasterol I, suprasterol II and 5,6-transvitamin D₃. While 5,6-transvitamin D₃ can be hydroxylated to an activated vitamin D analogue, the other substances are biologically inert and are sloughed off (Webb et al, 1989).
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Once vitamin D₃ is removed from the lipid membrane by the VDBP, it is taken to the liver and perhaps adipose tissue for storage (Mawer, 1972 #464). Vitamin D can be stored in the liver for many months (Tsiaras and Weinstock, 2011).

Figure 1.1 Vitamin D synthesis in the skin
Introduction

1.2.3 Activation and inactivation of vitamin D

To become the activated form, vitamin D undergoes two steps of hydroxylation. First in the liver to form 25-hydroxyvitamin D and then 1α-hydroxylation, the majority of which occurs in the kidney, to form 1,25-dihydroxyvitamin D (Jones et al, 1998).

25-hydroxylation

The regulation of 25-hydroxylation is only loosely controlled, the main limiting factor being available substrate (Jones et al, 1998). The liver has been identified as the main site of 25-hydroxylation (Zhu and DeLuca, 2012). The ‘25-hydroxylase’ enzyme has not been identified with certainty. A number of cytochrome p450 enzymes maybe responsible.

CYP27A1 was one of the first enzymes to be identified as having the ability to 25 hydroxylate vitamin D3 in vitro (Jones et al, 1998). However, it must not be the only enzyme, because it has no activity on vitamin D2. Furthermore, in the rare genetic condition of cerebrotendinous xanthomatosis in which CYP27A1 is absent, vitamin D absorption is reduced (due to abnormal bile salt) but 25-hydroxylation is not affected in the presence of adequate substrate (Federico et al, 1993, Martini et al, 2013).

A genetic mutation of another liver enzyme, CYP2R1, has been associated with vitamin D-dependent rickets in a small number of families from Saudi Arabia and Nigeria (Cheng et al, 2004, Al Mutair et al, 2012). CYP2R1 knockout mice have greatly reduced serum 25-hydroxyvitamin D3 concentrations compared to wild-type mice. The mice were healthy and had detectable serum 1,25-dihydroxyvitamin D3 (Zhu et al, 2013). Therefore, CYP2R1 appears to be an important 25-hydroxylase, but not the only one.

A number of other liver enzymes have been found to have 25-hydroxylase activity such as CYP2J3 in rats (Yamasaki et al, 2004). It is not present in humans, but a similar p450 enzyme CYP2J2, while having a weaker affinity for vitamin D than CYP2J3, has been shown to have 25-hydroxylase activity on vitamin D2. It is present in the liver but is more densely expressed in the heart where its role is in the oxidation of arachidonic acid. It may have an assisting role, particularly in the 25-hydroxylation of vitamin D2 (Zhu and DeLuca, 2012). Similarly CYP2D25 in pigs and CYP2D11 in male rats have high vitamin D3 and D2 25-hydroxylase activity (Zhu and DeLuca, 2012). The only member of the CYP2D subgroup in humans is CYP2D6. Although very similar in structure to the enzymes above, recombinant CYP2D6 did
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not show any 25-hydroxylase activity when in transfected insect cells (Hosseinpur and Wikvall, 2000).

1α-hydroxylation

In contrast, 1α-hydroxylation is carried out by one enzyme and is under tight control. CYP27B1 (also called 1α-hydroxylase) is most densely expressed in the kidney although it is found in a variety of tissue types (Zehnder et al, 2001). These include macrophages, pancreatic β-cells, keratinocytes, placenta, lung and colon.

25-hydroxyvitamin D is taken up into the renal proximal tubules by megalin receptors in the cell membrane that are expressed in response to parathyroid hormone (Nykjaer et al, 1999). Here 25-hydroxyvitamin D is converted to 1,25-dihydroxyvitamin D by CYP25B1. 1,25-dihydroxyvitamin D then circulates to its main target tissue, the intestinal epithelium.

CYP27B1 synthesis in the kidney is driven by parathyroid hormone. Parathyroid hormone is released in response to low serum calcium detected by the calcium-sensing receptor in the parathyroid cell. It then acts upon the cells in the proximal tubule of the kidney, the main site of 1α-hydroxylation (Prosser and Jones, 2004). 1,25-dihydroxyvitamin D self-regulates by reducing the expression of CYP27B1-hydroxylase and secretion of parathyroid hormone. Furthermore, it up-regulates the transcription of 24-hydroxylase, the inactivating enzyme, in all of its target cells (Haussler et al, 1998).

Other cell types that activate 25-hydroxyvitamin D via CYP27A1 hydroxylation such as monocytes, adipocytes and beta-cells in the pancreas do not have megalin receptors (Marzolo and Farfán, 2011, Abboud et al, 2014). This has given rise to the theory that the free portion, not bound to the VDBP, is the physiological important form for extra-renal 25-hydroxyvitamin D activation (Chun et al, 2010). This is supported by the finding that, unlike in those with normal kidneys, anephric patients’ serum concentrations of 1,25-hydroxyvitamin D are proportionate to the concentration of 25-hydroxyvitamin D (Lambert et al, 1982).

Inactivation by 24-hyroxylation

24-hydroxylation is the first step in the catabolism of 1,25-dihydroxy vitamin D to calcitroic acid and finally clearance in bile. It is carried out by CYP24 (24-hydroxylase), the transcription of which is up-regulated by 1,25-dihydroxyvitamin D (Haussler et al, 1998). Both 25-hydroxy and 1,25 hydroxyvitamin D (in D2 and D3 forms) are substrates producing
Introduction

inactive metabolites. Half of CYP24A1 null mice succumb to the toxic effects of vitamin D before weaning. Interestingly, the hypercalcaemic mice had reduced ossification of bone which is yet to be explained (St Arnaud, 1999).

CYP3A4 may be an alternative but minor contributor to catabolism. CYP3A4 is the most abundant cytochrome p450 in the liver and intestine of humans and is responsible for the metabolism of many drugs. While it is capable of 25-hydroxylation of 1-hydroxyvitamin D₃, 1-hydroxyvitamin D₂ and vitamin D₂ (although not vitamin D₃), its preference is 24-hydroxylation. It may have a role in first pass metabolism of dietary vitamin D, as it has been shown that expression of CYP3A4 is up regulated by its presence (Zhu and DeLuca, 2012).

1.2.4 Biological Function

Vitamin D is well-known for its role in calcium homeostasis and bone mineralisation. Other functions of vitamin D are less well understood. These vary widely and include immunomodulatory effects, skeletal muscle health, vascular protective effects, glucose lowering properties and induction of cell differentiation in epithelial cells. The role in glucose metabolism is discussed in more detail in section 3 of this Chapter.

The Vitamin D receptor

The actions of 1,25-hydroxyvitamin D are mediated by the nuclear vitamin D nuclear receptor (VDR). The VDR binds 1,25-dihydroxyvitamin D with high affinity then undergoes phosphorylation (Jurutka et al, 1993) and heterodimerisation with the retinoid X receptor (RXR) (Yu et al, 1991). This renders the retinoid X receptor incapable of binding its ligand 9-cis retinoic acid. Conversely, if 9-cis retinoic acid binds to RXR first, it is not able to heterodimerise with VDR. The VDR-RXR heterodimer binds through zinc-finger mediated DNA binding to vitamin D responsive elements (VDRE) in gene promoter regions (Haussler et al, 1995). The VDR also down regulates gene transcription by direct binding to the transcription factor of target genes and nullifying its effect (Mi-sun et al, 2007).

Often where the VDR and VDREs are found, the activating enzyme CYP27B1-hydroxylase is present also (Zehnder et al, 2001). Examples include macrophages, dendritic cells, parathyroid cells, osteoblasts, osteoclasts, keratinocytes, mammary epithelial cells, enterocytes, prostate epithelial cells, vascular endothelial cells, pancreatic β cells and renal tubular cells (Adams et al, 1983, Bikle et al, 1986, Norman, 2006).
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A notable exception is in adipose tissue. Adipose tissue contains the VDR, VDRE’s and CYP24A1 (the enzyme that inactivates 1,25-hydroxyvitamin D) but has not been shown to synthesise CYP27B1-hydroxylase. *In vitro* the VDR activates the transcription of inhibitory factors in the adipocyte resulting in reduced adipogenesis and reduced lipid accumulation in adipocytes (Dang *et al.*, 2012).

*Intestinal absorption of calcium*

Upon binding 1,25-dihydroxyvitamin D, the VDR of the intestinal epithelium increases transcription of transient receptor potential channel vaniloid 6, increasing calcium influx from the intestinal lumen into the cell (Hoenderop *et al.*, 1999). Calcium is transported to the basal membrane by calbindin-D9k which is also upregulated by 1,25dihydroxyvitamin D (Darwish *et al.*, 1987). Calcium exits the basal membrane via the plasma membrane calcium ATPase (PMCA1b); there is no evidence that this process is under VDR regulation (Bouillon *et al.*, 2003).

*Immune system*

Granuloma-forming macrophages produce CYP27B1-hydroxylase with the capacity to produce enough 1,25-dihydroxyvitamin D to be secreted by the cell (Adams *et al.*, 2014). At this time it appears that the main role of vitamin D in the macrophage is the production of cathelicidin.

Cathelicidin is an endogenous antimicrobial molecule that is synthesised in macrophages in response to VDR activation (Adams *et al.*, 2014). Synthesis of cathelicidin begins with activation of toll-like receptors by pathogen associated molecular pattern (PAMPs) in the macrophage. The macrophage responds by secreting interleukin 15 (IL-15). IL-15 has two roles. Firstly, it up-regulates CYP27B1-1α-hydroxylase and VDR expression in the macrophage, secondly, it activates T-helper 1 cells. Cathelicidin gene transcription is increased in response to ligand-VDR-RXR, binding resulting in improved efficacy of microbial killing of intracellular pathogens. Activated TH1 cells further accentuate the vitamin D response via interferon γ (Edfeldt *et al.*, 2013).

The feed-forward loop is regulated by IL-1β also expressed by macrophages in response to PAMPs. IL-1β activates T-helper 2 lymphocytes; the TH2 cells in turn express IL-4 and interferon β. These cytokines block 1α-hydroxylation and stimulate the production of CYP24A1-hydroxylase. 1,25-dihydroxyvitamin D also self-regulates the feedback loop.
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When released into the extracellular space it inhibits TH1 proliferation and promotes TH2 proliferation (Adams et al, 2014). In 25-hydroxyvitamin D deficient states, the cathelicidin response and the ability to kill mycobacterium tuberculosis in macrophage is reduced (Liu et al, 2006).

1.2.5 Evaluation of vitamin D status

Vitamin D status is determined by the concentration of serum or plasma 25-hydroxyvitamin D. 25-hydroxyvitamin D is the predominant circulating form of vitamin D. Unlike 1,25-dihydroxyvitamin D, hydroxylation to 25-hydroxyvitamin D is substrate dependent and not regulated by plasma calcium or parathyroid hormone concentrations (Stryd et al, 1979). It also has a long half-life of about a month (Heaney et al, 2003). The majority of circulating total 25-hydroxyvitamin D is 25-hydroxyvitamin D₃; 25-hydroxyvitamin D₂ contributes a varying proportion depending upon diet or supplementation.

There is no global consensus on the definition of vitamin D deficiency. Various authorities have chosen different 25-hydroxyvitamin D concentration thresholds. The Institute of Medicine (now the National Academy of Medicine) recommends a target concentration of 50 nmol/L to cover the requirements of bone mineralisation for 97.5% of the population (Institute of Medicine et al, 2011). The American Endocrine Society recommends a target of 75 nmol/L based upon the 25-hydroxyvitamin D concentration at which parathyroid hormone concentration plateaus (Holick et al, 2012). They define vitamin D deficiency as 50 nmol/L and insufficiency between 50 and 75 nmol/L.

Sample collection for 25-hydroxyvitamin D analysis is straight forward. 25-hydroxyvitamin D is stable in both plasma and serum after standing at room temperature for 4 hours and after storage at -80°C and -20°C after four years (Ocke et al, 1995, Colak et al, 2013). It can be analysed from serum or plasma collected with heparin or EDTA as the anti-coagulant (Zhang et al, 2014).

Liquid chromatography coupled to mass spectrometry (LC-MS/MS) has been used to detect vitamin D and its metabolites from the time they were first discovered (Holick et al, 1972). However, due to the cost of the required equipment and the technical skills required to develop LC-MS methods, until recently most laboratories opted to use immunoassays.

Immunoassays reduce the inter-laboratory variability if the same immunoassay kit is used. However, it is the less desirable method of analysis for a number of reasons. 25-
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hydroxyvitamin D₂ does not bind to the immunoglobulin with the same affinity as 25-hydroxyvitamin D₃ so that total 25-hydroxyvitamin D can be under-estimated (Hollis, 2000, Bedner et al, 2013). This is most problematic when vitamin D₂ is used as a supplement. Cross-reactivity with 24,25 dihydroxyvitamin D can result in an over-estimation especially if present in high concentrations (Cashman et al, 2015).

Since the establishment of external quality control collaborations such as the Vitamin D Metabolites Quality Assurance program (DEQAS) and the wider availability of capable equipment, LC-MS is once again the preferred method. It is a more sensitive method (Lorencio et al, 2013). 25-hydroxyvitamin D₂ and D₃ are differentiated due to their differing molecular mass and even the c3-epimer of 25-hydroxyvitamin D₃ can be chromatographically separated (van den Ouweland et al, 2014) although its significance is questionable. DEQAS is discussed in more detail on page 64.

VDBP polymorphisms may be an influencing factor upon vitamin D status. For example, it has been observed that African-Americans have, on average, lower serum 25-hydroxyvitamin D concentrations than White Americans, but higher bone density (Cauley et al, 2005, Schwartz et al, 2014). A recent study in a population cohort from Baltimore, USA, demonstrated that for the same parathyroid hormone concentration, African-Americans had lower total serum 25-hydroxyvitamin D and VDBP, indicating that calcium homeostasis was maintained at lower concentrations. The authors calculated that ‘bioavailable’ unbound 25-hydroxyvitamin D was the same in both black and white populations. The lower VDBP concentrations were associated with a genetic variant in the majority of African-Americans in the study (Powe et al, 2013).
1.3 Section Three: Vitamin D, Glycaemia and Pregnancy

1.3.1 The Relationship between glycaemia and vitamin D; animal studies

Many of the studies investigating the mechanisms by which vitamin D may influence glycaemia were carried out in the 1980s. It was discovered early in that decade that the vitamin D nuclear receptor (VDR) was present in beta cells of the pancreas (Clark et al., 1980). The same authors went on to investigate the action of vitamin D in this tissue. They found when vitamin D deficient rats were supplemented with 1,25-dihydroxyvitamin D₃, fasting insulin levels rose significantly compared to baseline and in comparison to untreated vitamin D deficient rats (Clark et al., 1981).

Severely vitamin D deficient rats have reduced food intake compared to vitamin D replete rats, and it was believed by some that insulin release from the pancreas improved when vitamin D was reintroduced to the diet because of increased food intake. It was shown that vitamin D replete rats pair-fed with vitamin D deficient rats had impaired insulin secretion to the same degree as the vitamin D deficient rats. When vitamin D was reintroduced to the vitamin D deficient rats their food intake increased. When food intake of both groups increased, insulin secretion from the pancreas increased also (Chertow et al., 1983).

Other contemporaneous studies supported the earlier findings of an effect of 1,25 dihydroxyvitamin D on insulin secretion independent of caloric intake. A group in Japan infused vitamin D deficient rats with either 1,25-hydroxyvitamin D₃ or placebo. The rats were then fasted for 20 hours. Fasting insulin concentrations were significantly higher in rats treated with 1,25-dihydroxyvitamin D₃ (2.0 ± 0.2 ng/mL compared to 1.0 ± 0.1 ng/mL) with no opportunity for increased food intake (Ishida et al., 1983). In another study of pair-fed rats the effect of 1,25 dihydroxyvitamin D on insulin secretion was additional to what was found with an increase in caloric intake (Kadowaki and Norman, 1985).

An influx of calcium via voltage gated Ca²⁺ channels is the critical step in insulin release from pancreatic β cells (Rorsman and Renstrom, 2003). It has been hypothesised that vitamin D may be exerting effects on β cells by improving intracellular calcium. However, one study demonstrated that when hypocalcaemic vitamin D deficient rabbits were treated either with vitamin D supplementation or calcium infusions before a glucose challenge test, vitamin D but not calcium improved insulin release (Nyomba et al, 1984). This suggests 1,25 dihydroxyvitamin D was having an effect independent of calcium.
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These early studies have been supported by more recent findings. Vitamin D nuclear receptor knockout mice supplemented with calcium and phosphate had equal food intake to wild-type mice but 60% less insulin release when glucose challenged (Zeitz et al, 2003). The presence of the vitamin D nuclear receptor (VDR) and the vitamin D activating enzyme CYP25B1 have also been confirmed in the human pancreas (Johnson et al, 1994, Bland et al, 2004).

The glycaemic properties of vitamin D are thought not to be limited to the pancreas. Vitamin D response elements have been found in the promoter region of the human insulin receptor gene (Maestro et al, 2003). Human promonocytes have a dose-responsive increase in insulin receptor mRNA, insulin receptor capacity and glucose uptake after incubation with 1,25-dihydroxyvitamin D$_3$ (Maestro et al, 2000). 1,25-dihydroxyvitamin D$_3$ sensitizes rat skeletal muscle to insulin and leucine with demonstrable increased synthesis of the insulin receptor (mRNA and protein) (Salles et al, 2013). Incubation with 1,25-hydroxyvitamin D$_2$ has also been shown to up-regulate plasma membrane glucose transporters (Castro et al, 2013). In contrast, in rat adipocytes it was demonstrated that 1,25-dihydroxyvitamin D$_3$ reduced glucose uptake (Huang et al, 2002).

1.3.2 The Relationship between glycaemia and vitamin D; studies in humans

Given the findings in animal models, investigators sought to determine if there is an association between vitamin D and type 2 DM. Many observational studies support a relationship between low serum 25-hydroxyvitamin D and impaired glucose tolerance.

In a study in Beijing with 518 participants (180 with type 2 DM, 178 with impaired glucose tolerance and 160 participants with normal glucose tolerance), serum 25-hydroxyvitamin D concentrations correlated with fasting insulin, insulin sensitivity index and beta cell function after adjustment for BMI, waist-hip ratio, total cholesterol, triglycerides, LDL and HOMA-IR (Guo et al, 2013). In obese adolescents with fatty liver disease, but not type 2 DM, low serum 25-hydroxyvitamin D concentration was associated with insulin resistance (Pirgon et al, 2013). In a study of 126 white Americans, serum 25-hydroxyvitamin D concentration was strongly correlated with insulin sensitivity index (Chiu et al, 2004). In a study of participants with a BMI > 40 kg/m$^2$, a relationship was demonstrated between 25-hydroxyvitamin D concentration and HbA1c, basal insulin level, and oral glucose challenge stimulated insulin level but not glucose concentration (Bellan et al, 2014). Multivariate regression analysis demonstrated that 25-hydroxyvitamin D concentration was predictive of HbA1c level. In participants of a National Health and Nutrition Examination carried out in the United States,
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25-hydroxyvitamin D concentrations of < 50 nmol/L was associated with an increased risk of type 2 DM in women (OR 1.2 95% CI 1.203 to 1.210) and men (OR 1.736 95% CI 1.731 to 1.740) (Kabadi et al, 2013).

A systematic review and meta-analysis of prospective cohort studies totalling 59,328 participants found that those who had lower serum 25-hydroxyvitamin D concentrations were more likely to develop type 2 DM over time (Forouhi et al, 2012).

Randomised controlled trials of vitamin D supplementation have demonstrated mixed results on glycaemic control. Three studies from Iran found favourable improvements with vitamin D supplementation. In a study of 100 people with type 2 DM, 8 weeks of weekly supplementation with 50,000 IU of vitamin D (by intramuscular injection) resulted in a reduction of fasting plasma glucose, insulin and improved insulin sensitivity (Talaei et al, 2013). In another study in people with type 2 DM, those randomised to vitamin D fortified yogurt for 12 weeks also had improved fasting serum glucose, HbA1c, and HOMA-IR compared to those receiving plain yogurt (Nikooyeh et al, 2011). Finally, 43 obese adolescents were randomised to receive either 300,000 IU of vitamin D₃ weekly or placebo. The intervention group had a significant improvement in insulin resistance and a reduction in triglycerides after 12 weeks of supplementation (Kelishadi et al, 2014). Outside of Iran, in a study from Boston, USA, 16 weeks of supplementation with 2000 IU vitamin D₃ daily or 400 mg of calcium daily, vitamin D supplementation improved pancreatic beta cell function as measured by the disposition index with vitamin D supplementation, but HbA1c was similar between groups. (Mitri et al, 2011).

Other studies have not found a beneficial effect with vitamin D supplementation, despite significant increases in plasma 25-hydroxyvitamin D. A study of 36 people with type 2 DM randomised to receive either 40,000 IU of vitamin D₃ weekly or placebo for 6 months found no improvement in fasting glucose, HOMA-IS, HOMA-IR or HbA1c (Jorde and Figenschau, 2009). The participants of this small study were vitamin D replete at baseline (60 nmol/L ± 14) and may not have benefited from supplementation. However, studies in more deficient populations have also failed to find benefits. A study in Korea, also in people with type 2 DM, restricted participation to those with baseline 25-hydroxyvitamin D concentrations < 50 nmol/L (Ryu et al, 2014). There was no improvement in HbA1c, and HOMA-IR in the intervention group compared to placebo after 24 weeks of supplementation with 2000 IU of vitamin D₃ daily. In another study in 86 adults with type 2 DM, with lower baseline 25-
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hydroxyvitamin D concentrations (median 50 nmol/L), there was no improvement in HbA1c, HOMA-IR and plasma glucose in the group supplemented with 1904 IU of vitamin D daily (Strobel et al., 2014). This was despite an association between baseline 25-hydroxyvitamin D concentrations < 50 nmol/L and a higher HbA1c at the beginning of the study. Another study also found baseline 25-hydroxyvitamin D concentrations were negatively associated with HbA1c well as HOMA-IR (Grimnes et al., 2011). However, after 6 months of supplementation with 20,000 IU of vitamin D twice weekly or placebo there was no difference plasma glucose, HbA1c, and HOMA-IR between groups. This was despite a large difference in serum 25-hydroxyvitamin D concentrations between supplementation and placebo groups (142.7 ± 25.7 compared to 42.9 ± 17.3 nmol/L). In an Asian population living in London, also with pre-diabetes, and with a high prevalence of vitamin D deficiency (95% with concentrations < 25 nmol/L), vitamin D supplementation made no difference to glucose tolerance (Boucher et al., 1995).

One randomised placebo-controlled trial, in 89 obese African-Americans with pre-diabetes or recently diagnosed type 2 DM found insulin sensitivity decreased in the group supplemented with vitamin D and improved in the placebo group (Harris et al., 2012).

A meta-analysis combining four studies investigating vitamin D supplementation in participants with impaired glucose tolerance demonstrated a small but significant reduction in fasting glucose (-0.32mmol/L 95% confidence interval -0.57 to –0.07) and a small improvement in insulin resistance (-0.5 95% CI -0.48 to -0.03) (George et al., 2012). There was no difference in normoglycaemic patients and when all studies were combined (total of 8 studies) and there was no difference in HbA1c between placebo and vitamin D groups.

The RECORD trial is a large randomized controlled trial of vitamin D supplementation in adults over 70 years of age who had previous osteoporotic fractures, to reduce future fractures (The Record Trial Group, 2005). 5,292 participants received either 800 IU of vitamin D3 and 1g of calcium or calcium alone or placebo. In a sub-study, it was found that there was no difference between those supplemented in the development of type 2 diabetes over the period of the study (mean follow-up 2 years) between those supplemented with vitamin D and those not (Avenell et al., 2009).

A systematic review and meta-analysis that included 3 cohort observational studies and 7 randomized controlled trials also investigated whether supplementation was associated with a reduction in incident type 2 DM. When the 3 cohort studies were combined,
supplementation of > 500 IU vitamin D daily was associated with a reduced risk of developing type 2 diabetes compared to supplementation < 200 IU daily (RR 0.87 95% CI 0.76-0.99) (Mitri et al, 2011). However, when the 7 randomised controlled trials were combined there was no improvement in glycaemia and no reduction in incident type 2 diabetes risk over the period of the study (OR 0.68 0.40-1.16).

While the weight of evidence suggests that optimal vitamin D status may improve glycaemic control, controversy remains. These studies have been carried out in a wide range of populations, including negative trials from vitamin D deficient participants. The limitation of all of these studies is their small size, except the RECORD trial, designed to investigate another outcome, and they may be underpowered. It is also possible that vitamin D is having multiple effects that cancel each other out, for example, reducing insulin sensitivity while increasing insulin release, resulting in an overall neutral effect on glycaemia.

1.3.3 The role of vitamin D in pregnancy

A number of changes occur in pregnancy that effect 25-hydroxyvitamin D concentrations. A study of women who underwent serial 25-hydroxyvitamin D, VDBP and albumin analysis during pregnancy at 7 intervals during pregnancy demonstrates these changes (Zhang et al, 2014). Circulating 25-hydroxyvitamin D concentrations fell even during the summer months. Plasma albumin concentrations also fell, reaching a nadir at 36 weeks gestation while VDBP increases to reach a peak at 32 weeks gestation. The result was a net percentage reduction in free 25-hydroxyvitamin D.

The VDR is expressed in the human placenta (Pospechova et al, 2009) and activation of 25-hydroxyvitamin D has been demonstrated in the placenta in vitro (Edgard E et al, 1985). It was demonstrated that calcium uptake increases in syncytiotrophoblasts in a dose dependent manner in response to 1,25-dihydroxyvitamin D₃ (Tuan et al, 1991). Other actions within the placenta are not known. The placenta of VDR -/- mice pups differentially express mRNA of genes related to a variety of functions, including vitamin D deactivation, cytoskeletal modification and cell signalling, in comparison to VDR +/+ placentas (Wilson et al, 2015).

VDR mRNA expression is up-regulated in extravillous trophoblasts and feto-placental endothelial cells from women with GDM compared to normoglycaemic controls (Knabl et al, 2015). In fetal growth restriction, VDR mRNA and protein expression have been shown to be reduced compared to gestational age matched controls (Nguyen et al, 2015). Plasma 25-
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hydroxyvitamin D concentrations were not analysed in the women from either of these studies.

1.4 Summary

Gestational diabetes mellitus is becoming increasingly common and is associated with short and long term consequences for mother and offspring. Preventing GDM may be part of the solution to childhood and adult obesity, an epidemic that has spread across the globe.

Vitamin D supplementation may prevent GDM. There are plausible underlying mechanisms by which vitamin D may improve glycaemia. Vitamin D supplementation is inexpensive and safe in pregnancy therefore there is potential for widespread uptake of supplementation.
Chapter 2  The Relationship between vitamin D and GDM

Two databases were used for searching the literature, Medline and SCOPUS. A Medline search for ‘vitamin D’ AND ‘gestational diabetes mellitus’ returned 91 results on the 5th of January 2014 and 120 results from SCOPUS. Further studies were identified from regular email alerts and later searches in both databases. In the last search on the 11th of November 2016 there were an additional 100 in results Medline and an additional 111 results in SCOPUS from January 2014. Systematic reviews and Google Scholar were used to make sure studies had not been missed in previous searches. From these search results 27 observational studies investigating the relationship between vitamin D and gestational diabetes mellitus were identified. Five intervention trials were identified of vitamin D supplementation in pregnancy to prevent GDM.
2.1 Observational studies

In 2008 the first two observational studies were published investigating the relationship between vitamin D status and GDM (Clifton-Bligh et al., 2008, Maghbooli et al., 2008). In both studies 25-hydroxyvitamin D concentrations were analysed between 24-28 weeks at the time of GDM screening. They differed in the ethnicity of the study population (Iranian and white Australian), with distribution of vitamin D status (70.5% with concentrations < 25 nmol/L compared to 11%) and in GDM diagnostic criteria (Carpenter & Coustan and ADIPS respectively). They also differed in their findings. The study of Iranian women found a difference in mean 25-hydroxyvitamin D concentration amongst those with GDM compared to controls (16.49 ± 10.44 vs 22.97 ± 18.25 nmol/L \( p = 0.009 \)). In multivariate linear regression after adjustment for BMI and parity, 25-hydroxyvitamin D concentration was negatively associated with insulin sensitivity assessed by homeostasis model assessment index (HOMA) \( (p = 0.001) \). The Australian study also found a significant difference in mean 25-hydroxyvitamin D concentration between GDM cases and controls (48.6 ± 24.9 nmol/L vs 55.3 ± 23.3 nmol/L \( p = 0.04 \)). However in multivariate analysis adjusting for age and BMI, vitamin D deficiency (< 25 nmol/L) was not associated with increased odds of subsequent development of GDM (odds ratio 1.92, 95% CI 0.89 to 4.17).

Of 27 observational cohort and case control studies published, 11 found an association between vitamin D status and GDM (Maghbooli et al., 2008, Zhang et al., 2008, Soheilykhah et al., 2010, Parlea et al., 2012, Bener et al., 2013, Cho et al., 2013, Parildar et al., 2013, Tomedi et al., 2013, Lacroix et al., 2014, Zhou et al., 2014, Flood-Nichols et al., 2015). This includes one study in which the prevalence of GDM was higher amongst women with 25-hydroxyvitamin D concentrations ≥ 75 nmol/L analysed at 16 – 20 weeks’ (adjusted OR 1.017 95% CI 1.002 – 1.033) (Zhou et al., 2014). Another four studies found negative associations with other measures of glycaemia and vitamin D such as HOMA index (McLeod et al., 2012, Walsh et al., 2013), HbA1c (El Lithy et al., 2014) and plasma glucose 2 hour post load (Makgoba et al., 2011), but not GDM. 11 studies found no association between vitamin D and GDM or other measures of glycaemia (Clifton-Bligh et al., 2008, Farrant et al., 2009, Savvidou et al., 2011, Baker et al., 2012, , Fernández-Alonso et al., 2012, Zuhur et al., 2013, Kramer et al., 2014, Park et al., 2014, Schneuer et al., 2014, Whitelaw et al., 2014, Rodriguez et al., 2015). While Burris et al did not find associations with GDM they did find associations with glucose concentrations at 1 hour post a 50g glucose load. Mean blood glucose at 1 hour was 114 ± 0.7
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mg/dL. Women with 25-hydroxyvitamin D concentrations of <25 compared to >25 nmol/L had higher glucose concentrations (8.1 mg/dL 95% CI 1.3-14.9 mg/dL) (Burris et al, 2012).

These studies have analysed 25-hydroxyvitamin D concentrations at various stages in pregnancy from 5 weeks gestation to delivery and used a variety of diagnostic criteria for GDM. A comparison of the main features of the studies is shown in Table 2.1. A table of the characteristic of individual studies can be found in Appendix I.

It can be seen in Table 1. that more studies finding a relationship between GDM and vitamin D status come from Middle Eastern countries (Iran, Turkey and Qatar) and North America, while no studies from Europe (United Kingdom, Ireland, Spain) or Australia found a relationship.

The prevalence of vitamin D deficiency (defined as < 25nmol/L) varies widely between study populations from 70.6% in an Iranian cohort (Maghbooli et al, 2008) to less than 5% in a mostly white population from Boston, USA (Burris et al, 2012). The vitamin D status of the study population and the gestation when 25-hydroxyvitamin D concentrations were analysed do not appear to be different between the studies that found an association between vitamin D status and GDM and those that did not.

The prevalence of GDM also varies widely, from 30% in populations selected for their high risk of GDM (Clifton-Bligh et al, 2008, Kramer et al, 2014) to 2% in a general obstetric population (Flood-Nichols et al, 2015). Neither of the studies from the high-risk populations found a relationship between vitamin D status and GDM, and the overall prevalence of GDM is not higher in the studies that did find a relationship with vitamin D status, than those that did not.
### Table 2.1: Comparison of 27 cohort and case-control studies investigating the relationship between vitamin D and GDM

<table>
<thead>
<tr>
<th>Study design</th>
<th>Association with GDM</th>
<th>No association with GDM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td><strong>Cohort</strong></td>
<td>6 (55)</td>
<td>11 (69)</td>
</tr>
<tr>
<td><strong>Case-control</strong></td>
<td>5 (45)</td>
<td>5 (31)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of participants</th>
<th>Association with GDM</th>
<th>No association with GDM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>&lt; 250</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>250 to &lt; 1000</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>&gt; 1000</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Geographical location</th>
<th>Association with GDM</th>
<th>No association with GDM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Middle East/North Africa</td>
<td>4 (36)</td>
<td>2 (12)</td>
</tr>
<tr>
<td>North America</td>
<td>5 (45)</td>
<td>3 (19)</td>
</tr>
<tr>
<td>Asia</td>
<td>2 (18)</td>
<td>2 (12)</td>
</tr>
<tr>
<td>Europe</td>
<td>0</td>
<td>6 (38)</td>
</tr>
<tr>
<td>Australia</td>
<td>0</td>
<td>3 (19)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Prevalence of GDM</th>
<th>Association with GDM</th>
<th>No association with GDM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>&lt; 5%</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5 – 10%</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>&gt; 10%</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Population vitamin D status¹</th>
<th>Association with GDM</th>
<th>No association with GDM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Low</td>
<td>2 (18)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Moderate</td>
<td>3 (27)</td>
<td>10 (63)</td>
</tr>
<tr>
<td>High</td>
<td>6 (55)</td>
<td>5 (31)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gestation vitamin D status assessed²</th>
<th>Association with GDM</th>
<th>No association with GDM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Before 14 weeks</td>
<td>2 (18)</td>
<td>7 (39)</td>
</tr>
<tr>
<td>14 to &lt; 22 weeks</td>
<td>4 (36)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>22 to 30 weeks</td>
<td>3 (27)</td>
<td>8 (44)</td>
</tr>
<tr>
<td>&gt; 30 weeks</td>
<td>2 (18)</td>
<td>2 (11)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Included in meta-analyses</th>
<th>Association with GDM</th>
<th>No association with GDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poel et al 2012</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Aghajafari et al 2013</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Wei et al 2013</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

¹ Low – population mean or median <25 nmol/L, Moderate – population mean or median 25 to < 50 nmol/L, High – population mean or median > 50 nmol/L
² Studies collecting samples at multiple time points considered an individual study for each time point.
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There have been three systematic reviews published on this literature (Poel et al, 2012, Aghajafari et al, 2013, Wei et al, 2013). The selection criteria between the studies were similar, and the difference in the studies included is due to the year in which the reviews were published. On meta-analyses, all of these studies found low maternal vitamin D status in pregnancy is associated with an increased likelihood of developing GDM. The most recent reported an increased likelihood of developing GDM with 25-hydroxyvitamin D concentrations < 50 nmol/L combined odds ratio of 1.38 (95% CI 1.12 to 1.70) (Wei et al, 2013). The main limitation of this review is that, due to differences and limitations in the included studies, the review authors were not able to consistently adjust for likely confounders. Most included studies have made an adjustment at minimum for BMI. This is the difference between a significant or non-significant result in many cases. For example, in one included study, a 25-hydroxyvitamin D concentration < 25 nmol/L was associated with an increased likelihood of developing GDM (odds ratio 3.6, 95% CI 1.7-7.8) but after adjustment for BMI this was no longer significant (odds ratio 2.2, 95% CI 0.9 – 5.7) (Burris et al, 2012).

The other two meta-analyses include adjusted analysis that also show an increased likelihood of developing GDM with serum or plasma 25-hydroxyvitamin D concentrations of < 25 nmol/L (odds ratio 1.57, 95% CI 1.11 – 2.22 (Poel et al, 2012) and odds ratio 1.98, 95% CI 1.23 – 3.23 (Aghajafari et al, 2013)). While an adjusted pooled odds ratio was calculated, some studies that did make adjustments for BMI and ethnicity were not included in this analysis (Maghbooli et al, 2008, Makgoba et al, 2011). The reason for this is not clear.

An additional 16 observational studies have been published following the meta-analyses above, which were published in 2012 to 2013. In all there are now 14 studies that report 25-hydroxyvitamin D means and standard deviations in women with and without GDM. Figure 3.2 shows a meta-analysis of these 14 studies, using a random effects model due to high heterogeneity (I\(^2\) = 81%) (Review Manager (RevMan) Version 5.3, 2014). The overall mean 25-hydroxyvitamin D concentration was lower by a small amount in women who developed GDM (mean difference -4.37, 95% CI -7.59 to – 1.15).
### Literature Review

**Figure 2.1 Meta-analysis of the association between maternal serum 25-hydroxyvitamin D concentration and GDM.**

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>GDM Cases</th>
<th>Control</th>
<th>Mean Difference</th>
<th>Mean Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Total</td>
<td>Weight</td>
</tr>
<tr>
<td>1.1.1 Before 22 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zhang 2008</td>
<td>60.5</td>
<td>21.3</td>
<td>57</td>
<td>75.3</td>
</tr>
<tr>
<td>Makkoba 2011</td>
<td>47.2</td>
<td>26.7</td>
<td>90</td>
<td>47.6</td>
</tr>
<tr>
<td>Baker 2012</td>
<td>97</td>
<td>29</td>
<td>60</td>
<td>86</td>
</tr>
<tr>
<td>Parlee 2012</td>
<td>56.3</td>
<td>19.4</td>
<td>116</td>
<td>62</td>
</tr>
<tr>
<td>Tomedi 2013</td>
<td>39.1</td>
<td>17.9</td>
<td>47</td>
<td>42.9</td>
</tr>
<tr>
<td>Zhou 2014</td>
<td>67.8</td>
<td>19.8</td>
<td>331</td>
<td>87.6</td>
</tr>
<tr>
<td>Latron 2014</td>
<td>57.5</td>
<td>17.2</td>
<td>53</td>
<td>63.5</td>
</tr>
<tr>
<td>Park (first trimester) 2014</td>
<td>35.3</td>
<td>16.5</td>
<td>23</td>
<td>32</td>
</tr>
<tr>
<td>Subtotal (95% CI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterogeneity: Tau² = 24.99; Chi² = 33.16, df = 7 (P &lt; 0.0001); I² = 79%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for overall effect: Z = 1.12 (P = 0.26)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.1.2 22 to 28 weeks

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>GDM Cases</th>
<th>Control</th>
<th>Mean Difference</th>
<th>Mean Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Total</td>
<td>Weight</td>
</tr>
<tr>
<td>Clifton-Bilgh 2008</td>
<td>48.6</td>
<td>24.9</td>
<td>78</td>
<td>55.3</td>
</tr>
<tr>
<td>Maghbochi 2008</td>
<td>16.5</td>
<td>10.4</td>
<td>52</td>
<td>23</td>
</tr>
<tr>
<td>McLeod 2012</td>
<td>132</td>
<td>59.9</td>
<td>46</td>
<td>183</td>
</tr>
<tr>
<td>Parlar 2013</td>
<td>48.8</td>
<td>23.3</td>
<td>44</td>
<td>57.3</td>
</tr>
<tr>
<td>Zhu 2013</td>
<td>138</td>
<td>16.3</td>
<td>168</td>
<td>38</td>
</tr>
<tr>
<td>Park (second trimester) 2014</td>
<td>46.7</td>
<td>18.4</td>
<td>23</td>
<td>46.5</td>
</tr>
<tr>
<td>Subtotal (95% CI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterogeneity: Tau² = 0.00; Chi² = 3.56, df = 5 (P = 0.61); I² = 0%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for overall effect: Z = 5.52 (P &lt; 0.00001)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.1.3 Later than 30 weeks

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>GDM Cases</th>
<th>Control</th>
<th>Mean Difference</th>
<th>Mean Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Total</td>
<td>Weight</td>
</tr>
<tr>
<td>Cho 2013</td>
<td>29.1</td>
<td>22.8</td>
<td>20</td>
<td>86.2</td>
</tr>
<tr>
<td>Park (third trimester) 2014</td>
<td>49.4</td>
<td>19.4</td>
<td>23</td>
<td>47</td>
</tr>
<tr>
<td>Subtotal (95% CI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterogeneity: Tau² = 1660.41; Chi² = 33.74, df = 1 (P &lt; 0.00001); I² = 97%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for overall effect: Z = 0.93 (P = 0.35)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total (95% CI) 1233 6217 100.0% 4.37 [-7.59, -1.15]
2.2 Randomised controlled trials

Five studies investigating whether vitamin D supplementation in pregnancy prevents gestational diabetes mellitus have been identified. A summary of these studies is given in Table 2.2. The largest of these studies, with 250 participants receiving 50,000 IU of vitamin D fortnightly from 12 weeks gestation and 250 participants receiving 400 IU daily found a reduction in the prevalence of GDM in the higher dose group compared to the low dose group (6.7% vs 13.4% \( p = 0.01 \)) (Mojibian et al, 2015). The other four trials found no reduction in GDM with vitamin D supplementation.

Only one of these studies compared vitamin D supplementation to placebo (Sablok et al, 2015). Participants in the intervention group were stratified according to their baseline 25-hydroxyvitamin D concentration as sufficient (>50nmol), insufficient (25 – 50 nmol/L) or deficient (< 25 nmol/L). The intervention differed depending on their vitamin D status. ‘Sufficient’ women were given 60,000 IU of vitamin D in two doses, at 20 weeks and 24 weeks, ‘insufficient’ women were given 120,000 IU at 20 weeks and 24 weeks, and ‘deficient’ women were given 120,000 IU at 20, 24, 28 and 32 weeks (only 2 doses prior to GDM diagnosis). The different intervention groups were analysed as one and compared to placebo. The prevalence of GDM was 0.9% in the intervention group, and 1.8% in the placebo group (\( p = 0.9 \)). The criterion used to diagnose GDM was not given, and it is not clear whether all women were screened. This may contribute to the low prevalence of GDM in this study. As discussed in Chapter 1, a significant proportion of women with GDM may not be diagnosed on the basis of high risk screening alone. This study was carried out in India where the prevalence of GDM in many studies is above 15% (Kragelund Nielsen et al, 2016, Mohan and Chandrakumar, 2016).
### Table 2.2 Randomised controlled trials of vitamin D supplementation to prevent GDM

<table>
<thead>
<tr>
<th>Country</th>
<th>Wagner</th>
<th>Hobbsain</th>
<th>Yap</th>
<th>Sabloki</th>
<th>Mojibiani</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intervention</strong></td>
<td>USA</td>
<td>Pakistan</td>
<td>Sydney</td>
<td>India</td>
<td>Iran</td>
</tr>
<tr>
<td><strong>Dose n</strong></td>
<td>400 IU daily 110</td>
<td>2000 IU daily 201</td>
<td>4000 IU daily 193</td>
<td>Calcium only 89</td>
<td>400 IU daily 90</td>
</tr>
<tr>
<td><strong>Intervention started</strong></td>
<td>12–16 weeks</td>
<td>18–20 weeks</td>
<td>&lt;20 weeks</td>
<td>20 weeks</td>
<td>12–16 weeks</td>
</tr>
<tr>
<td><strong>GDM diagnostic criteria</strong></td>
<td>Not given</td>
<td>O’Sullivan</td>
<td>ADIPS</td>
<td>Not given</td>
<td>ADA</td>
</tr>
<tr>
<td><strong>GDM prevalence (%)</strong></td>
<td>400 IU daily 7.3</td>
<td>2000 IU daily 8.0</td>
<td>4000 IU daily 5.2</td>
<td>Calcium only 6.45</td>
<td>400 IU daily 10.52</td>
</tr>
<tr>
<td><strong>Outcome with supplementation</strong></td>
<td>No reduction in GDM</td>
<td>No reduction in GDM</td>
<td>No reduction in GDM</td>
<td>No Reduction in GDM</td>
<td>Reduction in GDM</td>
</tr>
</tbody>
</table>

1 Refer to Table 1.3 for the diagnostic criteria
2 0.9% prevalence of GDM in the intervention groups combined

Abbreviations – ADIPS – Australasian diabetes in pregnancy in society, ADA – American Diabetes Association
Two studies compared low dose vitamin D supplementation (400 IU daily) to high dose supplementation, one in the USA the other in Australia (Wagner et al, 2013, Yap et al, 2014). In the Australian study, the intervention group were given 5000 IU units daily starting prior to 20 weeks gestation. The prevalence of GDM was not different between the high dose supplementation group and the low dose group (8% vs 13%). However, the authors did find an inverse relationship between baseline 25-hydroxyvitamin D concentration at less than 20 weeks gestation and both fasting plasma glucose and 2 hours post glucose load at between 26 – 28 weeks gestation (both \( p < 0.001 \)). The diagnostic criterion for GDM was not given in the study from the US population.

The remaining study compared calcium supplementation alone to calcium supplementation with 4000 IU of vitamin D daily from between 18 to 20 weeks gestation to delivery (Hossain et al, 2014). 100 women from a single centre in Pakistan were randomised to each group. The baseline vitamin D status was low in both groups (median 11.9 nmol/L in the vitamin D and calcium group, 13.3 nmol/L in the calcium alone group) and rose significantly with vitamin D supplementation. The prevalence of GDM was similar between groups, 6.5% in the calcium only group and 10.5% in the vitamin D and calcium group (\( p = 0.4 \)).

The study finding a reduction in the prevalence of GDM with vitamin D supplementation was not only the largest, with 500 participants, it was different from the other studies in a number of ways (Mojibian et al, 2015). All participants underwent a fasting OGTT, the intervention started at an earlier gestation, (12 to 16 weeks’ compared to 18 to 20 weeks’) and it is also the only study on a Middle Eastern population.

The study size is very likely to be important. Using the Australian study as an example, taking the low dose group as the background population prevalence of GDM (13%) a study size of 1178 would be required to find the 8% prevalence in the high dose group a significant reduction (80% power and significance of \( p = 0.05 \)). That is almost 10 times the size of the study (159 participants).

It may also be important that the study was carried out in Iran. Many of the observational studies finding a significant relationship between vitamin D status and GDM have been carried out in the Middle Eastern nations.
Table 2.1). Other randomised studies from Iran have shown a favourable effect of vitamin D supplementation on glycaemia in pregnancy. A study comparing 200 IU of vitamin D daily to 50,000 IU monthly or fortnightly from less than 12 weeks gestation found a reduction in plasma insulin and reduction in Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) in the high dose groups (Soheilykhah et al, 2013). Another found a reduction in high sensitivity C-reactive protein and triglycerides with 400 IU of vitamin D daily compared to placebo (Asemi et al, 2013). GDM was not reported as an outcome in either of these studies.

Other studies from Iran have found favourable outcomes in relation to vitamin D supplementation in women supplemented after they are diagnosed with GDM. Three randomised controlled trials from the same investigators found improvement in a variety of outcomes (Asemi et al, 2013, Asemi et al, 2014, Asemi et al, 2015). Supplementation started at the time of GDM diagnosis (placebo vs two doses of 50,000 IU of vitamin D with or without calcium depending on the study) and outcomes were assessed at delivery. Improved outcomes with supplementation included reductions in fasting plasma glucose, insulin, HOMA-IR, low density lipoprotein, the incidence of polyhydramnios, and new born hospitalisation and an increase in high density lipoprotein and Quantitative Insulin Sensivity Check Index (QUICKI).

A possible explanation for the number of positive studies coming from this region is due to the high prevalence of vitamin D deficiency amongst pregnant women in that region (Maghbooli et al, 2007). This may allow the effects of vitamin D to be more obvious with supplementation. However, the two other trials from regions with a high prevalence of vitamin D deficiency, Pakistan (Hossain et al, 2014) and India (Sablok et al, 2015) did not find a reduction in GDM prevalence.

Another possible explanation is that genetic factors that determine a favourable response to vitamin D supplementation maybe more common in that region. Variants of VDBP have been shown to influence the degree of response to vitamin D supplementation as determined by a rise in 25-hydroxyvitamin D concentration (Fu et al, 2009, Nimithpong et al, 2013). Variants in the vitamin D nuclear receptor have also been found to influence the rise in 25-hydroxyvitamin D concentration with supplementation (Elizabeth et al, 2014). Whether these variations results in an improved response to supplementation is not known.
2.3 Summary

Cohort and case control studies investigating the relationship between developing GDM and low vitamin D status have produced conflicting results, some finding a strong association, while others have found none. There have been only five randomised controlled trials of vitamin D supplementation in pregnancy to prevent GDM. These studies are small, and of varying study design and quality. Information so far is not conclusive either for or against vitamin D supplementation for the prevention of GDM.

GDM confers metabolic risk to mother and offspring. If vitamin D supplementation prevents GDM there are potential lifelong benefits to both. Furthermore, vitamin D supplementation is safe and inexpensive. Therefore the question of whether vitamin D prevents GDM deserves serious consideration.
2.4 Thesis Objectives

The aim of this thesis is to investigate whether vitamin D supplementation in pregnancy improves glycaemia in pregnancy thereby preventing the development of GDM and improving long term metabolic outcomes for their children.

Specific Aims

To establish a liquid chromatography mass spectrometry method for the analysis of 25-hydroxyvitamin D$_3$ and 25-hydroxyvitamin D$_2$.

In Chapter 1 section 2.5 it was shown that LC-MS/MS is the preferred method for analysing 25-hydroxyvitamin D. Chapter 3 provides the details of the method developed.

To investigate the relationship between GDM and maternal vitamin D status in a prospective cohort study.

Chapter 2 section 1 described studies investigating the relationship between vitamin D and GDM and discussed the limitations of these studies. Chapter 4 describes a prospective study from a well phenotyped pregnancy cohort.

To investigate the relationship between maternal vitamin D status in early pregnancy and adiposity in childhood.

In Chapter 1 section 1.1 it was shown that GDM is associated with an increased likelihood of obesity and type 2 DM in offspring. It is not known whether low maternal vitamin D status is associated with an increased likelihood of obesity and type 2 DM. Chapter 5 describes a study investigating the relationship between maternal vitamin D status in pregnancy and measures of adiposity in children.
Literature Review

To investigate the relationship between maternal vitamin D status in early pregnancy and outcomes in mothers 6 years post-partum including developing metabolic syndrome and anthropomorphic measures.

In Chapter 1 section 1.1 it was shown that GDM has been associated with metabolic dysfunction in the long-term. It is not known whether vitamin D status in pregnancy is associated with an increased. Chapter 6 describes a study investigating the relationship between maternal vitamin D status in pregnancy and long-term metabolic outcomes.

To investigate whether vitamin D supplementation from early pregnancy improves glycaemia and alters the plasma metabolome after 19 weeks of supplementation.

Chapter 2 section 2 described the limitations of randomised controlled trials investigating whether vitamin D supplementation in pregnancy prevented GDM. Chapter 7 describes a study investigating glycaemia and plasma metabolome of participants in a large randomised placebo controlled trial of vitamin D supplementation in pregnancy.
Chapter 3  Methods

3.1  Introduction

This section outlines in greater detail than provided in the manuscripts the methods used to investigate the aims of this thesis.

Samples from two studies were used: Screening for Pregnancy Endpoints (SCOPE) study and the Maternal Vitamin D Osteoporosis (MAVIDOS) study. Two major laboratory methodologies were employed in carrying out this work both using mass spectrometry technology.

3.2  Screening for Pregnancy Endpoints (SCOPE) STUDY

3.2.1  Participants

The Screening for Pregnancy Endpoints (SCOPE) study is a multinational collaboration established to investigate early pregnancy predictors of late pregnancy outcomes. There were 5,690 women participants from 6 centres across 4 countries. The aims can be found at www.scopestudy.net and are as follows:

1. Identify novel molecular markers that predict in early pregnancy women who will subsequently develop late pregnancy complications
2. Test and validate combinations of key clinical, known and novel molecular markers to predict each disease
3. Develop predictive tests that offer first time mothers an accurate, personalised risk rating for each disease

Auckland was the first site to begin recruitment in 2004 and completed recruitment in 2008. Participation in the study included an interview, anthropomorphic measurements and sample collection at 14-16 weeks gestation, and again at 19-21 weeks gestation with an ultrasound at 23-25 weeks to measure foetal growth and placental blood flow. Women were visited by study nurses as soon as possible at home or in hospital if any complications developed during pregnancy and all women were visited at home soon after delivery. Data collected were entered directly at the time of interview onto a secure internet accessed central database with a complete audit trail shared by all study sites (MedSciNet AB Stockholm, Sweden). Further
Methods

Information on the data collected is included in chapter 4 and a flow diagram of participation is shown in Figure 4.1 Cohort flow diagram of Auckland SCOPE participants from recruitment at 15 weeks gestation.

Samples used were serum that had been collected at 15 weeks gestation (2004–2008). Serum samples were collected in plain 6mL glass tubes (with no anticoagulant or other additives) and were kept on ice for 30 minutes to allow clotting. Samples were centrifuged at 4°C at 2400 g for 10 minutes (Eppendorf 5810R centrifuge Hamburg, Germany). Serum was transferred to a second spin tube and centrifuged again at 3200 g for 10 minutes at 4°C. Serum was then pipetted into cyrotubes in 250 μL aliquots. Each sample was given a unique identifier number and barcode. Sample identification and location were scanned into the online central database.

Children of SCOPE

Women who had consented to be followed up were contacted between 5 and 6 years after delivery and were invited to participate in the Children of SCOPE follow-up study. The study was approved by The Health and Disability Commission, Northern X Regional Ethics Committee (NTX/10/10/106). Children of SCOPE seeks to identify early life modifiable determinates of obesity and insulin resistance. Children were excluded if they had a major chromosomal abnormality or a major congenital abnormality that would interfere with growth. Further information on the data collected is included in Chapter 5.

Maternal SCOPE follow-up study

Women were also invited to participate in follow-up. The aim of the maternal follow-up study was to investigate predictors of long-term metabolic health from pregnancy. Anthropomorphic measures were taken as well as blood samples that were stored for later analysis. Fasting blood samples were collected when possible.

3.2.2 25-hydroxyvitamin D liquid chromatography mass spectrometry analytical method

As noted in Chapter 1 (Section 1.2.5), liquid chromatography system coupled to a tandem mass spectrometer (LC-MS/MS) is the preferred method for the analysis of 25-hydroxyvitamin D₃ and 25-hydroxyvitamin D₂. The method described here was developed by the author and Mr Eric Thorstensen for the analysis of the SCOPE serum samples.
Methods

LC-MS grade methanol and acetonitrile were supplied by Merck (Kenilworth, NJ, USA). 25-hydroxyvitamin D standards and d6 25-hydroxyvitamin D₃ internal standard were supplied by Isosciences (Prussia, PA, USA)
Methods

Standard curve

The standard curve was made by diluting concentrated 25-hydroxyvitamin D stock solutions. First dilutions were made in methanol. To reflect expected serum concentrations, the standards contained a higher concentration of 25-hydroxyvitamin D₃ than 25-hydroxyvitamin D₂ and epi-25-hydroxyvitamin D₃. 80 μL of 25-hydroxyvitamin D₃ (99.22 μg/mL), 40 μL 25-hydroxyvitamin D₂ (100.2 μg/mL) and 16 μL of epi-25-hydroxyvitamin D₃ (101.1 μg/mL) were added to 864 μL of methanol to make 1000 μL of concentrated standard H. Concentrated standard H was diluted by degrees to make concentrated standards G to A. The dilution to make each of these concentrated standards is shown in Table 3.1

<table>
<thead>
<tr>
<th>Concentrated Standard</th>
<th>25-(OH) vitamin D₃ μmol/L</th>
<th>25-(OH) vitamin D₂ μmol/L</th>
<th>epi-25-(OH) vitamin D₃ μmol/L</th>
<th>Volume of previous standard</th>
<th>Volume of methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.1</td>
<td>0.05</td>
<td>0.02</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>B</td>
<td>0.2</td>
<td>0.1</td>
<td>0.04</td>
<td>400</td>
<td>600</td>
</tr>
<tr>
<td>C</td>
<td>0.5</td>
<td>0.25</td>
<td>0.1</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>0.5</td>
<td>0.2</td>
<td>400</td>
<td>600</td>
</tr>
<tr>
<td>E</td>
<td>2.5</td>
<td>1.25</td>
<td>0.5</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>F</td>
<td>5</td>
<td>2.5</td>
<td>1</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>G</td>
<td>10</td>
<td>10</td>
<td>2</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>H</td>
<td>20</td>
<td>10</td>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Concentrated 25-hydroxyvitamin D was found to be unstable in methanol at -20°C. Therefore final concentrations were made in one batch by diluting the concentrated standards 100 times in 4% bovine serum albumin (BSA) (MP Biomedicals, Auckland, New Zealand) in phosphate buffered saline. Once diluted to their final concentration, standards were stored in 150 μL aliquots at -80°C. Final concentrations of the standard curve solutions are given in Table 3.2. An example of the standard curves generated by the assay is given in Figure 3.1.

The concentration of 25-hydroxyvitamin D in the standards was confirmed by spectrophotometry. The concentration was calculated using Beer’s law

\[ c = \frac{A}{\varepsilon b} \]
where \( c \) is concentration, \( A \) is absorbance, \( \epsilon \) is molar absorptivity, \( b \) is path length. The molar absorptivity for both 25-hydroxyvitamin D₃ and 25-hydroxyvitamin D₂ is 18,300. A wavelength of 264 nm was used.

BSA was chosen to make up the standard because it provided a complex matrix more similar to plasma than an organic solvent alone such as methanol. In addition, BSA did not contain any detectable endogenous 25-hydroxyvitamin D, whereas both plasma from sheep and stripped human plasma contained endogenous 25-hydroxyvitamin D.

**Table 3.2 Final concentration of the 25-hydroxyvitamin D standard curve for LC-MS/MS method**

<table>
<thead>
<tr>
<th>Standard</th>
<th>25-hydroxyvitamin D₃ nmol/L</th>
<th>25-hydroxyvitamin D₂ nmol/L</th>
<th>epi-25-hydroxyvitamin D₃ nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>D</td>
<td>10</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>E</td>
<td>25</td>
<td>12.5</td>
<td>5</td>
</tr>
<tr>
<td>F</td>
<td>50</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>G</td>
<td>100</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>H</td>
<td>200</td>
<td>100</td>
<td>40</td>
</tr>
</tbody>
</table>
Methods

Figure 3.1 Standard curves generated by the 25-hydroxyvitamin D LC-MS/MS assay

Area ratio – the ratio of analyte to the internal standard
Methods

Sample extraction

The following sample extraction method was adapted from the manufacturer’s instructions for use of Phree™ Phospholipid Removal Plates (Phemenex, Torrance, CA, USA). All liquid addition steps utilised the EpMotion 5070 pipetting robot (Eppendorf). Differences from the manufacturer’s method include the addition of internal standard to the extraction solvent and use of the centrifuge to draw the extraction solvent and sample solution through the solid phase rather than applying a vacuum.

50 μL of internal standard (deuterated 25-hydroxyvitamin D₃ d₆) was added to 250 mL of extraction solvent acetonitrile:methanol 85:15.

Samples were analysed in duplicate, therefore when space was made for blanks, the standard curve and quality controls, 68 samples (from 34 individuals) were able to be extracted in each 96 well plate.

1. 300 μL of 85:15 acetonitrile:methanol with internal standard was added to the wells of the Phree phospholipid removal plate
2. 80 μL of standard curve or sample were added to each well
3. The plate was shaken for 2 minutes, then centrifuged for 5 minutes at 1000g (Thermo Scientific, Asville, NC, USA)
4. A further 200 μL of 85:15 acetonitrile:methanol with internal standard was added to each sample in the plate. The plate was shaken again for 2 minutes, then centrifuged for 5 minutes at 1000g as above.
5. The plate was dried for 12 hours in a Savant SC250EXP speed-vac concentrator with a Savant RVT4104 Refrigerated Vapor Trap (Thermo Scientific, Ashville, NC, USA).
6. To prepare for injection the dried residues were resuspended in 100 μL of 70:30 methanol:water, shaken for 5 minutes and centrifuged for 1 minute at 500g.
**Methods**

*Liquid chromatography and mass spectrometry conditions*

The LC-MS/MS conditions are given in Table 3.3. The high performance liquid chromatography (HPLC) column used for the sample analysis was a Kinetex pentafluorophenyl 100 x 3.0 mm 2.6μm column (Phenomenex). Epi-25-hydroxyvitamin D₃ is a stereoisomer of 25-hydroxyvitamin D₃. This means they have the same mass to charge ratio. A number of columns were trialled before separation of epi-25-hydroxyvitamin D₃ from 25-hydroxyvitamin D₃ was achieved. These included Kinetex 100 x 3.0 mm 2.6μm C18 (Phenomenex), Luna C18 (Phenomenex), Synergi hydro (Phenomenex and Hypersil Gold 100 x 2.1mm 1.9 μm (Thermo Fisher).

<table>
<thead>
<tr>
<th><strong>Table 3.3 LC-MS/MS conditions</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Column Dimensions</td>
</tr>
<tr>
<td>Mobile Phase</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Column Temperature</td>
</tr>
<tr>
<td>Flow rate</td>
</tr>
<tr>
<td>Gradient</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Injection volume</td>
</tr>
<tr>
<td>Detection</td>
</tr>
<tr>
<td>Compound</td>
</tr>
<tr>
<td>25-hydroxyvitamin D₃</td>
</tr>
<tr>
<td>epi-25-hydroxyvitamin D₃</td>
</tr>
<tr>
<td>25-hydroxyvitamin D₂</td>
</tr>
<tr>
<td>25-hydroxyvitamin D₃ d₃ (Internal standard)</td>
</tr>
</tbody>
</table>

Quality control was ensured through commercial quality controls (Quantimetrrix, Redondo Beach, CA, USA) and through participation in the Vitamin D external Quality Assessment Scheme (DEQAS) based at Charing Cross Hospital, London, UK (see appendix at the end of this chapter). The intra-assay coefficient of variation was 7.3% for 25-hydroxyvitamin D₃ and
Methods

8.2% for 25-hydroxyvitamin D\textsubscript{2}. The inter-assay coefficient of variation was 4.9% for 25-hydroxyvitamin D\textsubscript{3} and 5.8% for 25-hydroxyvitamin D\textsubscript{2}.

3.2.3 Vitamin D binding protein immunoassay and albumin analysis

Vitamin D binding protein (VDBP) and albumin were analysed in a subgroup of participants (32 GDM cases and 128 matched controls) so that free 25-hydroxyvitamin D could be calculated. This was to investigate whether there was an association between free 25-hydroxyvitamin D at 15 weeks gestation and the development of GDM and whether this was a better predictor of GDM than total 25-hydroxyvitamin D.

An ELISA kit from R&D Systems, Quantikine ® (Minneapolis MN, USA) was used to analyse VDBP. All reagents were provided in the kit and accompanying instructions were followed unchanged. The following is taken directly from the manufacturer’s instructions. From our own assays, the coefficient of variation was 3.4% for the low concentration and 3.1% for the high concentration.

7. Samples were diluted 2000 fold in Calibrator Diluent RD6-11.
8. The VDBP standard was reconstituted in 1.0 mL millipore water to make a concentration of 1000 ng/mL. 250 μL of diluted standard was added to 750 μL of Calibrator Diluent RD6-11 to make the high concentration standard, 250 ng/mL. This was diluted 4 more times to make 5 standard concentrations.
9. 100 μL of Assay Diluent RD1-19 was added to each well of a 96 well plate coated with mouse monoclonal antibody against VDBP
10. 50 μL of standard, control or sample to each well. The plate was covered with the adhesive strip provided and incubated for 1 hour at room temperature on a microplate shaker set at 500 rpm
11. Each well was aspirated and washed four times with the Wash Buffer. Remaining Wash Buffer was removed by aspiration. The paper was inverted and blotted against clean paper towel.
12. 200 μL of Vitamin D BP Conjugate was added to each well. The plate was covered with a new adhesive strip and incubated for 2 hours at room temperature on the shaker.
13. The plate was again aspirated and washed 4 times.
14. 200 μL of Substrate Solution was added to each well. The plate was covered by aluminium foil to protect it from light and it was incubated at room temperature
Methods

15. 50 μL of Stop Solution was added to each well. The plate was inserted into a microreader set at 450 nm. Sample concentrations were derived from the standard curve after the blank standard optimal density was subtracted.

Albumin was analysed on a Hitachi 902 clinical chemistry analyser (Hitachi High-Technologies Corporation) using a BCG analytical kit purchased from Roche (Mannheim, Germany). The coefficient of variation was 2.6% for the low concentration and 2.0% for the high concentration.

3.2.4 Calcium and parathyroid hormone

Calcium and parathyroid hormone were analysed in 15 week serum samples in a subgroup of participants to confirm an expected relationship with 25-hydroxyvitamin D analysed by the method described. Parathyroid hormone was analysed by Elecsys 2010 (Hitachi High-Technologies Corporation, Tokyo, Japan) using a COBAS parathyroid hormone analytical kit (Roche). The coefficient of variation was 1.2% at the low quality control concentration and 1.2% for the high quality control concentration. Calcium was analysed on a Hitashi 902 chemical analyser using a COBAS CA2 calcium Gen.2 analytical kit (Roche). The coefficient of variation was 1.3% for the low quality control concentration and 0.5% for the high quality control concentration.

![Figure 3.2](image_url)

Figure 3.2: The relationship between parathyroid hormone and total 25-hydroxyvitamin D, and between calcium and total 25-hydroxyvitamin D

As expected, 25-hydroxyvitamin D was negatively correlated with parathyroid hormone (-0.4 $p = <0.001$) and positively correlated with calcium (0.2 $p = 0.003$) (Figure 3.2).
Methods

3.2.5 Statistical analysis

All statistical analysis was performed in SPSS statistics (IBM) version 21. Normality of continuous data were assessed by visualising the histogram and using tests for skewness and kyphosis. Specific tests for outcomes of interest are given in Chapters 4, 5 and 6.

The 25-hydroxyvitamin D concentration of an individual varies significantly with season, in a sinusoidal pattern from a peak at the end of summer to a nadir at the beginning of spring. Seasonal adjustments were made in two ways. Firstly by including a season as a categorical variable in multivariate analysis (summer, December to February; autumn, March to May; winter, June to August; and spring, September to November). This allows an investigation into effect of season including, but not be limited to, variation in vitamin D status.

However, the sinusoidal increase and decrease of 25-hydroxyvitamin D concentration throughout the year does not exactly line up with the “official” change of the seasons. For this reason 25-hydroxyvitamin D concentrations were also converted to z-scores calculated for each month. This calculation is independent of other seasonal effects. A further option would be to map each vitamin D concentrations to monthly percentiles of an external reference dataset, however no such local data were available.
Methods

3.3 The Maternal Vitamin D Osteoporosis (MAVIDOS) study

3.3.1 Participants

The Maternal Vitamin D Osteoporosis (MAVIDOS) study is a multicentre, double blind placebo-controlled trial of vitamin D supplementation in pregnant women. The trial was registered with the International Clinical Trials Registry, ISRCTN82927713). The study design and results have been published (Harvey et al, 2012, Cooper et al, 2016). In brief, the primary aim was to investigate whether maternal vitamin D supplementation during pregnancy improves infant bone mineral content in comparison to the children of women not supplemented in pregnancy.

Participants were recruited from three centres in the United Kingdom (University Hospital Southampton, Oxford University Hospital and Sheffield Hospital) between October 2008 and February 2014. Women were recruited at 6-19 weeks gestation. Plasma samples were collected at that time and 25-hydroxyvitamin D concentrations were analysed in local laboratories. Women with 25-hydroxyvitamin D concentrations between 25-100 nmol/L were invited to continue in the study. The MAVIDOS trial outline is shown in Figure 3.3. The primary outcome was neonatal bone mineral content assessed by DEXA.

In total 1134 women were randomised to receive either 1000 international units of vitamin D₃ daily or placebo and 836 neonates underwent DEXA. At 30-38 weeks gestation plasma samples were collected from participants and stored at -80°C. These samples have been used for the study below.
Methods

Mothers invited \( n = 3200 \)

Mothers recruited \( n = 1600 \)

Vitamin D status checked

25 nmol/L < 25D < 100 nmol/L

Anthropometry, DEXA \( n = 477 \) in each arm

25D < 25 nmol/L

Supplementation \( n = 300 \)

25 nmol/L < 25D < 100 nmol/L

Randomisation \( n = 1200 \)

25D > 100 nmol/L

Ineligible \( n = 100 \)

1000 IU vitamin \( D_3 \) \( n = 600 \)

Placebo \( n = 600 \)

Repeat Vitamin D status checked and plasma samples stored

Figure 3.3 Study design of the MAVIDOS trial

Diagram adapted from figure 1 in (Harvey et al, 2012)

25D = 25 hydroxyvitamin D concentration
Methods

3.3.2 Metabolite profiling gas chromatography mass spectrometry analytical method

Metabolomics refers to the study of the low molecular weight (<1500 Da) molecules present in an organism or tissue (Holmes et al). Analytical techniques aim to detect and identify the maximum possible number of compounds to make a metabolite profile. Metabolomics has found a range of applications in environmental, agricultural and human research.

There are a number of analytical platforms that can be used for the non-targeted analysis of the metabolome, most commonly nuclear magnetic resonance spectroscopy (NMR), gas-chromatography coupled to mass spectrometry (GCMS) and liquid chromatography coupled to mass spectrometry (LC-MS). Each analytical method has advantages and disadvantages. For example, while NMR is more restricted in the number of metabolites that can be detected (Chan et al, 2009), metabolite identification is simpler than in mass spectrometry-based technologies (Beckonert et al, 2007). Thousands of metabolites are detected by LC-MS (Fan et al, 2016) making it ideal for untargeted metabolomics, however there are two major caveats. Firstly, it is difficult to identify metabolites with certainty without specific standards and two steps of ionisation (tandem mass spectrometry) (Evans et al, 2009) (De Vos et al, 2007). Secondly, due to large data outputs with current analytical techniques there is problematic loss of statistical power due to multiple testing.

For this study, GCMS was chosen as the analytical platform. While features numbering in the hundreds rather than thousands can be detected, metabolites relevant to changes in glucose metabolism are readily detected and methods for their identification are well established (Villas-Bôas et al, 2006, Dunn et al, 2013). The disadvantage of GCMS is sample preparation particularly the process of derivatization. Polar compounds must be derivatized to become more volatile. The process of derivatization replaces groups such as hydroxyl, carboxylic acids and thiols with non-polar ones. Only metabolites that are derivatized are detected.

Two methods of derivatization were employed in this study, methylchloroformate (MCF) and trimethyl silyl (TMS). TMS is the more commonly employed method and commercial libraries are more widely available (Dunn et al, 2008, Kumari et al, 2011). MCF has the advantage of being able to detect a larger number of metabolites found in plasma and is more stable than TMS therefore has better reproducibility (Villas-Bôas et al, 2003). Employing both methods allowed a larger number of metabolites to be detected and identified.

Plasma metabolite extraction for gas-chromatography mass spectrometry
Methods

Plasma extraction was performed following the protocol below. The protocol for plasma extraction for samples derivatized by methylchloroformate (MCF) and trimethylsilyl (TMS) were similar and are described here together. For MCF samples, liquid transfer steps were pipetted by hand and samples were vortexed individually. For TMS samples, a liquid handling robot (Eppendorf) was used and samples were mixed using a Geno/Grinder (SPEX Sample Prep, Metuchen, NJ, USA). Automation of these processes improved sample processing time.

1. Samples were defrosted on ice, then mixed for 1 minute.
2. 150 μL of plasma was transferred into a 1.5mL microcentrifuge tube (Eppendorf).
3. 20 μL of internal standard was added to the sample. For MCF derivatized samples this was 10mM d4-alanine. For TMS derivatized samples, the internal standard was 10mM ribitol (Sigma-Aldrich, St Louis, MO, USA). Samples were mixed for 30 seconds after addition of internal standard.
4. Samples were dried using a Savant SC250EXP speed-vac concentrator with a Savant RVT4104 Refrigerated Vapor Trap (Thermo Scientific, Ashville, NC, USA) for 4 hours and then immediately transferred to the -80°C freezer to await further processing.
5. Samples were taken from the -80°C freezer and immediately placed on dry ice.
6. 500 μL of 50% cold methanol in water (also kept on dry ice) was added to each sample and mixed for 2 minutes.
7. Samples were centrifuged at -4°C at 3500 rpm for 5 minutes (Eppendorf 5430 R centifuge).
8. The supernatant was collected and transferred to another 1.5mL microcentrifuge tube.
9. 500 μL of 80% cold methanol in water was added to what remained in the sample microcentrifuge tube and mixed for 2 minutes.
10. Samples were centrifuged at -4°C at 3500 rpm for 5 minutes.
11. The supernatant was collected and added to the supernatant removed previously.
Quality control (QC) samples were prepared by pooling aliquots from all extracted serum samples.
12. Samples and QC samples were dried in the speed vac for 4 hours then stored at -80°C.

Methylchloroformate (MCF) derivatization

The following method is based upon the method developed by Silas Villas Boas (Villas-Bôas et al, 2003). It has been modified to improve derivatization of plasma sample metabolites with
Methods

A doubling of all liquid additions except chloroform. Pyridine and anhydrous sodium sulphate were purchased from Sigma-Aldrich (St Louis, MO, USA). Methyl chloroformate and N-methyl-N (trimethylsilyl) trifluoroamide (MSTFA) were purchased from Merck (Darmstadt, Germany). Methoxyamine hydrochloride was purchased from Fluka (Steinheim, Switzerland).

13. Extracted plasma samples were resuspended in 400 μL of 1 molar sodium hydroxide and mixed with 334 μL of methanol and 68 μL of pyridine.
14. 20 μL of MCF was added and mixed for 30 seconds. A further 20 μL of MCF was again added and again mixed for 30 seconds.
15. 400 μL of chloroform was added and mixed for 10 seconds.
16. 800 μL of 50 millimolar sodium bicarbonate was added to the reaction tubes and mixed for 10 seconds.
17. Samples were centrifuged for 5 minutes at 2500 rpm. The upper layer containing water and proteins was discarded. 10mg of anhydrous sodium sulfate was added to remove residual water.
18. The chloroform layer was transferred to a GC vial, ready for injection.

Trimethyl silyl derivatization

Trimethyl silyl (TMS) is the most widely used derivatization method. This automated method was established at the Centre for Genomics, Proteomics and Metabolomics at the University of Auckland by Erica Zarate. The method described below and a validation of this method for plasma samples has recently been published (Zarate et al., 2017).

19. Methoxyamine hydrochloride in pyridine (2g/100ml) was added to a GC vial and MSTFA was added to another GC vial. The two vials were placed in the cooler tray on the autosampler.
20. Extracted plasma samples were loaded into the GC tray.

The following steps are automated

21. Vials were moved from the GC tray to the agitator.
22. Extracted samples were resuspended in 40 μL of methoxyamine hydrochloride in pyridine (2g/100mL).
23. Samples were mixed for 90 minutes at 750 rpm.
24. 40 μL of MSTFA was added to the sample vials and mixed again for 30 minutes.
Methods

25. Samples were set aside for two hours before injection.

Gas chromatography mass spectrometry conditions

Derivatized samples were injected into the chromatography column using a CTC PAL autosampler into a glass split/splitless 4mm ID straight inlet liner packed with deactivated glass wool (Supelco, Bellafonte, PA, USA). The column used was a fused silica Zebron ZB-1701 (Phenomenex), 30 m x 250 μm (internal diameter) by 0.15 μm (film thickness), stationary phase (86% dimethylpolysiloxane, 14% cyanopropylphenyl, Phenomenex) with 5 m guard column.

Samples were analysed using an Agilent GC 7890A coupled to a MS 5975C (Agilent Technologies, USA) with a quadrupole mass selective detector.

An example of the injection sequences is given in
Methods

Table 3.4. These sequences contain standard mixes, and quality control samples. The standard mix includes (given in table) and provides an aid to metabolite identification. Metabolites detected by both methods and the co-efficient of variation for the quality control samples is given in Appendix table 7 and Appendix table 8.
Table 3.4 Gas chromatography mass spectrometry injection sequence

<table>
<thead>
<tr>
<th>MCF injection sequence</th>
<th>TMS injection sequence 1</th>
<th>TMS injection sequence 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>Alkane mix</td>
<td>Alkane mix</td>
</tr>
<tr>
<td>Alkane mix</td>
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</tr>
<tr>
<td>Blank</td>
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<td>QC</td>
</tr>
<tr>
<td>Standard Mix with d4 alanine</td>
<td>QC</td>
<td>Sample</td>
</tr>
<tr>
<td>QC</td>
<td>Sample</td>
<td>Sample</td>
</tr>
<tr>
<td>QC (repeat injection)</td>
<td>Sample</td>
<td>Sample</td>
</tr>
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<td>Sample</td>
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<td>Sample</td>
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<tr>
<td>QC</td>
<td>Standard mix 2</td>
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<td>Sample</td>
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<td>Sample</td>
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<td>Sample</td>
<td>Sample</td>
<td>Sample</td>
</tr>
<tr>
<td>QC</td>
<td>Standard mix 3</td>
<td>Sample</td>
</tr>
</tbody>
</table>

**QC** – quality control
Methods

Data extraction

Raw GC-MS data were deconvoluted using the Automated Mass Spectral Deconvolution and Identification System (AMDIS) (online software distributed by the National Institute of Standards and Technology, USA – http://www.amdis.net/) (Stein, 1999). Data were normalised by internal standard, and batch-corrected using quality control samples. An in-house-R-based software data mining and analysis platform (open source, version 3.1.2 https://www.r-project.org/) was used for metabolite identification and peak integration using peak height for relative quantification (Aggio et al, 2011).

3.3.3 Fructosamine analysis

Fructosamine was analysed by ROCHE Cobas c 311 chemical analyser (Hitachi High Technologies Corporation, Tokyo, Japan) using a Roche analytical kit. The coefficient of variation for the low concentration quality control was 0.9%. The coefficient of variation for the high concentration quality control was 1.2%.

3.3.4 Statistical analysis

Data extraction and statistical analysis methods are provided in detail in Chapter 7
Methods

3.4 Appendix DEQAS participation

The Vitamin D External Quality Assessment Scheme (DEQAS) is the largest proficiency testing scheme for vitamin D metabolites. It was established in 1989 to improve the reliability of 25-hydroxyvitamin D analysis (Carter et al, 2004). It has since expanded to include 1,25-dihydroxyvitamin D and most recently 24,25 dihydroxyvitamin D (Cashman et al, 2015).

DEQAS is operated from the Charing Cross Hospital London Endocrinology Laboratory and administered through an online portal at www.deqas.org. Samples are sent out to participating laboratories four times a year. Each batch includes five samples, four of which are used in proficiency testing. Samples used are pooled serum, collected from patients undergoing venesection for haemochromatosis.

A report from DEQAS shows the performance of your laboratory compared to other participating laboratories. Figure 3.4 shows the report from April 2014, the first time results were submitted from the LC-MS/MS method described. The symbol ▼ indicates where our laboratory results lies. It can be seen that our result was 3 standard deviations above the mean in all samples. At this time concentrated stock solutions of the standard curve in methanol were being stored at -20°C. The stock solutions were not stable and the 25-hydroxyvitamin D concentrations were gradually declining.

Figure 3.5. shows the DEQAS results at the time the SCOPE samples were analysed. At this time the issues with the standard curve had been solved by making the standard curve in the way described above (page 45).
Methods

Figure 3.4 First DEQAS report April 2014
Methods

Figure 3.5 DEQAS report at the time of the SCOPE serum samples
Chapter 4 The relationship between maternal vitamin D status and Gestational Diabetes Mellitus

4.1 Preface

The following section contains a published original research manuscript “The relationship between 25-hydroxyvitamin D concentration in early pregnancy and pregnancy outcomes in a large prospective cohort”. The article was published in the British Journal of Nutrition in 2016.

In chapter 2 a large number of observational studies investigating the relationship between maternal vitamin D status and the development of GDM were discussed. The number of studies finding an association between vitamin D and GDM number approximately the same as those that find no relationship.

At the time when laboratory analysis for the study presented in this chapter was underway, it was larger than any previously published investigating the relationship with vitamin D status before 20 weeks gestation. One study with 1,873 participants had investigated the relationship between GDM and vitamin D status during the third trimester (Bener et al, 2013). They found women with 25-hydroxyvitamin D concentrations < 75 nmol/L were more likely to develop GDM in multivariate analysis (odds ratio 1.4 95% CI 1.1 – 1.8).

Bener et al used data collected from hospital medical records for clinical purposes. The disadvantage of using medical records is that data may be missing, the data collected less accurate and relevant variables missing because they are not collected as part of routine clinical practice. The advantage of a retrospective study of medical records over a prospective study is a large saving of cost and other large studies have used data collected for medical records. This includes the largest study to date with 5,109 participants (Schneuer et al, 2014); participants were randomly selected from the women attending first trimester Down’s syndrome screening. However, in this study of Schneuer et al it is not clear whether all women were screened for GDM.

One reason for the disparate findings from the observational studies may be due to limitations in study design.
Chapter 4

The aim of this chapter is to investigate the relationship between 25-hydroxyvitamin D concentration at 15 weeks gestation and the development of GDM in a large prospective pregnancy cohort. Other pregnancy outcomes have been included in this chapter because the association between maternal vitamin D status and other pregnancy outcomes may be of interest to British Journal of Nutrition readers.
Chapter 4

4.2 Maternal vitamin D status and pregnancy outcomes

The relationship between 25-hydroxyvitamin D concentration in early pregnancy and pregnancy outcomes in a large prospective cohort (2016)

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3. Institute of Natural and Mathematical Sciences, Massey University, New Zealand

4. The Department of Obstetrics and Gynaecology and South Auckland Clinical School The University of Auckland, New Zealand

5. The Irish Centre for Fetal and Neonatal Translational Research (INFANT) and the Department of Obstetrics and Gynaecology, University College Cork, Ireland

6. The College of Medicine, Biosciences and Psychology, University of Leicester, UK
Chapter 4

Abstract

Vitamin D insufficiency and deficiency have been associated with an increased risk of adverse pregnancy outcomes. Controversy remains as findings have been inconsistent between disparate populations. The aim of this study was to investigate the relationship between vitamin D status and pregnancy outcomes in a large prospective pregnancy cohort. 25-hydroxyvitamin D concentration was analysed in serum samples collected at 15 weeks gestation from 1710 New Zealand women participating in a large observational study. Associations between vitamin D status and pre-eclampsia, preterm birth, small for gestational age and gestational diabetes were investigated.

Mean 25-hydroxyvitamin D concentration was 72.9 nmol/L. 23% had a 25-hydroxyvitamin D concentration less than 50 nmol/L and less than 25 nmol/L in 5% of participants. Women with a 25-hydroxyvitamin D concentration less than 75nmol/L at 15 weeks gestation were more likely to develop GDM than those with concentrations above 75nmol/L (OR 2.3 95% CI 1.1-5.1). However, this effect was not significant when adjustments were made for BMI and ethnicity (OR 1.8 95% CI 0.8-4.2). 25-hydroxyvitamin D concentration at 15 weeks was not associated with development of pre-eclampsia, spontaneous preterm birth or small for gestational age infants. Pregnancy complications were low in this largely vitamin D replete population.
4.2.2 Introduction

Much progress has been made in the screening, diagnosis and management of important pregnancy disorders but little progress has been made in prevention. Pre-eclampsia remains the second most common cause of maternal death worldwide (Say et al, 2014). Prematurity, mostly due to spontaneous preterm birth, is the leading cause of death amongst infants worldwide (You et al, 2015). This is followed by growth restriction in term infants (Morisaki et al, 2013). Furthermore infants born preterm or with a low birth weight have an increased risk of cardiovascular and metabolic disease later in life, the leading cause of death in adulthood (Abitbol and Rodriguez, 2012). The incidence of gestational diabetes mellitus (GDM) is increasing rapidly worldwide (Lawrence et al, 2008) and is associated with lifelong risks of metabolic disease for both mother and baby (Gunderson et al, 2007, Whincup et al, 2008).

An association between low vitamin D and adverse pregnancy outcomes was first identified in the early 2000’s (Sabour et al, 2006) and consequently, vitamin D has been postulated as a possible intervention strategy to reduce pregnancy complications. A recent meta-analysis found that low vitamin D, defined variably by the authors of included studies, was associated with an increased odds of pre-eclampsia (OR 1.79 95% CI 1.25-2.58), small-for-gestational age (SGA) babies (OR 1.85 95% CI 1.52 – 2.26) and GDM (OR 1.49 95% CI 1.18 – 1.89) (Aghajafari et al, 2013). Preterm birth was not included as an outcome. However, most included studies did not correct for important confounders, and those that did were small. In addition, vitamin D deficiency was variably defined between 35 and 80 nmol/L, or analysed in late pregnancy (Aghajafari et al, 2013).

Late pregnancy may not be the optimum time to measure vitamin D, as it is believed that the origins of most late pregnancy disorders are in early pregnancy. Moreover, the expression of both CYP25B1 (the vitamin D activating enzyme) and the vitamin D nuclear receptor (Zehnder et al, 2002) is highest during the first trimester. In the current study, factors that may alter the development of pregnancy conditions were investigated at the beginning of the second trimester, at a time when critical vascular development of the placenta is occurring (Pijnenborg et al, 1983).

The aim of this study was to investigate the relationship between early pregnancy 25-hydroxyvitamin D concentration and the subsequent development of pre-eclampsia, spontaneous preterm birth, SGA and GDM in a large, well-phenotyped prospective cohort.
4.2.3 Methods

Participants

Samples were obtained from Auckland participants recruited to the Screening for Pregnancy Endpoints study (SCOPE) between 2005 and 2008. SCOPE is an international prospective pregnancy study of nulliparous women which aims to identify early pregnancy predictors of late pregnancy complications (Chappell et al., 2013). The study was approved by the Auckland University Ethics Committee (AKX/02/00/364).

Non-fasting serum samples were collected at 15 weeks of gestation and stored at -80°C. At the time of sample collection participants were asked whether they took multivitamins and which brand. Data on socioeconomic status (Davis et al., 1997) were recorded and participants were asked whether they smoked and to rate their exercise (over the previous three months) as vigorous, moderate, recreational walking or no physical activity, and the frequency at which they engaged in physical activity. Data were entered into an internet-accessed, password protected centralised database with complete audit train (MedSciNetAB, Stockholm, Sweden) (McCowan et al., 2007).

Pre-eclampsia was diagnosed according to the International Society for the Study of Hypertension in Pregnancy criteria (Brown et al., 2001) as gestational hypertension (systolic blood pressure of $\geq 140$ mmHg and/or diastolic blood pressure of $\geq 90$ mmHg on at least two occasions) with proteinuria or any multisystem complication of pre-eclampsia (North et al., 2011). SGA was defined as a weight below the 10th customised birth weight centile (McCowan and Stewart, 2004), and spontaneous preterm birth as delivery before 37 weeks gestation. Women were screened for GDM between 24 to 28 weeks gestation with a non-fasting 50g polycose challenge in community laboratories, according to the Auckland District Health Board Guidelines. Those with a positive polycose test (defined as a one hour post-challenge glucose of $\geq 7.8$mmol/L) were further tested with a fasting 75g oral glucose tolerance test (OGTT). 44 women considered at high risk of GDM underwent an OGTT without a polycose test. GDM was diagnosed if a fasting glucose was greater or equal to 5.5nmol/L or a two hour post glucose challenge was greater than or equal to 9.0mmol/L.
Sample Analysis

Serum samples were prepared using Phree\textsuperscript{TM} phospholipid removal cartridges (Phenomenex, Torrance, California, USA) after addition of the internal standard deuterated 25-hydroxyvitamin D3. Separation of 25-hydroxyvitamin D3 and 3-epi-25-hydroxyvitamin D3 was achieved with a Kinetex\textsuperscript{TM} pentafluorophenyl (PFP) 100x3mm 2.6\textmu m HPLC column (Phenomenex, USA). 25-hydroxyvitamin D3, c3-epi 25-hydroxyvitamin D3 and 25-hydroxvitamin D2 were analysed by liquid chromatography tandem mass spectrometry (Finnigan TSQ Quantum Ultra AM triple quadrupole mass spectrometer Thermo Electron Corporation, San Jose, Ca, USA). Total 25-hydroxyvitamin D was calculated by the sum of these three compounds. The intra- and inter-assay coefficient of variation was 7.3\% and 4.9\% for 25-hydroxyvitamin D3 and 8.2\% and 5.8\% for 25-hydroxyvitamin D2. Quality control was ensured through participation in the DEQAS program operating out of Charing Cross Hospital, London (Carter et al, 2010) and the use of commercially available control materials of known vitamin D concentration. Vitamin D binding protein was analysed using an ELISA from R&D systems.

Statistical Analysis

All statistical analyses were performed in SPSS (IBM) v21. Normality was tested by assessing the histogram and tests for skewness and kyphosis. Continuous variables are reported as mean ± standard deviation. Chi-squared tests were performed for categorical variables and t-test for continuous variables. Fishers exact test was performed when Chi squared was not appropriate. The dates of sample collection were categorised into seasons as follows; summer: December to February, autumn: March to May, winter: June to August, spring: September to November. Logistic regression analyses were performed to determine whether 25-hydroxyvitamin D concentration was associated with the development of GDM, pre-eclampsia, SGA and spontaneous preterm birth. Only participants screened for GDM were used in the analysis for GDM. Multiple logistic regression with backwards selection using the likelihood ratio criteria was used to incorporate known risk factors for adverse pregnancy outcomes including age, body mass index (BMI), ethnicity, smoking and socioeconomic status. The analysis was performed with 25-hydroxyvitamin D as a continuous variable and vitamin D level as a categorical variable. Vitamin D categorical variables were created with cut offs at 25 nmol/L, 50 nmol/L and 75 nmol/L. These cut-offs were decided upon based on the Institute of Medicine definition of deficiency (<50 nmol/L) (Ross et al, 2011)and Endocrine Society clinical practice guidelines definition of insufficiency (<75
nmol/L) and severe deficiency (<25 nmol/L) (Holick et al., 2011). Hosmer-Lemeshow goodness of fit was performed to test the adequacy of the model; (a value of 1 is indicates perfect correspondence between the model predicted and observed risks).

### 4.2.4 Results

2065 women were recruited to the SCOPE study in Auckland. Of those women, 355 did not have 15 week serum or plasma available for analysis. 1710 women were included in the final analysis. 166 of those participants were not screened for GDM and 1544 women were included in the final analysis for that outcome. 32 (2.1%) were diagnosed with GDM, 73 (4.2%) women with pre-eclampsia, 70 (4.1%) women spontaneously delivered preterm infants and 170 (9.9%) women delivered SGA infants Figure 4.1. The majority of women were of NZ European ethnicity (83.8%). The remaining ethnic groups were Asian (n = 91 5.3%), Maori (n = 57 3.3%), Indian (n = 65 3.8%), Pacific Island (n = 34 2.0% and African (n = 30 1.8%) (combined as “other ethnicities” in Table 4.1).

![Figure 4.1 Cohort flow diagram of Auckland SCOPE participants from recruitment at 15 weeks gestation](image)
55% of women took a multivitamin supplement containing vitamin D. One brand contained vitamin D2 and was taken by 20 women. The dose of vitamin D supplement ranged from 5μg/day to 20μg/day. Women who took multivitamin supplements containing vitamin D had significantly higher 25-hydroxyvitamin D concentrations than those who did not (93.6 ± 29.6 nmol/L vs 75.8 ± 32.6 nmol/L, \( p < 0.001 \)). The proportion of women who took multivitamin supplements containing vitamin D was lower amongst those who developed GDM, although this was not statistically significant (38% vs 55% \( p = 0.4 \)).

Concentration of 25-hydroxyvitamin D was approximately normally distributed (skewness -0.03 standard error 0.05, kurtosis -0.3 standard error 0.1). Just over half of women (53%) had a 25-hydroxyvitamin D concentration less than 75 nmol/L, 22.7% with concentrations less than 50 nmol/L and 4.4% with concentrations less than 25 nmol/L. There was a significant difference in the mean BMI between those women who developed GDM and pre-eclampsia and those who did not (\( p = 0.001 \)) (Table 4.1). The prevalence of GDM and pre-eclampsia varied significantly depending, upon ethnicity (\( p = 0.02 \) and \( p = 0.05 \) respectively) with Indian women having the highest prevalence of GDM and NZ European women the lowest. Pacific Island women had the highest prevalence of pre-eclampsia. The mean total serum 25-hydroxyvitamin D concentration was lower in those who developed GDM than those who did not (61.6 ± 23.9 nmol/L as compared to 72.9 ± 27.0 nmol/L, \( p = 0.03 \)) but not lower in women who developed pre-eclampsia (68.1 ± 27.8 nmol/L), preterm birth (73.7 ± 27.5 nmol/L) or with SGA infants (70.6 ± 29.0 nmol/L) (Table 4.1). Of the women who developed GDM, 72% had a concentration less than 75 nmol/L and 22% < 50 nmol/L. Women of European decent were the largest ethnic group; there was no significant difference in mean total 25-hydroxyvitamin D in those who developed GDM and in those who did not (70.6 nmol/L vs 75.9 nmol/L, \( p = 0.35 \)).

There was an increased likelihood of developing GDM if total 25-hydroxyvitamin D was less than 75 nmol/L (OR 2.3 95%CI 1.1-5.1). However, after adjustment for BMI and ethnicity, this difference was not significant (adjusted OR 1.8 95% CI 0.8-4.2). There was no increased likelihood of developing any of the adverse pregnancy outcomes at concentrations of < 50 nmol/L and < 25 nmol/L Table 4.2.
Table 4.1 Maternal Characteristics at 15 week’s gestation

<table>
<thead>
<tr>
<th>Age years (SD)</th>
<th>All n=1710</th>
<th>Pre-eclampsia n = 72</th>
<th>sPTB* n = 73</th>
<th>SGA n = 170</th>
<th>GDM n = 32</th>
</tr>
</thead>
<tbody>
<tr>
<td>30.3 (4.7)</td>
<td>30.0 (5.0)</td>
<td>30.8 (4.8)</td>
<td>31.1 (4.8)</td>
<td>30.8 (5.1)</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BMI kg/m² (SD)</th>
<th>Pre-eclampsia n = 72</th>
<th>sPTB* n = 73</th>
<th>SGA n = 170</th>
<th>GDM n = 32</th>
</tr>
</thead>
<tbody>
<tr>
<td>24.8 (4.2)</td>
<td>26.9 (4.8)</td>
<td>24.9 (4.5)</td>
<td>25.4 (5.1)</td>
<td>27.4 (5.2)</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Ethnicity n (%)</th>
<th>Pre-eclampsia n = 72</th>
<th>sPTB* n = 73</th>
<th>SGA n = 170</th>
<th>GDM n = 32</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZ European</td>
<td>1433 (83.8)</td>
<td>54 (3.8)</td>
<td>67 (4.7)</td>
<td>137 (9.6)</td>
</tr>
<tr>
<td>Other ethnicities</td>
<td>277 (16.2)</td>
<td>18 (6.5)</td>
<td>6 (2.1)</td>
<td>33 (11.9)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Season at sample collection n (%)</th>
<th>Pre-eclampsia n = 72</th>
<th>sPTB* n = 73</th>
<th>SGA n = 170</th>
<th>GDM n = 32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summer</td>
<td>452 (26.4)</td>
<td>12 (2.7)</td>
<td>22 (4.9)</td>
<td>28 (6.2)</td>
</tr>
<tr>
<td>Autumn</td>
<td>397 (23.2)</td>
<td>21 (5.3)</td>
<td>16 (4.0)</td>
<td>46 (11.6)</td>
</tr>
<tr>
<td>Winter</td>
<td>510 (29.8)</td>
<td>19 (3.7)</td>
<td>20 (3.9)</td>
<td>53 (10.4)</td>
</tr>
<tr>
<td>Spring</td>
<td>351 (20.5)</td>
<td>20 (5.7)</td>
<td>15 (4.3)</td>
<td>43 (12.3)</td>
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</table>

<table>
<thead>
<tr>
<th>Mean total 25-hydroxyvitamin D nmol/L (SD)</th>
<th>Pre-eclampsia n = 72</th>
<th>sPTB* n = 73</th>
<th>SGA n = 170</th>
<th>GDM n = 32</th>
</tr>
</thead>
<tbody>
<tr>
<td>72.9 (27.0)</td>
<td>68.1 (27.8)</td>
<td>73.7</td>
<td>70.6 (29.0)</td>
<td>61.6 (23.9)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total 25(OH) vitamin D level n (%)</th>
<th>Pre-eclampsia n = 72</th>
<th>sPTB* n = 73</th>
<th>SGA n = 170</th>
<th>GDM n = 32</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;25nmol/L</td>
<td>292 (17.1)</td>
<td>15 (5.1)</td>
<td>14 (4.8)</td>
<td>35 (12.0)</td>
</tr>
<tr>
<td>25-49.9nmol/L</td>
<td>508 (29.7)</td>
<td>24 (4.7)</td>
<td>16 (3.1)</td>
<td>43 (8.5)</td>
</tr>
<tr>
<td>50-74.9nmol/L</td>
<td>834 (48.8)</td>
<td>29 (3.4)</td>
<td>39 (4.7)</td>
<td>80 (9.6)</td>
</tr>
<tr>
<td>&gt;75nmol/L</td>
<td></td>
<td></td>
<td></td>
<td>9 (1.1)</td>
</tr>
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</table>

* Spontaneous preterm birth
Table 4.2 Pregnancy outcomes associations with vitamin D as a continuous variable and at cut-offs < 50 nmol/L (compared with ≥ 50 nmol/L) and < 75 nmol/L (compared with ≥ 75 nmol/L), unadjusted and adjusted for BMI and ethnicity (Odds Ratio (OR) and 95% confidence intervals)

<table>
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<tr>
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<th>Unadjusted</th>
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<tr>
<td></td>
<td>Continuous</td>
<td>&lt; 25 nmol/L</td>
<td>&lt; 50 nmol/L</td>
<td>&lt; 75 nmol/L</td>
</tr>
<tr>
<td></td>
<td>OR 95% CI</td>
<td>OR 95% CI</td>
<td>OR 95% CI</td>
<td>OR 95% CI</td>
</tr>
<tr>
<td>Pre-eclampsia</td>
<td>0.99 0.99, 1.00</td>
<td>0.8 0.3, 2.2</td>
<td>1.3 0.8, 2.2</td>
<td>1.4 0.9, 2.2</td>
</tr>
<tr>
<td>Spontaneous preterm birth</td>
<td>1.00 0.99, 1.01</td>
<td>0.8 0.3, 2.2</td>
<td>1.2 0.7, 2.2</td>
<td>0.8 0.5, 1.3</td>
</tr>
<tr>
<td>Small for gestational age</td>
<td>1.00 0.99, 1.00</td>
<td>0.6 0.2, 1.1</td>
<td>1.4 0.9, 2.2</td>
<td>1.1 0.8, 1.5</td>
</tr>
<tr>
<td>Gestational diabetes</td>
<td>1.00 1.01, 1.03</td>
<td>1.5 0.2, 11.7</td>
<td>1.3 0.6, 2.9</td>
<td>2.3 1.1, 5.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Adjusted</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Continuous</td>
<td>&lt; 25 nmol/L</td>
<td>&lt; 50 nmol/L</td>
<td>&lt; 75nmol/L</td>
</tr>
<tr>
<td></td>
<td>OR 95% CI</td>
<td>OR 95% CI</td>
<td>OR 95% CI</td>
<td>OR 95% CI</td>
</tr>
<tr>
<td>Pre-eclampsia</td>
<td>0.99 0.99, 1.01</td>
<td>0.9 0.3, 2.9</td>
<td>0.95 0.53, 1.73</td>
<td>1.2 0.7, 1.9</td>
</tr>
<tr>
<td>Spontaneous preterm birth</td>
<td>1.00 0.99, 1.01</td>
<td>1.7 0.5, 5.5</td>
<td>1.46 0.82, 2.58</td>
<td>0.9 0.6, 1.5</td>
</tr>
<tr>
<td>Small for gestational age</td>
<td>1.00 0.99, 1.00</td>
<td>1.6 0.8, 3.3</td>
<td>1.33 0.91, 1.96</td>
<td>1.0 0.7, 1.4</td>
</tr>
<tr>
<td>Gestational diabetes</td>
<td>1.00 0.99, 1.02</td>
<td>0.2 0.1, 1.8</td>
<td>0.97 0.42, 2.25</td>
<td>1.8 0.8, 4.2</td>
</tr>
</tbody>
</table>
On logistic regression, 25-hydroxyvitamin D concentration as a continuous variable was associated with an increased likelihood of developing GDM with a 1% increase in risk for every 1 nmol/L reduction in 25-hydroxyvitamin D (OR 1.01 95% CI 1.01-1.03 p = 0.03) (Table 2). Following inclusion of BMI and ethnicity in the model, the relationship between 25-hydroxyvitamin D concentration and GDM was no longer significant. Using the International Association of Diabetes in Pregnancy criteria for the diagnosis of GDM increased the number of GDM cases (n = 48) but did not increase the significance of the relationship with 25-hydroxyvitamin D.

In a logistic regression model including vitamin D and season, vitamin D was not a significant contributor. After adjustment for BMI and ethnicity, season remained a significant risk factor for GDM (adjusted odds ratio 4.2 95% CI 1.3-13.0 p = 0.04). Season was also a significant predictor of developing pre-eclampsia (adjusted odds ratio 2.2 95% CI 1.0 – 4.5 p = 0.04) and SGA (adjusted odds ratio 2.1 95% CI 1.3 – 3.5 p = 0.003). After adjustment for BMI and ethnicity, exercise (p = 0.1), age (p = 0.7), socioeconomic status (p = 0.8) and smoking (p = 0.2) were not statistically associated with GDM and are not included in the model shown in Table 2.

Vitamin D binding protein (VDBP) was analysed and free 25-hydroxyvitamin D calculated on a subgroup of participants (32 GDM with 4 BMI and ethnicity matched controls). VDBP concentration was significantly correlated with 25-hydroxyvitamin D concentration (p = 0.03). No correlation between calculated free 25-hydroxyvitamin D and GDM was found.

### 4.2.5 Discussion

In this large, well-phenotyped cohort, we found that 25-hydroxyvitamin D concentration was not lower at 15 weeks gestation in women who later developed pre-eclampsia, spontaneous preterm birth or who had SGA infants. Concentrations were lower in women who developed GDM but did not predict GDM when adjusted for BMI and ethnicity.

This cohort differs from most other pregnancy cohorts in that it is relatively vitamin D replete. While 23% of Auckland SCOPE participants were vitamin D deficient (25-hydroxyvitamin D < 50 nmol/L) half to more than 90% of pregnant women have been found to be deficient in previous studies (Song et al, 2013, Park et al, 2014, Flood-Nichols et al, 2015). The SCOPE Auckland cohort may be more vitamin D replete in comparison to the obstetric Auckland population and wider New Zealand population. Auckland is in the northern region of New Zealand and New Zealand generally has lower levels of sunlight than other regions.
Zealand where it is estimated that 24 minutes of hand and face exposure to the sun daily is adequate. This is not the case for much of New Zealand’s geographic distribution (Nowson et al., 2012). NZ European women had the highest 25-hydroxyvitamin D concentrations by ethnicity, and while they represented 83.8% of Auckland Scope, at the time of sample collection they represented 49.0% of the women delivering at Auckland City Hospital (National Womens Health, 2008) and in the most recent National Women’s report, 44% of women delivering at Auckland city hospital (National Womens Health, 2014).

In the SCOPE cohort there are a limited proportion of women with vitamin D concentrations less than 25nmol/L (4.4%). We believe this has not significantly altered the association of other outcomes given that previous studies investigating the relationship between 25-hydroxyvitamin D concentration and pregnancy outcomes have reported both positive and negative associations in populations with high and low prevalence of vitamin D deficiency (summarized in Table 3).

In some vitamin D replete populations of pregnant women, significant associations with GDM (Lacroix et al., 2014) and SGA (Schneuer et al., 2014) have been found. In a similarly replete population, investigators found no association between vitamin D status and pregnancy outcomes including pre-eclampsia, SGA, preterm birth and GDM (Flood-Nichols et al., 2015). While in vitamin D deficient populations no association between 25-hydroxyvitamin D concentration and SGA(Akcakus et al, 2006) and GDM (Park et al, 2014) (Table 3).

The main limitations to our study were the comparatively low prevalence of late pregnancy disorders particularly GDM. This is due to the purpose for which SCOPE was established; that is, to investigate predictors of late pregnancy disorders in early pregnancy in low risk nulliparous women (Chappell et al, 2013). We do not have data on sunlight exposure, the factor that contributes most to 25-hydroxyvitamin D concentration. Given the lack of an association between vitamin D and pregnancy outcomes in this study, we do not believe this data would have altered the outcome.

Our findings are supported by a recent meta-analysis on trials of vitamin D supplementation in pregnancy and pregnancy outcomes (Pérez-López et al, 2015). There was no difference in the incidence of pre-eclampsia, preterm birth, low birth weight and GDM with vitamin D supplementation in pregnancy. However, a recently updated Cochrane systematic review on vitamin D supplementation in pregnancy found a reduced incidence of pre-eclampsia, preterm birth, and low birth weight (<2500g) (De-Regil et al, 2016). These results were sensitive to
the inclusion, or not, of trials using low dose vitamin D supplementation. This highlights the need for pregnancy outcomes to be evaluated in ongoing randomised trials of vitamin D supplementation in pregnancy.

There may be a number of reasons for the inconsistency in the literature. The aetiology of pre-eclampsia, preterm birth, SGA and GDM are not fully understood and likely to be multifactorial. Vitamin D deficiency may be an important predictor in some populations and not others. For example, because vitamin D increases intestinal absorption of calcium, in a population where dietary calcium is particularly low, vitamin D status may be an important predictor for developing pre-eclampsia but not when dietary calcium is adequate (Hofmeyr et al, 2014). Genetic polymorphisms of the vitamin D nuclear receptor and vitamin D binding protein have been associated with a wide variety of conditions (Gnagnarella et al, 2014, Koplin et al, 2015), and may explain why a study in the USA found vitamin D deficiency (<37.5nmol/L) was a risk factor for SGA in white women (adjusted OR 7.5 95% CI 1.8–31.9) but not black women (adjusted OR 1.5 95% CI 0.6–3.5) (Bodnar et al, 2010). This study found a non-linear association between 25-hydroxyvitamin D concentration, and SGA, with an increased risk of SGA with concentrations above 75nmol/L (OR 2.2 95% CI 1.2 – 3.8).

Vitamin D may be a surrogate marker for other causative factors that vary between populations, particularly those with a seasonal variation. We found a significant variation in GDM, pre-eclampsia and SGA with season that was not explained by 25-hydroxyvitamin D concentration. Factors that vary with season may be population specific, especially dietary patterns. A study of New Zealand pregnant found significant variations in macro and micronutrient intake according to season including lower dietary calcium consumption in winter and summer (Watson and McDonald, 2007). A recent study has demonstrated seasonal variation in gene expression in human lymphocytes and adipocytes (Dopico et al, 2015). These cell types play important roles in insulin sensitivity and inflammation. While it is not yet known how cell function is affected, these variations in gene expression may be relevant to GDM and pre-eclampsia risk. Others have found a significant variation in the prevalence of pre-eclampsia with season (TePoel et al, 2011). In our data, the association of season and GDM remains significant after adjustment for BMI and ethnicity, consistent with greatest risk if early pregnancy occurs during the winter and early spring, when 25-hydroxyvitamin D concentrations are lowest.
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In this vitamin D replete pregnancy cohort 25-hydroxyvitamin D concentration did not predict pregnancy outcomes including pre-eclampsia, SGA, spontaneous preterm birth and GDM when adjustments were made for confounders. Season was identified as a significant predictor for GDM. This does not exclude a potential contribution of vitamin D to GDM risk, but also raises the possibility of other pertinent seasonal factors.
4.3 Additional results and discussion

Additional data not included in the published manuscript are presented in this section. In section 4.4.2, descriptive data is presented to provide greater context to the Auckland SCOPE study. The spread of 25-hydroxyvitamin D concentration in the whole cohort is seen in Figure 4.2. The seasonal variation of 25-hydroxyvitamin D is seen in Figure 4.3. The difference in the spread of 25-hydroxyvitamin D concentration between ethnic groups is seen in Figure 4.4.

Sections 4.4.3 and 4.4.4 provide additional analysis. It is mentioned in the manuscript that a sensitivity analysis was performed using the IADPSG diagnostic criteria for GDM. The results of this analysis are presented in full here. Additional analysis on the participants who underwent an OGTT after a positive polycose test and the relationship with 25-hydroxyvitamin D concentration at 15 weeks gestation is also presented.

Section 4.4.5 contains a case control study investigating the relationship between free 25-hydroxyvitamin D and GDM. This study is referred to briefly in the main text of the manuscript above and here the findings are presented in full.
4.3.2 Additional descriptive data

Figure 4.2 Histogram of 1,710 Auckland SCOPE participants
Figure 4.3 Mean 25-hydroxyvitamin D concentration throughout the year (± 2 SD)
Figure 4.4 25-hydroxyvitamin D concentration by ethnicity
4.3.3 The relationship between vitamin D status at 15 weeks gestation and GDM by the International Association of Diabetes and Pregnancy Study Groups diagnostic criteria for GDM

Of the 1544 women with known vitamin D status at 15 weeks gestation who underwent a polycose screening test for GDM, 210 women met the criteria for a positive test and 44 women underwent an OGTT without polycose screening, resulting in 258 women with OGTT plasma glucose results. Compared to women with a negative polycose screening test women who underwent an OGTT were older (31.1 ± 4.2 vs 30.2 ± 4.8 years \( p = 0.005 \)), had higher BMI’s (26.2 ± 5.3 vs 24.6 ± 4.0, \( p < 0.001 \)) and lower 15 week 25-hydroxyvitamin D concentrations (68.1 ± 26.3 vs 72.6 ± 24.6, \( p = 0.02 \)). The OGTT was performed at a mean of 30 weeks gestation (SD ± 3.7).

Thirty two women met the threshold for GDM based on New Zealand guidelines (fasting plasma glucose \( \geq 5.5 \) mol/L, 2 hour post glucose load \( \geq 9.0 \) mmol/L). An additional 48 participants met the criteria based on the IADPSG guidelines (fasting plasma glucose \( \geq 5.1 \) mmol/L, or 1 hour post glucose challenge \( \geq 10.0 \), or 2 hour post glucose challenge \( \geq 8.5 \) mmol/L) (Table 4.3).

The mean 25-hydroxyvitamin D concentration was lower in women who met the IADPSG GDM criteria than women who did not (65.8 nmol/L vs 72.2 nmol/L, \( p = 0.045 \)). In multivariate regression analysis, including BMI and ethnicity in the model, there was no association between 25-hydroxyvitamin D concentration or vitamin D level at three cut-off concentrations, 25 nmol/L, 50 nmol/L and 75 nmol/L (Table 4.3).

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted</th>
<th></th>
<th>Adjusted</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds Ratio</td>
<td>95% CI</td>
<td>Odds Ratio</td>
<td>95% CI</td>
</tr>
<tr>
<td>Continuous</td>
<td>0.99</td>
<td>0.98, 1.00</td>
<td>1.00</td>
<td>0.99, 1.01</td>
</tr>
<tr>
<td>&lt; 25 nmol/L</td>
<td>1.93</td>
<td>0.46, 8.01</td>
<td>3.6</td>
<td>0.80, 16.20</td>
</tr>
<tr>
<td>&lt; 50 nmol/L</td>
<td>1.49</td>
<td>0.90, 2.46</td>
<td>0.92</td>
<td>0.52, 1.63</td>
</tr>
<tr>
<td>&lt; 75 nmol/L</td>
<td>1.44</td>
<td>0.90, 2.30</td>
<td>1.05</td>
<td>0.64, 1.74</td>
</tr>
</tbody>
</table>

The stricter IADPSG diagnostic criteria increased the number of participants meeting the diagnostic criteria for GDM increasing the power to find a significant association. In fact, the already weak association was weakened further with no significant association between vitamin D level at all thresholds prior to adjustment for BMI and ethnicity.
4.3.4 The relationship between vitamin D status at 15 weeks and plasma glucose concentrations

Univariate and multivariate linear regression analysis was performed to investigate the relationship between fasting, 1-hour post glucose load and 2 hour post glucose load plasma glucose, and 25-hydroxyvitamin D concentration in 258 SCOPE participants who underwent an oral glucose tolerance test.

25-hydroxyvitamin D concentration at 15 weeks gestation negatively correlated with fasting plasma glucose (\(-0.14, p = 0.02\) and 2 hour plasma glucose (\(-0.17, p = 0.006\)) but not 1 hour plasma glucose. In multivariate regression analysis the association between fasting plasma glucose was no longer significant (\(p = 0.2\)). On the other hand a 10 nmol/L increase in 25-hydroxyvitamin D was associated with a 0.1 mmol/L decrease in 2 hour plasma glucose (95% CI \(-0.28, -0.02, p = 0.01\)) after adjustment for BMI and ethnicity.

One other study found an association between vitamin D status in the first trimester (11 – 13 weeks gestation) and 2 hour post glucose load plasma glucose concentrations but not an association with GDM (Makgoba et al, 2011). In this study of 258 women, of whom 90 met the WHO criteria for GDM, a 1 ng/mL increase in 25-hydroxyvitamin D (2.5 nmol/L) was associated with a 0.737 mmol/L reduction in GTT 2 hour plasma glucose (95% CI \(-1.67, -0.008, p = 0.048\)). Two other studies found an association between fasting plasma glucose and 25-hydroxyvitamin D concentration early second trimester (Walsh et al, 2013) and at the time of GDM screening (McLeod et al, 2012) without associations with GDM.

The meaning of these findings is difficult to interpret. They either indicate that these studies including the SCOPE study are not powered to detect the real but small effect of plasma glucose by vitamin D, or there is no genuine association and the inconsistent findings reflect type 1 error.

4.3.5 The relationship between free 25-hydroxyvitamin D concentration and gestational diabetes mellitus; a case control study

The free hormone hypothesis states that the unbound portion of a circulating hormone is the bioactive form. This theory was proposed in the 1950’s when clinicians observed that patients with nephrotic syndrome were euthyroid despite low circulating thyroid hormone and has been validated for steroid hormones (Laurent et al, 2016). Because of the structural similarity
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steroids it has been proposed that unbound 25-hydroxyvitamin D may be the physiologically important portion.

Indeed, many tissues that can activate and respond to vitamin D in vitro lack the megalin receptor, the cell membrane protein that takes VDBP bound 25-hydroxyvitamin D into the renal cells for activation in response to parathyroid hormone.

VDBP polymorphisms are known to be strongly associated with 25-hydroxyvitamin D concentrations (Sinotte et al, 2009). Common variations contribute to the varying prevalence of vitamin D deficiency between ethnic groups.

A criticism of studies not finding a relationship between vitamin D status and GDM has been that free 25-hydroxyvitamin D is more important than total 25-hydroxyvitamin D. The purpose of this case control study is to investigate whether there is an association between free 25-hydroxyvitamin D and GDM.

Method

Each case of GDM was matched with four controls. Controls were matched for BMI and ethnicity and were selected by an independent administrator.

The analysis of 25-hydroxyvitamin D is described in Chapter 3.2.2. Details of albumin and vitamin D binding protein analyses are given in Chapter 3.2.3

Free 25-hydroxyvitamin D was calculated by the following equation (Zhang et al, 2014)

\[
\text{Free 25 hydroxyvitamin D} = \frac{\text{Total 25 hydroxyvitamin D}}{1 + (6 \times 10^3 \times \text{albumin}) + (7 \times 10^8 \times \text{VDBP})}
\]

Student’s \(t\) test was performed to compare mean calculated free 25-hydroxyvitamin D concentration between the GDM and control group. Multivariate linear regression analysis with backwards selection using the likelihood ratio criteria as performed in the main study was performed to determine the relationship between free 25-hydroxyvitamin D.

Results

32 cases of GDM were matched with 148 controls. Of the GDM cases 21 were New Zealand, six Indian and two Asian women developed GDM. Only one person from the Maori, Pacific Island and African ethnic groups developed GDM.
Mean free 25-hydroxyvitamin D was not different between GDM cases and controls ($p = 0.1$) (Table 4.4).

<table>
<thead>
<tr>
<th></th>
<th>GDM $n = 32$</th>
<th>Controls $n = 148$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>30.1 ± 5.1</td>
<td>30.4 ± 4.7</td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td>27.7 ± 5.0</td>
<td>27.1 ± 5.4</td>
</tr>
<tr>
<td><strong>Total 25-hydroxyvitamin D nmol/L (± SD)</strong></td>
<td>61.6 ± 6</td>
<td>55.7 ± 24.7</td>
</tr>
<tr>
<td><strong>Free 25-hydroxyvitamin D pmol/L (± SD)</strong></td>
<td>28.2 ± 20.9</td>
<td>23.1 ± 15.0</td>
</tr>
</tbody>
</table>

**Discussion**

Consistent with the findings presented in the main body of this chapter, free 25-hydroxyvitamin D at 15 weeks gestation was not associated with the development of GDM.

This study was limited by the small number of GDM cases as discussed in the main text of this chapter. The other important limitation of this study was that the calculation of free 25-hydroxyvitamin D was made on the assumption that all participants had the same VDBP genotype. This is important because it is known that VDBP genotypes have varying affinity for 25-hydroxyvitamin D (Powe et al., 2013) and knowing the genotype of an individual would alter the calculation for free 25-hydroxyvitamin D.

The prevalence of VDBP variants has not been studied in a New Zealand population since 1989 (White et al., 1989). The frequencies of three common alleles were studied in an international survey including 54 Maori, 40 Pacific Island New Zealanders and 172 New Zealand Europeans. This is not likely to capture the diversity of genotypes in the New Zealand population given there are over a hundred known alleles and variations are present in homogenous populations for example, a recent study of 770 children from one ethnic group identified 24 variants (Fu et al., 2016).

The findings of this case control study suggest free 25-hydroxyvitamin D does not have a stronger association with GDM than total 25-hydroxyvitamin D.
Chapter 5  The relationship maternal vitamin D status in pregnancy and offspring outcomes in childhood

5.1 Preface

The following section contains a manuscript that is under review by the International Journal of Obesity, titled “The relationship between maternal 25-hydroxyvitamin D status in pregnancy and childhood adiposity and allergy: An observational study”.

By the time a woman has been diagnosed with GDM the fetus has been exposed to hyperglycaemia for several weeks. There is evidence that maternal hyperglycaemia may predispose the fetus to developing obesity later in life. Preventing GDM, therefore, may reduce childhood adiposity.

In the previous chapter no relationship was found between maternal vitamin D status at 15 weeks gestation and GDM. However, glycaemia is a continuum, and the effects of maternal glycaemia are known to have an effect on pregnancy outcome before the threshold for GDM is reached (The HAPO Study Cooperative Research Group, 2008).

While not directly related to the hypothesis of this thesis, asthma and eczema are included as outcomes in this study. As with childhood obesity, these conditions are on the rise (Asher et al, 2006), coinciding with a re-emergence of vitamin D deficiency (Mithal et al, 2009). Exposures in utero are known to be associated with asthma risk, for example, maternal smoking is associated with an increased likelihood of developing asthma in childhood after controlling for post-natal exposure (Neuman et al, 2012). Cells of the immune system are able to both activate (Townsend et al, 2005) and respond to vitamin D (Nagpal et al, 2005).

The enzyme that activates vitamin D in immune cells, 1-α hydroxylase, is not regulated by parathyroid hormone in immune cells, and may therefore be more sensitive to local substrate concentrations. Amongst a number of immunomodulatory effects, vitamin D promotes T helper 2 cell (Th2) proliferation and maturation (Boonstra et al, 2001). Th2 cells regulate eosinophils, basophils mast cells and IgE producing B cells (Toscano et al, 2007).

Critical steps in the development of the immune systems occur during pregnancy (Chidgey and Boyd, 2001). Furthermore, an association between vitamin D status in children and
developing asthma (Rajabbik et al, 2014) and vitamin D deficiency has been found to be more prevalent in children with eczema than in those without eczema (Wang et al, 2014). Low maternal dietary intake of vitamin D during pregnancy has been associated with a higher likelihood of developing asthma in childhood (Camargo et al, 2007).
5.2 Maternal vitamin D status and offspring outcomes

The relationship between maternal 25-hydroxyvitamin D status in pregnancy and childhood adiposity and allergy: An observational study

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10. The College of Medicine, Biosciences and Psychology, University of Leicester, UK
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Abstract

Background

Vitamin D insufficiency (defined as <75 nmol/L) is widespread amongst pregnant women around the world and has been proposed to influence offspring outcomes in childhood and into adult life, including adiposity and allergy. Disorders including asthma and eczema are on the rise amongst children. Our aim was to investigate the relationship between maternal 25-hydroxyvitamin D status in pregnancy and offspring adiposity, asthma and eczema in childhood.

Subjects and Methods

Maternal 25-hydroxyvitamin D concentrations were analysed in serum samples collected at 15 weeks gestation from 1710 participants of the prospective Screening for Pregnancy Endpoints (SCOPE) cohort study. The offspring of 1208 mothers were followed up at age 5 - 6 years. Data collected included height, weight, percentage body fat (PBF, measured by bioimpedence) and history of asthma and eczema. Multivariate analysis controlled for maternal BMI, age and sex of the child and season of serum sampling.

Results

Complete data were available for 922 mother-child pairs. Each 10 nmol/L increase in maternal 25-hydroxyvitamin D concentration at 15 weeks gestation was associated with a decrease in offspring PBF of 0.2% (95% CI 0.04 - 0.36% p=0.01) after adjustment for confounders, but was not related to child BMI z-score. Maternal mean (±SD) 25-hydroxyvitamin D concentration was similar in children who did and did not have asthma (71.7 ± 26.1 vs 73.3 ± 27.1 nmol/L p = 0.5), severe asthma (68.6 ± 28.6 vs 73.3 ± 26.8 nmol/L p = 0.2) and eczema (71.9 ± 27.0 vs 73.2 ± 27.0 nmol/L p = 0.5).

Conclusions

The finding of a relationship between maternal vitamin D status and adiposity in childhood is important, particularly because vitamin D insufficiency in pregnancy is highly prevalent. The association between maternal vitamin D supplementation in pregnancy and adiposity in the offspring merits examination in randomised controlled trials.
5.2.1 Introduction

Many critical processes in fetal development occur at the end of the first trimester and beginning of the second trimester of pregnancy and these could be influenced by maternal vitamin D status. Between 10 and 17 weeks gestation processes in immune cell development include negative selection to remove auto-reactive cells (Chidgey and Boyd, 2001) and migration from primitive generation sites such as the fetal liver to the bone marrow and lymph tissue. It is at this time that the vitamin D activating enzyme, 1-α hydroxylase, is at the highest concentration in the placenta (Zehnder et al, 2002).

Many tissues besides kidney and intestine are able to activate and respond to vitamin D, including adipose precursor cells (Nimitphong et al, 2012) and cells of the immune system (Nagpal et al, 2005, Townsend et al, 2005). In tissues outside of the kidney, 1-α hydroxylase is not regulated by parathyroid hormone, and may therefore be more sensitive to local substrate concentrations.

Obesity in childhood is on the rise (Stamatakis et al, 2010) coinciding with a re-emergence of vitamin D deficiency (Mithal et al, 2009). Obese children will most likely stay obese into adulthood (Serdula et al, 1993) and some will suffer from the consequences of obesity related disease, such as metabolic syndrome in childhood (Steinberger et al, 2001). We are beginning to understand how early life conditions predispose to obesity (Reilly et al, 2005) and how adipose tissue is an active participant in the regulation of food intake and metabolic regulation (Jung and Choi, 2014). Vitamin D promotes adipocyte maturation and inhibits adipocyte proliferation (Nimitphong et al, 2012). An in utero insult such as vitamin D deficiency may therefore set a life-course towards fat accumulation.

Asthma and eczema are also on the increase (Asher et al, 2006), and there are mechanisms by which vitamin D deficiency may play a role. Vitamin D has a number of immunomodulator effects (Correale et al, 2009) and promotes T helper 2 cell (Th2) proliferation and maturation (Boonstra et al, 2001). Th2 cells regulate eosinophils, basophils mast cells and IgE producing B cells (Toscano et al, 2007). Vitamin D has been shown to reduce B cell proliferation and reduce IgG production in adult autoimmune disease, and alter immune cell differentiation and signalling between mature immune cells. (Mattner et al, 2000, Chen et al, 2007). An association between maternal 25-hydroxyvitamin D concentration during pregnancy and the development of the autoimmune disease type 1 diabetes mellitus in childhood has been found.
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(Sørensen et al., 2012). Exposure to vitamin D deficiency in utero may also predispose to allergic hypersensitivity in childhood.

The aim of this study was to investigate the relationship between vitamin D status in pregnancy at the beginning of the second trimester of pregnancy in a cohort of low risk first time mothers and the development of allergic disease and obesity in their children between 5 and 6 years of age.

5.2.2 Methods

Participants – Mothers

The Screening for Pregnancy Endpoints Study (SCOPE) is an international prospective cohort study which aimed to identify early pregnancy predictors of late pregnancy complications in women in their first ongoing pregnancy (North et al., 2011). Only SCOPE Auckland participants were included in this study. Auckland participants were recruited between 2005 and 2008. The study was approved by the Health and Disability Ethics Committee (AKX/02/00/364).

Women were interviewed at the first SCOPE research visit at 15 weeks gestation. Data collected included weight and height to the nearest kg and cm from which BMI was calculated. Non-fasting serum samples were also collected and stored at -80°C for later processing. SCOPE study data collection has been described in detail elsewhere (North et al., 2011).

Participants - Children

Women were asked at the first visit whether they were happy to be contacted for a follow-up study (Children of SCOPE). These women were then contacted again 5-6 years postpartum to seek consent to participate in the follow up study of their children. Children aged between 5 and 6.25 years were eligible to participate if they did not meet any of the exclusion criteria. The exclusion criteria were major chromosomal abnormality such as Down’s syndrome, a major congenital abnormality such as cyanotic heart disease or diaphragmatic hernia, or other chronic illness that interferes with growth such as type 1 diabetes mellitus or inflammatory bowel disease. The study was approved by the Health and Disability Ethics Committee (NTX/10/10/106).
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The aim of the follow up study was to identify early life modifiable determinants of obesity and insulin resistance in children, with emphasis on factors occurring during pregnancy. The age of 5 years was identified as the follow-up period because, unlike at a younger age, parameters such as adiposity are predictive of long term health outcomes (Guo et al., 1994). It is also early enough that health trajectories may be malleable to change with intervention. Data were collected at a single visit and entered into a secure internet-accessed auditable database (MedSciNetAB, Stockholm, Sweden). Data is accessible following application to the SCOPE consortium (www.scopestudy.net). Parents were interviewed and children were examined.

Outcome variables

Children’s height was measured by stadiometer (SECA 213) to the nearest 1mm. Weight was measured in light clothing without shoes to the nearest 0.1kg and BMI calculated (weight/height squared). BMI was converted to a z-score according to the LMS method (Cole et al., 1995). Lean body mass was estimated using bioelectrical impedance (ImpediMed Imp SFB7). As the equation used in the calculations in the SFB7 software is not validated on children we extracted the data from the 50Khz reading (as used in previous versions of the ImpediMed machines) and calculated fat free mass (FFM) according to a validated equation (Schaefer et al., 1994). The equation used to calculate FFM was: FFM = 0.65(height^2/impedance) + 0.68 x age + 0.15. Percentage body fat (PBF) was calculated using (weight-FFM/weight x 100).

Current wheezing was identified by a positive response to the question: “In the past 12 months, has your child/have you had wheezing or whistling in the chest?” (Asher et al., 1995). If the participant answered ‘yes’ further questions followed to determine the severity of asthma:

- In the last 12 months:
  - How many attacks of wheezing has your child had?
  - Has wheezing ever been severe enough to limit your child’s speech to only one or two words at a time between breaths?
  - How often, on average, has your child’s sleep been disturbed due to wheezing?

Severe asthma was defined by fulfilling one of the following criteria:
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4 or more attacks of wheezing in the past 12 months

Wheezing limiting speech to one or two words at a time between breaths in the past 12 months

Sleep disturbance due to wheezing, occurring one or more nights per week in the past 12 months

Current eczema was identified by a positive answer to each of the following questions:

“Has your child ever had an itchy rash, which was coming and going for at least 6 months?” and

“If yes, has your child had this itchy rash at any time in the past 12 months?” and

“If yes, has this itchy rash at any time affected any of the following places – the folds of the elbows, behind the knees, in front of the ankles, under the buttocks, or around the neck, ears, or eyes?”

25-hydroxyvitamin D analysis

25-hydroxyvitamin D$_3$ and 25-hydroxyvitamin D$_2$ were analysed by liquid chromatography tandem mass spectrometry (Finnigan TSQ Quantumn Ultra AM triple quadrupole mass spectrometer, Thermo Electron Corporation, San Jose, CA, USA). Samples were prepared using Phree phospholipid removal plates (Phenomenex, Torrance, CA, USA). Chromatographic separation of the 25-hydroxyvitamin D$_3$ stereoisomers was achieved with a pentafluorophenyl (PFP) 100 x 3mm 2.6 µm Kinetex HPLC column (Phenomenex, Torrance, CA, USA). The intra- and inter-assay coefficients of variation were 7.3% and 4.9% for 25-hydroxyvitamin D$_3$ and 8.2% and 5.8% for 25-hydroxyvitamin D$_2$, respectively. Quality control was through participation in the DEQAS program operating out of Charing Cross Hospital, London (Carter et al, 2010) and the use of commercially available control materials of known vitamin D concentration.
**Chapter 5**

*Statistical analysis*

Continuous variables are reported as mean ± standard deviation. Total 25-hydroxyvitamin D was analysed as a continuous and categorical variable. Cut-offs for vitamin D as a categorical variable were 50nmol/L and 75nmol/L based upon the Institute of Medicine and the Endocrine Society clinical practice guideline definitions of vitamin D deficiency and insufficiency, respectively (Holick *et al.*, 2011, Ross *et al.*, 2011).

T-tests were performed to investigate the relationship between maternal 25-hydroxyvitamin D concentration as a continuous variable and asthma and eczema, and between vitamin D as a categorical variable and child (PBF) and child BMI z-scores. Linear regression analysis was performed to investigate the relationship between 25-hydroxyvitamin D as a continuous variable and i) child PBF and ii) child BMI z-score. Multivariate linear regression was performed to control for maternal BMI at 15 weeks gestation, ethnicity, maternal smoking during pregnancy, socioeconomic status, sex of the child and age at sampling, and season of sample collection.

### 5.2.3 Results

The SCOPE study was completed by 2065 Auckland women. Of these, 4% were excluded due to loss to follow up (n=23), fetal or neonatal death (n=27) or major congenital abnormalities (n=34). The remaining 1981 children were eligible for the Children of SCOPE follow-up study. From this group 1208 children were followed up at 5 to 6 years of age (58%). Maternal serum samples were available for 25-hydroxyvitamin D analysis from 1715 women, child BMI z-scores from 1207 children and PBF from 1176 children (Figure 5.1). Mothers whose children participated in Children of SCOPE were older than those who participated in SCOPE but were not followed up at 6 years by an average of 1.6 years ($p = 0.001$), a smaller proportion were smokers (9.4% vs 15.9% $p = <0.0001$) and a greater proportion were NZ European (87.5% vs 78.8% $p = 0.001$) (Table 5.1). The mother’s BMI at 15 weeks gestation was similar in those who were and were not followed up (24.7 ± 4.2 and 24.9 ± 4.3 kg/m², $p = 0.46$). Infant birth weight was higher in children followed up than in those who were not (3445 ± 533 vs 3345 ± 728 g $p = 0.0004$) but customised birth centile was similar (49.0 ± 28.1 vs 48.2 ± 30.3 $p = 0.55$) ( 
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Table 5.2).
2065 Auckland SCOPE participants

1710 25-hydroxyvitamin D analysed at 15 weeks gestation serum samples

1208 children followed up at 6 years of age

1208 child BMI z-score

1176 child percentage body fat

922 child BMI z-score and maternal 25-hydroxyvitamin D

900 child percentage body fat and maternal 25-hydroxyvitamin D

Figure 5.1 Children of SCOPE study flow diagram
Table 5.1 Characteristics comparing mothers whose children undertook the Children of SCOPE assessment compared to those not taking part in the follow-up study

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Participants of Children of SCOPE n = 1208</th>
<th>Those not participating in follow-up n = 857</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at child’s birth, years</td>
<td>31.0 ± 4.4</td>
<td>29.4 ± 5.0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Ethnicity n (%)</td>
<td></td>
<td></td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>- Asian</td>
<td>47 (3.9)</td>
<td>60 (7.3)</td>
<td></td>
</tr>
<tr>
<td>- NZ European</td>
<td>1058 (87.5)</td>
<td>649 (78.8)</td>
<td></td>
</tr>
<tr>
<td>- Maori</td>
<td>17 (1.4)</td>
<td>15 (1.8)</td>
<td></td>
</tr>
<tr>
<td>- African/other</td>
<td>34 (2.8)</td>
<td>33 (4.0)</td>
<td></td>
</tr>
<tr>
<td>- Indian</td>
<td>40 (3.3)</td>
<td>37 (4.5)</td>
<td></td>
</tr>
<tr>
<td>- Pacific Island</td>
<td>12 (1.0)</td>
<td>30 (3.6)</td>
<td></td>
</tr>
<tr>
<td>BMI at 15 weeks’ (kg/m²)</td>
<td>24.7 ± 4.2</td>
<td>24.9 ± 4.3</td>
<td>0.46</td>
</tr>
<tr>
<td>Smoked in pregnancy, n (%)</td>
<td>113 (9.4)</td>
<td>131 (15.9)</td>
<td>0.02</td>
</tr>
<tr>
<td>Multivitamin supplementation during pregnancy, n (%)</td>
<td>681 (56.9)</td>
<td>426 (52.2)</td>
<td>0.036</td>
</tr>
<tr>
<td>Mean 25-hydroxyvitamin D concentration at 15 weeks’ (nmol/L)</td>
<td>73.0 ± 26.9</td>
<td>70.1 ± 27.1</td>
<td>0.036</td>
</tr>
<tr>
<td>Vitamin D Status n (%)</td>
<td></td>
<td></td>
<td>0.16</td>
</tr>
<tr>
<td>&lt; 25nmol/L</td>
<td>36 (3.0)</td>
<td>40 (5.7)</td>
<td></td>
</tr>
<tr>
<td>25-49.9 nmol/L</td>
<td>167 (13.6)</td>
<td>125 (15.7)</td>
<td></td>
</tr>
<tr>
<td>50-74.9 nmol/L</td>
<td>302 (25.0)</td>
<td>205 (29.2)</td>
<td></td>
</tr>
<tr>
<td>≥75 nmol/L</td>
<td>502 (42.9)</td>
<td>333 (47.4)</td>
<td></td>
</tr>
</tbody>
</table>

Missing data – smoking n = 2, multivitamin supplementation n = 20, maternal 25-hydroxyvitamin D n = 483
Continuous variables presented as means ± standard deviation
BMI – body mass index
NZ – New Zealand
Table 5.2 Characteristics of Children of SCOPE participants for mothers with and without data on maternal vitamin D status

<table>
<thead>
<tr>
<th></th>
<th>Maternal Vitamin D data n = 921</th>
<th>No maternal vitamin D data n = 284</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight (g)</td>
<td>3445 ± 533</td>
<td>3345 ± 728</td>
<td>0.0004</td>
</tr>
<tr>
<td>Customised birth weight percentile</td>
<td>49.0 ± 28.1</td>
<td>48.2 ± 30.0</td>
<td>0.55</td>
</tr>
<tr>
<td>Age (years)</td>
<td>5.96 (0.19)</td>
<td>5.94 (0.19)</td>
<td>0.15</td>
</tr>
<tr>
<td>BMI z-score</td>
<td>0.15 ± 0.97</td>
<td>0.03 ± 0.88</td>
<td>0.07</td>
</tr>
<tr>
<td>Percentage body fat (%)</td>
<td>23.0 ± 6.5</td>
<td>22.1 ± 6.5</td>
<td>0.06</td>
</tr>
<tr>
<td>Asthma, n (%)</td>
<td>160 (17.4)</td>
<td>50 (17.5)</td>
<td>0.94</td>
</tr>
<tr>
<td>Severe asthma, n (%)</td>
<td>54 (5.9)</td>
<td>19 (6.7)</td>
<td>0.62</td>
</tr>
<tr>
<td>Eczema, n (%)</td>
<td>118 (12.7)</td>
<td>36 (12.6)</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Continuous variables presented as means ± standard deviation
Mean PBF was higher in the children with maternal 25-hydroxyvitamin D concentration < 50 nmol/L compared to ≥ 50 nmol/L (23.9% ± 6.8 vs 22.7% ± 6.3 vs p=0.02). It was similar between children with maternal 25-hydroxyvitamin D concentration < 75 nmol/L and ≥ 75 nmol/L (23.3% ± 6.6 vs 22.6% ± 6.3 p=0.09). In multivariate linear regression analysis, adjusting for maternal BMI, season of sample collection, child’s age and sex, each 10 nmol/L decrease in maternal 25-hydroxyvitamin D was associated with an increase in child PBF of 0.18% p = 0.03 (Table 5.3). Similarly, this association was seen with body fat (70g increase per 10 nmol/L decrease in maternal 25-hydroxyvitamin D concentration) but there were no associations with lean mass, weight, height or BMI z-score (Table 5.3). After adjustment for ethnicity this effect was no longer significant (10nmol/L decrease in maternal 25-hydroxyvitamin D associated with a 0.12% increase in child PBF 95% CI 0.28% increase to 0.05% decrease in PBF). Maternal smoking during pregnancy and socioeconomic status were not independently associated with child percentage body fat (p = 0.2 and p = 0.4 respectively) and were not included in the multivariate analysis.

| Table 5.3 Univariate and multivariate analyses of maternal vitamin D at 15 weeks gestation and child anthropometry at 6 years of age represented as change in child parameters for a 10 nmol/L increase in maternal 25-hydroxyvitamin D concentration |
|---|---|---|---|
| | Univariate (95% CI) | P value | Multivariate* (95% CI) | P value |
| Percentage body fat | | | | |
| (n=900) | -0.18 | 0.03 | -0.2 | 0.01 |
| | (-0.33, -0.02) | | (-0.36, -0.04) | |
| Body fat (kg) | | | | |
| (n=900) | -0.07 | 0.01 | -0.07 | 0.01 |
| | (-0.12, -0.02) | | (-0.12, -0.02) | |
| Lean mass (kg) | | | | |
| (n=900) | -0.01 | 0.79 | 0.01 | 0.55 |
| | (-0.05, -0.04) | | (-0.03, 0.06) | |
| BMI z-score | | | | |
| (n=922) | -0.01 | 0.23 | -0.01 | 0.41 |
| (n=922) | (-0.04, 0.01) | | (-0.01, 0.01) | |

*Multivariate model controls for maternal BMI at 15 weeks gestation, child age and sex, and season of serum sampling
Mean BMI z-score was similar in children with maternal 25-hydroxyvitamin D concentrations above and below 50 nmol/L (0.12 ± 0.90 vs 0.27 ± 1.13 p = 0.06) and likewise for concentrations above and below 75 nmol/L (0.11 ± 0.10 vs 0.19 ± 1.00 p = 0.2). Linear regression analysis showed no significant association between BMI Z-score and maternal total 25-hydroxyvitamin D analysed as a continuous variable (Table 5.3).

Mean maternal 25-hydroxyvitamin D was similar between children who had asthma and those who did not (71.7 ± 26.1 vs 73.3 ± 27.1 nmol/L p = 0.5) and between those with severe asthma and those who did not have severe asthma (68.6 ± 28.6 vs 73.3 ± 26.8 nmol/L p = 0.2). Likewise, mean maternal 25-hydroxyvitamin D concentration was not significantly different between children who had eczema and those who did not (71.9 ± 27.0 vs 73.2 ± 27.0 nmol/L p = 0.5).

5.2.4 Discussion

Adjusting for maternal BMI, and child age and sex, we found that lower maternal 25-hydroxyvitamin D concentration at 15 weeks gestation was associated with higher PBF in children aged 6 years. PBF in childhood predicts BMI and metabolic risk in adulthood (Serdula et al., 1993) and it is widely acknowledged that small changes at an individual level can result in important changes in disease incidence at a population level (Rose, 1981). We found no relationship between maternal vitamin D status and asthma or eczema in their children.

Our findings are consistent with others who have found an association between lower maternal 25-hydroxyvitamin D concentrations and greater fat mass in childhood (Crozier et al., 2012, Hrudey et al., 2015). Our study differs from Crozier et al in that we analysed our maternal samples at 15 weeks gestation, 6 months before term delivery, while they analysed 25-hydroxyvitamin D at 34 weeks gestation. This finding suggests that 25-hydroxyvitamin D concentration throughout pregnancy may be important in relation to childhood adiposity. While others have found an association between maternal vitamin D deficiency in the third trimester and greater adiposity at one year, this relationship did not remain significant at 4 years of age (Morales et al., 2015).

Vitamin D is best known for its role in increasing intestinal calcium absorption and the effects of vitamin D on percentage body fat (PBF) may be mediated through calcium. Studies in rat models of adult obesity have shown that high calcium diets reduce PBF (Nobre et al., 2011,
Lopes Nobre et al., 2012). Randomised trials of calcium supplementation in pregnancy have not reported neonatal or childhood adiposity; however the findings of a Cochrane systematic review found on pooled analysis of 19 trials an increase in birth weight with supplementation (mean difference 64.66 g 95% CI 15.75 to 113.58 g) (Buppasiri et al., 2011). Furthermore, it is uncertain whether vitamin D facilitates calcium transport across the placenta and vitamin D may be having a direct effect upon adipose tissue. The active form of vitamin D, 1,25 dihydroxyvitamin D has been shown to prevent adipogenesis in vitro by suppressing adipogenic factors such as PPARγ (Kong and Li, 2006) and lowering lipid accumulation (Ji et al., 2015, Kim et al., 2016).

We did not find a significant association between maternal vitamin D status and BMI z-score. The likely explanation for this is that there was a relationship between maternal 25-hydroxyvitamin D and child fat mass but not lean body mass. Lean body mass makes up between 70 - 80% of the weight of a 7 year old child. The contribution of lean body mass to total weight, blunted the relationship between maternal vitamin D status and BMI and BMI Z-score. BMI is dynamic through childhood (Goldhaber-Fiebert et al., 2013) and while standardising for age by calculating BMI z-scores improves applicability, PBF is a more direct measure of childhood adiposity (Field et al., 2003). The limitations of BMI as a measure of adiposity in children is well known (Mei et al., 2002). PBF as determined by DEXA is the gold standard for measuring adiposity (Heymsfield et al., 1990). Cost, machine availability and acceptability to participants (a significant proportion of parents decline DEXA because of the exposure to X-rays) considerations led us to measure body fat using bioimpedance. In children, body fat and percentage body fat as determined by bioimpedance has been shown to correlate more closely with body fat and percentage body fat as determined by DEXA than BMI and BMI z-score (Boeke et al., 2013, Ejlerskov et al., 2014).

We found no relationship between maternal vitamin D status and asthma. This is consistent with follow up studies from 2 recent randomised controlled trials. Participants of the Vitamin D Antenatal Asthma Reduction Trial (VDAART) were given either 4400 IU of vitamin D daily or 400 IU daily from 10 to 18 weeks gestation through pregnancy (Litonjua et al., 2016). In a trial in Copenhagen, women were randomised to receive 2800 IU of vitamin D or 400 IU daily from 24 weeks gestation (Chawes et al., 2016). Between these two studies 1391 children were followed up at three years of age. The prevalence of asthma or wheeze in these children was not statistically significant between those who received the higher and lower doses of vitamin D in either of those studies. A recent meta-analysis of observational studies
investigating the relationship between maternal vitamin D status in pregnancy and childhood asthma and wheeze at 5 to 6 years of age found no relationship in combined analysis (pooled OR 0.98 95% CI 0.94 - 1.02 and Pooled OR 1.00 95% CI 0.98 – 1.01) (Wei et al, 2016). An earlier observational study not included in the meta-analysis found associations between maternal vitamin D intake during pregnancy and childhood wheezing at 5 years but not at 2 years (Devereux et al, 2007).

The same meta-analysis reviewed studies investigating the relationship between maternal vitamin D status and offspring eczema (Wei et al, 2016). They found a reduced likelihood of childhood eczema for each unit increase in maternal 25-hydroxyvitamin D in children assessed between 1 and 5 years old (Pooled OR 0.944 95% CI = 0.831 – 0.983). We found no such relationship.

Findings from recent observational studies not included in the meta-analysis are also conflicting. Participants in a UK study who had a maternal 25-hydroxyvitamin D concentrations > 75nmol/L during pregnancy were more likely to have infants who had eczema at 9 months age compared to mothers with concentrations < 30nmol/L (OR 3.26, 95% CI 1.15-9.29) (Gale et al, 2007). The Generation R study found no correlation between maternal 25-hydroxyvitamin D concentration mid gestation and cord 25-hydroxyvitamin D and the development of eczema before age 4 (Gazibara et al, 2016). Another study found in children with a family history of allergic disease however a higher cord blood 25-hydroxyvitamin D concentration was associated with reduced eczema in childhood (Palmer et al, 2015). And finally, maternal dietary vitamin D intake during the first and second trimester of pregnancy was associated with a reduction in allergic rhinitis in school age children but there was no association between maternal 25-hydroxyvitamin D serum concentration or maternal vitamin D supplementation and allergic rhinitis (Bunyavanich et al).

The major limitation of this study is that we have no data to adjust for some possible additional confounding variables. These include maternal exercise, maternal diet and sunlight exposure during pregnancy. Multivitamin supplementation was not included in the analysis due to the data not being available at the time of writing the thesis. Additional factors known or suggested to affect childhood adiposity include the child’s own exercise levels and dietary patterns. Participants in SCOPE were self-selected and were not representative of all pregnant women in Auckland. While not all of the mothers and children from the SCOPE study were
followed up, because responders were more likely to be older, better educated, non-smokers we believe the findings are more likely to be conservative and likely to be at least as relevant to those who did not take part. Participants were also more likely to be NZ European (87%). Ethnicity influences vitamin D status in both skin pigmentation and cultural practice around sun exposure as well as influencing adiposity. Given the small numbers of individuals making up the remaining ethnic groups, and the diverse ways in which ethnicity interacts with vitamin D status and adiposity makes interpretation difficult. Ideally for the diagnosis of asthma and eczema in this study, all participants would have lung function tests and assessment by a physician. This is not always practical in large studies and because signs maybe transient. On the other hand self-reports and questionnaires have been shown to correlate well with formal examination for example, in a comparison to a respiratory physician diagnosis the ISAAC questionnaire had a sensitivity of 0.85 (95% CI 0.73, 0.93) and specificity of 0.81 (95% CI 0.76, 0.86) in children (Jenkins et al, 1996).

The finding that lower maternal 25-hydroxyvitamin D concentration is associated with higher PBF in children is important at a time when obesity is so prevalent. A small reduction in adiposity of the individuals has the potential for large population benefits. Our findings support the inclusion of formal measurements of adiposity in the children of participants of randomised trials of vitamin D supplementation in pregnancy (Cooper et al, 2016).
Chapter 6  

The relationship between maternal vitamin D status in pregnancy and maternal long-term metabolic outcomes

6.1 Preface

The following section contains a manuscript prepared for submission to be published “Vitamin D status in first pregnancy is associated with adiposity 6 years post-partum”.

The finding of a relationship between maternal vitamin D status in pregnancy and childhood adiposity has the potential to be of great importance at the population level. This finding lead to another investigation, described here, of a relationship between maternal vitamin D status in pregnancy and increasing adiposity in the following 5-6 years.

It has been estimated in the USA and the UK alone obesity related diseases will cost 48 to 66 billion US dollars a year. Most of this burden is a consequence of the metabolic disturbances that occur with increased adiposity. These conditions include hypertension, impaired glucose tolerance and type 2 DM, dyslipidaemia and when occurring together are called the metabolic syndrome (Alberti et al, 2006). The metabolic dysfunction that occurs with increased adiposity is a major contributor to the increased risk of mortality associated with obesity (Mark and Emmanuel, 2012).
6.2 Maternal vitamin D status in pregnancy and outcomes 6 years post-partum

Vitamin D status in first pregnancy is associated with adiposity 6 years post-partum

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*Joint last author
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Abstract

An inverse relationship between vitamin D status and adiposity has been described. This study investigates whether maternal vitamin D status in pregnancy is associated with subsequent BMI, BMI change, skin fold thickness and the development of later metabolic syndrome six years post-partum.

Methods

25-hydroxyvitamin D concentration was analysed at 15 weeks gestation in Auckland participants of the Screening for Pregnancy Endpoints (SCOPE) study. Women were followed up 6 years later at which time weight, waist circumference, skin fold thickness and blood pressure were measured and fasting glucose and lipids were analysed. Metabolic syndrome was categorised by the International Diabetes Federation definition.

Results

Data from 1011 women were available for analysis. In multivariate analysis vitamin D status in early pregnancy was significantly associated with subsequent BMI change (coefficient -0.2 95% CI -0.3, -0.03), and skin fold thickness at the triceps (-0.4 95% CI -0.8, -0.03), biceps (-0.4 95% CI -0.7, -0.1) and subscapular (-0.4 95% CI -0.8, -0.02) 6 years later. We did not detect an association between vitamin D status in pregnancy and the development of metabolic syndrome (p = 0.09).

Conclusion

We found a significant negative relationship between vitamin D status in pregnancy and BMI change 6 years post-partum. In the context of the obesity epidemic our findings raise the possibility that vitamin D supplementation in pregnancy should be explored as a strategy to reduce later obesity.
6.2.1 Introduction

It has long been recognised that increased adiposity is associated with lower plasma and serum concentrations of 25-hydroxyvitamin D, the measure by which vitamin D status is determined (Looker, 2005, Dong et al, 2010, Earthman et al, 2012). It was commonly thought that this inverse relationship was due to sequestering of fat soluble vitamin D in adipose tissue (Wortsman et al, 2000), and possibly differences in behaviour such as less outdoor activity and less skin exposure to sunshine in obese individuals (Hyppönen and Power, 2007).

Metabolic syndrome is a cluster of conditions that confer a greater risk of developing cardiovascular disease and type 2 diabetes mellitus (Alberti et al, 2009). These include, reduced plasma high density lipoprotein and elevated plasma triglycerides, waist circumference, blood pressure and fasting glucose. Metabolic syndrome has also been found to be associated with lower 25-hydroxyvitamin D concentrations in cross-sectional studies, with BMI matched controls (Ford et al, 2005).

The prevalence of obesity and metabolic syndrome is increasing. More than half of the adult population is overweight in more than half the countries of the world (International Food Policy Research Institute, 2016). An association between obesity and type 2 diabetes mellitus, cardiovascular disease and all-cause mortality has been established (Klein et al, 2004, Flegal et al, 2013). Indeed, obesity is associated with an increase in all-cause mortality, even without features of metabolic syndrome (Kuk and Ardern, 2009).

It has been demonstrated that a change in adiposity has an influence on vitamin D status. Weight loss without vitamin D supplementation can increase serum 25-hydroxyvitamin D concentrations (Mallard et al, 2016). It is not known whether vitamin D supplementation can influence adiposity.

In the large Women’s Health Initiative clinical trial of 36,282 women, those randomised to receive calcium and vitamin D supplementation gained less weight over 2 years than women taking placebo (mean difference -0.13kg 95% confidence interval -0.21 to -0.05) (Caan et al, 2007). In contrast, a meta-analysis of 26 clinical trials of vitamin D supplementation in women, that included the Women’s Health Initiative trial, did not show an overall lower weight in supplemented women (Chandler et al, 2015). The other studies included in the meta-analysis differed greatly from the Women’s Health Initiative in their size (between 52 and
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870 participants) and duration of follow-up (median 12 months IQR 6 – 36 months) but not in the age of participants or intervention dose of vitamin D.

The prevalence of vitamin D deficiency is also increasing (Ginde et al, 2009). In some urban populations over 90% of individuals have 25-hydroxyvitamin D concentrations considered deficient by the American Endocrine Society (<50nmol/L) (Woo et al, 2008) (Hill et al, 2006). Oral vitamin D supplementation is inexpensive, and is effective at increasing 25-hydroxyvitamin D concentrations and treating vitamin D deficient rickets and osteomalacia. At an individual level, the effect of vitamin D on adiposity is expected to be small (Vimaleswaran et al, 2013) and if there is a benefit it is more likely to be relevant at a population level. Moreover, it is at the population level that there is a greater need for successful interventions to prevent obesity.

The aim of this study was to investigate whether 25-hydroxyvitamin D concentration in early pregnancy in a prospective cohort study was associated with weight gain and development of metabolic syndrome at six years after delivery of their first child. This is the first study to investigate the relationship between vitamin D status in pregnancy and subsequent change in maternal adiposity and development of metabolic syndrome.

6.2.2 Methods

Participants

The Screening for Pregnancy Endpoints Study (SCOPE) is an international prospective cohort study of low risk first time mothers. The original aim of the study was to develop early pregnancy screening tests for late pregnancy complications (North et al, 2011). Only SCOPE Auckland participants were included in this study. Auckland participants were recruited between 2004 and 2008. The study was approved by the Health and Disability Ethics Committee (AKX/02/00/364).

The SCOPE study has been described in detail elsewhere (North et al, 2011). In brief, at 15 weeks gestation, participants were interviewed by a research midwife and data were collected including height and weight, socioeconomic status (as determined by the New Zealand Socio-Economic Index of Occupational Status (Davis et al, 1997)), whether they took multivitamin supplements and which brand, smoking status and the amount and vigorousness of exercise. Non-fasting serum samples were also collected and stored at -80ºC for later processing. No samples went through a freeze/thaw cycle prior to analysis.
Women who agreed to long-term follow-up were contacted six years after first pregnancy to seek consent for further participation and that of their child. Women were excluded if their child from their SCOPE pregnancy had a major congenital abnormality that interfered with growth, if they suffered a fetal or neonatal death, or if they were currently pregnant. Measures of height and weight were taken to the nearest 1 cm and 0.1 kg respectively and BMI calculated. Other measurements included arm, waist and hip circumference, blood pressure, skin fold thickness over the triceps, biceps, subscapular and suprailliac regions. Fasting and non-fasting plasma and serum samples were collected. The maternal follow up study was approved by the Health and Disability Ethics Committee (NTX/10/10/106).

Data from the pregnancy study and follow-up study were entered into a secure internet-accessed auditable database (MedSciNet<sup>AB</sup>, Stockholm, Sweden). Data is accessible following application to the SCOPE consortium Steering Committee (www.scopestudy.net).

"Metabolic syndrome"

Metabolic syndrome was defined according to the International Diabetes Federation definition (Alberti <i>et al</i>, 2006). A woman was confirmed to have metabolic syndrome if she met the following criteria: obesity (BMI ≥ 30 kg/m<sup>2</sup>) or central obesity (waist circumference ≥ 80 cm), and any two of the following seven factors:

- raised fasting triglycerides (≥ 1.7 mmol/L),
- reduced fasting HDL cholesterol (< 1.29 mmol/L),
- prescribed lipid-lowering medications
- raised blood pressure (≥ 130 mmHg systolic or ≥ 85 mmHg diastolic)
- prescribed blood pressure lowering medications
- pre-diabetes or diabetes (classified by a fasting glucose ≥ 5.5 mmol/L or ≥ 7 mmol/L)
- glucose lowering medications

<i>25-hydroxyvitamin D analysis and lipid analysis</i>

25-hydroxyvitamin D<sub>3</sub> and 25-hydroxyvitamin D<sub>2</sub> were analysed by liquid chromatography tandem mass spectrometry (Finnigan TSQ Quantum Ultra AM triple quadrupole mass spectrometer, Thermo Electron Corporation, San Jose, CA, USA). Samples were prepared
using Phree phospholipid removal plates (Phenomenex, Torrance, CA, USA). Chromatographic separation of the 25-hydroxyvitamin D₃ stereoisomers was achieved with a pentafluorophenyl (PFP) 100 x 3mm 2.6 µm Kinetex HPLC column (Phenomenex, Torrance, CA, USA). The intra- and inter-assay coefficients of variation were 7.3% and 4.9% for 25-hydroxyvitamin D₃ and 8.2% and 5.8% for 25-hydroxyvitamin D₂, respectively. Quality control was through participation in the DEQAS program operating out of Charing Cross Hospital, London (Carter et al., 2010) and the use of commercially available control materials of known vitamin D concentration.

Cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol and triglycerides concentrations were analysed on a Hitachi 902 autoanalyser (Hitashi High Technologies corporation, Tokyo, Japan) by an enzymatic colorimetric assay (Roche, Manheim, Germany)

Statistics

All statistical analysis was performed in SPSS (IBM) v21. Normality was assessed by viewing the histogram. Continuous variables are reported as mean ± standard deviation. Chi-squared tests were performed for categorical variables.

A seasonal adjustment was made for 25-hydroxyvitamin D concentration. Participants were divided into months of collection and the 25-hydroxyvitamin D concentration was converted to a z score for that month. Analysis was performed using these z-scores.

Outcome variables included for analysis were BMI at 6 years follow-up, BMI change from 15 weeks gestation to 6 years post-partum, skinfold thickness, blood pressure, waist circumference and metabolic syndrome. Linear regression was used to investigate possible confounders for investigating the relationship between vitamin D z-score and outcome variables. Linear regression analysis was performed to determine whether vitamin D z-score maintained a significant relationship with outcome variables when confounders were taken into account. Potential confounders included age, ethnicity, baseline BMI, vigour and frequency of exercise, socioeconomic status, time from initial assessment to follow-up, and number of pregnancies following the index SCOPE pregnancy. Binary logistic regression analysis was performed to investigate the relationship between developing metabolic syndrome and vitamin D status.
6.2.3 Results

From Auckland, 2032 women participated in the SCOPE pregnancy cohort study. Because of sample availability serum 25-hydroxyvitamin D was analysed on 1716 women at 15 weeks gestation. Women were followed up at a median 5.9 years after delivery of their SCOPE pregnancy (minimum 4.9, maximum 7.0 years). Of the 1208 women followed up 6 years later, 25-hydroxyvitamin D concentrations were available for 1013. Complete biometric measurements were not available for two participants, leaving 1011 included in the final analysis Figure 6.1. Table 6.1 details the characteristics of those from the original SCOPE study who participated in follow-up and those who did not. Women who did not participate were more likely to be smokers and on average younger, non-European with lower 25-hydroxyvitamin D concentrations.
Auckland participants in SCOPE

Vitamin D status known

Follow-up at 6 years

Complete biometric data available

Complete data available to assess metabolic syndrome

2013

297 no vitamin D status available

1716

703 not followed up at 6 years

1013

2 missing biometric data

1011

475 did not have fasting bloods at follow-up

536

Figure 6.1 SCOPE maternal follow-up study flow diagram
Table 6.1 Comparison of SCOPE participants who were and were not followed up at 6 years

<table>
<thead>
<tr>
<th></th>
<th>Participants (n = 1011)</th>
<th>Non-participants (n = 702)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at child’s birth (years)</td>
<td>31.0 ± 4.4</td>
<td>29.4 ± 5.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NZ European ethnicity n (%)</td>
<td>881 (87.0)</td>
<td>556 (79.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI at 15 weeks (kg/m²)</td>
<td>24.8 ± 4.2</td>
<td>24.8 ± 4.3</td>
<td>0.9</td>
</tr>
<tr>
<td>Smoked in pregnancy n (%)</td>
<td>100 (9.9)</td>
<td>110 (15.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Multi Vitamin supplementation during pregnancy n (%)</td>
<td>605 (60.0)</td>
<td>394 (56.1)</td>
<td>0.1</td>
</tr>
<tr>
<td>25-hydroxyvitamin D concentration at 15 weeks (nnmol/L)</td>
<td>74.0 ± 26.8</td>
<td>71.4 ± 27.3</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Continuous variables reported as mean ± SD
BMI – Body Mass Index
NZ – New Zealand

Fasting blood samples, required to determine the presence of metabolic syndrome, were available from 630 participants of whom 536 had known 15 weeks gestation 25-hydroxyvitamin D concentrations. There was no difference in BMI change between those with and without fasting samples (0.7 ± 2.7 vs 0.7 ± 2.5 p = 0.7). A comparison of participants with and without fasting found the fasting group tended to be older with higher systolic blood pressure Table 6.2.
Table 6.2 Characteristics of SCOPE participants at 6 year follow-up comparing those with and without fasting samples to assess lipid and fasting glucose

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Follow-up study participants with fasting specimen</th>
<th></th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No (n = 475)</td>
<td>Yes (n = 536)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>30.5 ± 4.4</td>
<td>31.3 ± 4.5</td>
<td>0.004</td>
</tr>
<tr>
<td>Number of children following SCOPE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>293</td>
<td>331</td>
<td>0.9</td>
</tr>
<tr>
<td>2</td>
<td>87</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Follow-up BMI (kg/m²)</td>
<td>25.3 ± 5.1</td>
<td>25.7 ± 5.7</td>
<td>0.2</td>
</tr>
<tr>
<td>BMI &gt; 30 kg/m²</td>
<td>65 (13.8%)</td>
<td>86 (16.0%)</td>
<td>0.3</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>85.0 ± 11.5</td>
<td>85.9 ± 12.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Waist ≥ 80 cm n</td>
<td>274 (57.7%)</td>
<td>322 (60.0%)</td>
<td>0.9</td>
</tr>
<tr>
<td>Diastolic BP ≥ 85mmHg</td>
<td>15 (3.2%)</td>
<td>22 (4.1%)</td>
<td>0.4</td>
</tr>
<tr>
<td>Systolic BP ≥ 130mmHg</td>
<td>34 (7.2%)</td>
<td>66 (12.3%)</td>
<td>0.006</td>
</tr>
<tr>
<td>Normotensive but on an antihypertensive medication n</td>
<td>4 (0.8%)</td>
<td>9 (1.7%)</td>
<td>0.2</td>
</tr>
<tr>
<td>Low HDL cholesterol &lt;1.29 mmol/L n</td>
<td></td>
<td>105 (17%)</td>
<td></td>
</tr>
<tr>
<td>High triglycerides ≥ 1.7 mmol/L</td>
<td></td>
<td>33 (5%)</td>
<td></td>
</tr>
<tr>
<td>Fasting glucose ≥ 5.6 mmol/L n</td>
<td></td>
<td>45 (7%)</td>
<td></td>
</tr>
<tr>
<td>Metabolic syndrome n</td>
<td></td>
<td>46 (7.3%)</td>
<td></td>
</tr>
</tbody>
</table>

Continuous variables reported as mean ± standard deviation
BMI – body mass index, BP – blood pressure, HDL – high density lipoprotein
Six years following their first pregnancy, 482 (47.7\%) women had increased their weight by more than 1 kg and 358 (35.4\%) women had lost more than 1kg compared to their weight at 15 weeks gestation. Twenty-five participants had been diagnosed with hypertension. Thirteen women were on antihypertensive medications, and of these eleven were normotensive and two were hypertensive on assessment. Five women were diagnosed and on medications for type 2 diabetes mellitus.

In linear regression analysis, first pregnancy vitamin D z-score was associated with BMI at follow-up and BMI change (Table 6.3). These remained significant after adjustment for BMI at 15 weeks gestation, ethnicity, exercise vigour and frequency, time to follow-up and socioeconomic status. Data on the number of subsequent pregnancies was missing on 174 participants and was not included in multivariate analysis. 25-hydroxyvitamin D concentration and vitamin D z-score was not different between women who had one, two, three or no children following the SCOPE index pregnancy ($p = 0.5$ and $p = 0.3$ respectively). First pregnancy vitamin D z-score was also associated with skin fold thickness in the triceps, biceps and subscapular regions but not in the suprailiac region. Vitamin D z-score was not correlated with blood pressure or waist circumference (Table 6.3). We did not detect a difference in the mean vitamin D z-score between the women who developed metabolic syndrome and those who did not (-0.31 vs -0.03 $p = 0.09$).
Table 6.3 Unit change in surrogate measures of adiposity for unit change in vitamin D z-score in univariate and multivariate linear regression analysis

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted</th>
<th></th>
<th>Adjusted*</th>
<th></th>
<th>R² of multivariate model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coefficient</td>
<td>(95% confidence interval)</td>
<td>Coefficient</td>
<td>(95% confidence interval)</td>
<td></td>
</tr>
<tr>
<td>BMI at follow-up (kg/m²)</td>
<td>-0.4</td>
<td>(-0.7, -0.04)</td>
<td>0.03</td>
<td>-0.2</td>
<td>(-0.3, -0.03)</td>
</tr>
<tr>
<td>BMI change (kg/m²)</td>
<td>-0.2</td>
<td>(-0.4, -0.05)</td>
<td>0.01</td>
<td>-0.2</td>
<td>(-0.3, -0.03)</td>
</tr>
<tr>
<td>Skin fold thickness - triceps (cm)</td>
<td>-0.5</td>
<td>(1.0, -0.08)</td>
<td>0.02</td>
<td>-0.4</td>
<td>(-0.8, -0.03)</td>
</tr>
<tr>
<td>Skin fold thickness - biceps (cm)</td>
<td>-0.5</td>
<td>(-1.0, -0.2)</td>
<td>0.006</td>
<td>-0.4</td>
<td>(-0.7, -0.1)</td>
</tr>
<tr>
<td>Skin fold thickness - subscapular (cm)</td>
<td>-0.7</td>
<td>(-1.2, -0.2)</td>
<td>0.005</td>
<td>-0.4</td>
<td>(-0.8, -0.02)</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>-0.05</td>
<td>(-1.3, 0.2)</td>
<td>0.1</td>
<td>-0.01</td>
<td>(-0.9, 0.6)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>-0.01</td>
<td>(-0.8, 0.6)</td>
<td>0.7</td>
<td>-0.01</td>
<td>(-0.8, 0.6)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>-0.02</td>
<td>(-0.7, 0.3)</td>
<td>0.5</td>
<td>-0.02</td>
<td>(-0.7, 0.4)</td>
</tr>
</tbody>
</table>

*adjusted for baseline BMI, age, ethnicity, exercise, socioeconomic index, time to follow-up
6.2.4 Discussion

We found a negative relationship between maternal vitamin D status at 15 weeks gestation in first pregnancy and BMI change 6 years post-delivery that persisted after adjustment for baseline BMI and other predictors. In multivariate analysis, a one standard deviation increase (26.4nmol/L in February) in 25-hydroxyvitamin D concentration at 15 weeks gestation BMI was associated with a 0.2 kg/m² lower BMI, a 0.5 kg difference for a woman 1.64 cm tall (the mean height of New Zealand women). Measurements of skin fold thickness at 6 years postpartum were also significantly associated with vitamin D status at 15 weeks’ of first pregnancy while waist circumference was not. This suggests a negative association with expanding peripheral adiposity in this study of relatively young women without risk factor for metabolic syndrome, with further adiposity gain an increase in central adiposity leading to metabolic syndrome may occur. We did not find any association between vitamin D status and blood pressure change or the subsequent development of metabolic syndrome.

In contrast, others have found an association between vitamin D status in non-pregnant adults at baseline and subsequent development of metabolic syndrome. A study of 301 men and women identified as having risk factors for developing metabolic syndrome and type 2 diabetes mellitus found that a one standard deviation increase in baseline 25-hydroxyvitamin D decreased the likelihood of developing metabolic syndrome at 3 years follow-up (OR 0.62 95% CI 0.45 – 0.85 p = 0.003) (Kayaniyi et al, 2014). In a population of 3,404 adults without type 1 or type 2 diabetes mellitus, a 10 nmol/L increase in 25-hydroxyvitamin D was associated with a decreased likelihood of developing metabolic syndrome (adjusted OR 0.85 95% CI 0.8 – 0.89 p = 0.001) (Pannu et al, 2016). The difference between the present study and these cohorts may be explained by the inclusion of both men and women who were older and more at risk for metabolic syndrome at baseline and were therefore adequately powered to find an effect.

To our knowledge, this is the first study to demonstrate an association between vitamin D status in pregnancy and post-partum weight gain. Early pregnancy may be an opportunistic time to identify women at risk for metabolic syndrome and for later excess weight gain. Screening is a routine part of antenatal care (NICE, 2008, The Royal Australian and New Zealand College of Obstetricians and Gynaecologists, 2016). It is a time when women who otherwise have no reason to see a health professional have a point of contact. At present many women are identified as having risk factors for developing metabolic syndrome in pregnancy,
such as insulin resistance and adiposity (Vilmi-Kerälä et al., 2015). Major lifestyle changes are the only intervention known to reduce long term risk (Orchard et al., 2005); unfortunately, even with intensive interventions the effects are frequently not lasting (Kukkonen-Harjula et al., 2005).

Vitamin D appears to play a complex and incompletely understood role in adipocyte development and function. In mouse and pig cells, vitamin D inhibits differentiation of immature adipocytes and inhibits fat accumulation in mature adipocytes, both effects resulting in lower body fat (Kong and Li, 2006, Zhuang et al., 2007, Kim et al., 2016). However, vitamin D nuclear receptor (VDR) knockout mice, devoid of any direct vitamin D effect, also show low body fat (Zinser and Welsh, 2004) even in the presence of a high fat diet (Narvaez et al., 2009, Weber and Erben, 2013, Chang and Kim, 2016).

Clearly there are non-vitamin D effectors of fat accumulation, and there are likely to be vitamin D effects that are not mediated by the VDR. A partial explanation of the apparently contradictory finding above may be offered by observations made in mouse 3T3-L1 adipocyte cell lines, VDR activation inhibits fat accumulation when ligand bound, and promotes fat accumulation when not ligand bound (Blumberg et al., 2006). In humans VDR variants are associated with percentage body fat, visceral adiposity and appetite regulating adiponectin circulating concentrations (Khan et al., 2016). It is not yet known whether these findings at a molecular level translate to clinically significant changes in humans. As shown above, data from clinical trials have been inconclusive (Chandler et al., 2015).

An alternative explanation is positive health behaviours, such as more outdoor exercise, are associated with higher 25-hydroxyvitamin D concentrations and therefore less adiposity gain. We adjusted for baseline adiposity and exercise in pregnancy, and adjusted for season. However, we did not have, information on individual sun exposure, skin colour or objective measures of exercise at the time of follow-up. Other potential confounding factors not included due to a lack of available data are smoking status and whether multivitamin supplements were taken.

A further limitation of our study is that 25-hydroxyvitamin D analysis was performed at one time point. Multiple measures over the years would allow a more precise investigation into the temporal relationship of vitamin D status and weight gain. Despite these limitations, our findings are important, given the context of a global obesity epidemic and widespread vitamin D deficiency.
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In conclusion, a significant inverse relationship between vitamin D status in pregnancy and BMI change over a 6 year post-partum period was found. Widespread obesity is one of the greatest challenges facing the current generation and at this time there are no effective interventions at the population level. In this context, our findings may be important and would support those carrying out randomised trials of vitamin D supplementation in pregnancy to investigate long term weight gain and adiposity as an outcome.
Chapter 7  
Vitamin D supplementation in pregnancy, glycaemia and metabolomics (MAVIDOS TRIAL)

7.1 Preface

Five randomised controlled trials investigating whether vitamin D supplementation improves glycaemia were discussed in Chapter 2. The largest was the only study to show a reduction in GDM, comparing vitamin D supplementation of 50,000 IU fortnightly to 400 IU daily (5600 IU fortnightly) (prevalence of GDM 6.7% and 13.4% respectively \(p = 0.01\)) (Mojibian et al, 2015). It is possible that the other studies were not adequately powered to detect a difference with supplementation.

The Maternal Vitamin D Osteoporosis (MAVIDOS) is larger than any of the above randomised trials and, although glycaemia was not a primary outcome, provides an opportunity to investigate the effects of vitamin D supplementation on glycaemia in pregnancy. Universal screening for GDM is not part of routine antenatal care in the United Kingdom, where the MAVIDOS trial was carried out. Maternal glycaemia was evaluated by analysing plasma fructosamine at 34 weeks gestation.

Fructosamine is a measure of glycated plasma proteins which undergo the same non-enzymatic glycation as haemoglobin. In the same way that HbA\(_1c\) increases with elevated plasma glucose, the proportion of protein that becomes glycated is elevated in diabetes mellitus (Lloyd and Marples, 1988, Tahara and Shima, 1995). Fructosamine has been shown to correlate well with HbA\(_1c\) and fasting plasma glucose (Juraschek et al, 2012). In patients with type 1 diabetes mellitus using continuous glucose monitoring, fructosamine and HbA\(_1c\) correlated equally well with hyperglycaemic AUC 180 (0.5 and 0.5 respectively) (Beck et al, 2011).

Both HbA\(_1c\) and fructosamine are affected by physiological changes in pregnancy, HbA\(_1c\) decreases until the end of the second trimester, then begins to rise again, while fructosamine, and the similar measure glycated albumin, continue to gradually decline (Hiramatsu et al, 2012). Even so fructosamine has been shown to be a good predictor of gestational diabetes mellitus. Using a threshold of 180 \(\mu\)mol/L, fructosamine had a positive predictive value of 85% and negative predictive value of 95% for GDM, compared to a 100g 3 hour oral glucose
tolerance test using the O’Sullivan criteria (Table 1.3) (Roberts et al, 1983). While an HbA1c of 5.4% (36mmol/L) had a positive predictive value of 42%, and negative predictive value of 91% compared to the ADIPS OGTT criteria for GDM (Khalafallah et al, 2016).
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7.2 Plasma fatty acid composition and fructosamine following vitamin D supplementation during pregnancy: a metabolomics study

Abstract

Vitamin D insufficiency is common in pregnant women and has been associated with adverse outcomes including gestational diabetes. The underlying mechanisms by which vitamin D may influence outcomes are not known. Characterising the effects of vitamin D supplementation on the plasma metabolome may suggest causal mechanisms. This study investigated whether vitamin D supplementation during pregnancy altered the plasma metabolome and fructosamine concentration (glycosylated plasma protein, a marker of maternal glycaemia).

Methods

Following 19 weeks of vitamin D3 1000 IU daily (n = 455) or matched placebo (n = 455), plasma fructosamine at 34 weeks gestation was analysed in participants in the MAVIDOS randomised, double-blind, placebo controlled trial of vitamin D supplementation in pregnancy. Gas chromatography coupled to mass spectrometry (GC-MS) was used to analyse plasma metabolites.

Results

At 34 weeks gestation mean 25-hydroxyvitamin D concentrations were higher in the intervention group compared to placebo (67.9 (SD 22.1) nmol/L compared with 43.6 (SD 22.4) nmol/L respectively, \(p\leq 0.001\)). Plasma fructosamine concentration was similar in the intervention and placebo group (194.7 (SD 12.6) \(\mu\)mol/L vs 193.9 (SD 11.7) \(p=0.5\)) and in multivariate linear regression analysis there was no association between fructosamine concentration and 25-hydroxyvitamin D concentration (\(p=0.8\)).

A total of 152 metabolites were detected by GC-MS, of which 109 were identified using in-house MS libraries. After correction for multiple testing, no individual metabolites were significantly different between groups. There appeared to be a global reduction in the relative abundance of free fatty acids at 34 weeks gestation in the vitamin D intervention group compared with placebo, while amino acids and other metabolite groups were not uniformly affected.

Conclusion

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Our data did not demonstrate a clear difference in the plasma metabolome and fructosamine concentration between placebo and vitamin D intervention groups. However, we observed a global reduction of free fatty acids in plasma in response to vitamin D supplementation. This observation suggests that vitamin D intake may influence lipid and fatty acid metabolism in late pregnancy.
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7.2.1 Introduction

Vitamin D deficiency is common amongst pregnant women and has been associated with pregnancy disorders including gestational diabetes mellitus (GDM) (Aghajafari et al., 2013). A reduction in glucose tolerance occurs in normal pregnancy and is considered an adaptive response to pregnancy (McIntyre et al., 2010). However, hyperglycaemia in pregnancy is associated with adverse pregnancy outcomes and the risk of pregnancy complications increases as a continuum rather than at a specific glucose threshold (The HAPO Study Cooperative Research Group, 2008).

It has been proposed that Vitamin D may improve glucose tolerance by improving insulin release (Rorsman and Renstrom, 2003) and insulin sensitivity (Maestro et al., 2000). It is unknown whether vitamin D supplementation lowers glycaemia in pregnancy. Small randomised trials have shown conflicting results. For example, in a trial of 56 women with GDM given vitamin D supplementation or placebo in pregnancy, vitamin D supplementation was associated with a reduction in fasting plasma glucose from baseline \( p < 0.001 \) (Asemi et al., 2014). In contrast, a study of 159 women comparing high dose vitamin D supplementation (5,000 IU daily) to the standard recommendation in their region (400 IU daily) did not find a difference between groups in fasting blood glucose or 2 hour blood glucose (Yap et al., 2014).

The MAVIDOS study is a large multi-centre randomised, double-blind, placebo-controlled trial of vitamin D supplementation in pregnancy (Cooper et al., 2016). At 34 weeks gestation, after 19 weeks of supplementation with 1000 IU of cholecalciferol daily more women were vitamin D replete compared with the placebo group (64% vs 17% of participants had a serum 25-hydroxyvitamin D concentration \( >50 \) nmol/L \( p < 0.0001 \)). This large randomised trial provides an opportunity to investigate the hypotheses that have been proposed regarding vitamin D supplementation and health outcomes.

One such hypothesis is that vitamin D improves glucose tolerance. Glucose tolerance in pregnancy is typically tested by fasting glucose and response to glucose load. Due to the day-to-day variation in glucose concentrations (Bonongwe et al., 2015), in a population of women who do not have diabetes mellitus, analysis of accumulated of mean glucose over time may offer more information. HbA\(_1c\) and fructosamine are established ways to estimate mean glucose over months or weeks respectively. Given pregnancy-related alterations to red cell metabolism (which affect HbA\(_1c\) but not fructosamine), and the relatively rapid changes to
insulin sensitivity during pregnancy, fructosamine may be better than HbA1c as an indicator of mean plasma glucose concentrations in pregnancy (Sugawara et al., 2016).

Glucose intolerance represents a disorder of energy availability and utilisation at a cellular level. Cells can derive energy from metabolising a variety of substrates including glucose, amino acids and fatty acids. The pattern of substrate use at any time can be detected in the metabolome, the total metabolites in an organism, tissue or extracellular fluid such as serum and plasma (Villas-Bôas et al., 2007).

Gas chromatography coupled to mass spectrometry (GC-MS) is one of the most established analytical platforms for metabolite profiling as it allows simultaneous detection of hundreds of compounds. With improved data extraction techniques and well developed in-house and commercial MS libraries, a large number of compounds, such as sugars, amino acids and fatty acids are readily detected and identified (Villas-Bôas et al., 2006, Dunn et al., 2013). For example, metabolite biomarkers such as N-acetylthreonine and tryptophan have been identified as predictors of declining renal function in type 2 diabetes mellitus (Solini et al., 2016).

In this study, we analysed plasma fructosamine concentration and characterised the late gestation metabolome of participants from the MAVIDOS trial. The aims were two-fold; to investigate whether vitamin D supplementation lowered plasma fructosamine as a measure of plasma glucose in pregnant women and to investigate changes in the plasma metabolome in pregnant women after vitamin D supplementation. To the best of our knowledge, this is the largest study investigating the plasma metabolome following vitamin D supplementation.

7.2.2 Method

Participants

The MAVIDOS trial is a multicentre, double blind, randomised placebo-controlled trial of vitamin D supplementation in pregnant women. Further detail on the study design has been published previously (Harvey et al., 2012, Cooper et al., 2016). Women were recruited from three centres in the United Kingdom, University Hospital Southampton National Health Service (NHS) Foundation Trust, Oxford University Hospital NHS Foundation Trust and Sheffield Hospitals NHS Trust and were randomised to receive either 1000 IU cholecalciferol daily or placebo. Women were included in the MAVIDOS study if their 25-hydroxyvitamin D concentration at 14 weeks gestation, measured on a local assay (by a DEQAS accredited
laboratory), was between 25 and 100 nmol/L. Study blood sampling was repeated at 34 weeks gestation. Weight and height were measured at 14 weeks and weight measured again at 34 weeks. Gestational weight gain and BMI change was calculated from these measurements. Reference plasma 25-hydroxyvitamin D analysis (Liaison RIA automated platform, Diasorin, Minnesota, USA), calcium, alkaline phosphatase, and albumin was undertaken centrally (MRC Human Nutrition Research, Cambridge, UK) in a single batch at the end of the study on samples at 14 and 34 weeks gestation. Details of assay performance and quality control through participation in DEQAS, NIST and NEQAS are given elsewhere (Sempos et al, 2012, Redmond et al, 2015). Metabolomic analysis was undertaken on samples at 34 weeks gestation.

Reagents

Reagents for fructosamine analysis were purchased from ROCHE diagnostics, Rotkreuz, Switzerland. Mass spectrometry grade methanol used for sample preparation was purchased from Global Science (Auckland, New Zealand). Pyridine and anhydrous sodium sulphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). The internal standards, 2,3,3,3-di-alanine (L-D4_alanine) and D4-ribitol, were also purchased from Sigma-Aldrich. Methyl chloroformate (MCF) and N-methyl-N (trimethylsilyl) trifluoroamide (MSTFA, derivatisation grade) were purchased from Merck (Darmstadt, Germany). Methoxyamine hydrochloride was purchased from Fluka (Steinheim, Switzerland). All solutions were prepared using Grade 1 water 77 (BARNSTEAD® NANOpure DIamondTM Water Purification System, Waltham, MA, USA).

Sample preparation and analysis

Plasma used was collected with EDTA anticoagulant. Fructosamine was analysed by ROCHE Cobas c 311 autoanalyser (Hitachi High-Technologies Corporation, Tokyo, Japan) using a Roche analytical kit (interassay coefficient of variation 0.9% and 1.2% for the low and high quality controls respectively).

Extraction of metabolites from plasma samples has been described in detail (Zarate et al, 2017). 20 µL of internal standard, L-alanine-2,3,3,3-d4 and D4-ribitol, was added to 150 µL of plasma prior to extraction. An aliquot from each extracted sample contributed to a single polled Quality control (QC) preparation. Samples from this preparation were run each day.

Metabolite profiling of plasma samples
We used two different derivatisation methods to obtain the metabolite profiles of plasma samples by gas chromatography coupled to mass spectrometry (GC-MS). An example of the injection sequence is included in Chapter 3 Table 3.4.

**Methylchloroformate (MCF) derivatisation**

Methylchloroformate (MCF) derivatisation of the plasma samples was carried out to determine the levels of amino and non-amino organic acids. Dried plasma samples were resuspended in 400 µL of 1 M NaOH. The MCF derivatisation method described previously (Villas-Bôas et al, 2003) has been modified with a doubling of all liquid addition volumes except chloroform. The derivatised samples were analysed using an Agilent GC 7890A coupled to a MS 5975C (Agilent Technologies, USA) with a quadrupole mass selective detector (electron ionization) with a split/splitless inlet according to a previously described protocol (Smart et al, 2010). The column was a fused silica Zebron ZB-1701 (Phenomenex), 30 m × 250 µm (internal diameter) by 0.15 µm (film thickness), stationary phase (86% dimethylpolysiloxane, 14% cyanopropylphenyl, Phenomenex) with 5 m guard column.

**Trimethyl silyl (TMS) derivatisation**

An automatic TMS derivatisation protocol was used to analyse the sugar, sugar alcohols and other derivatives in plasma samples, using GERSTEL MAESTRO 1.4 software. This protocol is described (Zarate et al 2017). The column details are provided in the MCF method. The column details are provided in MCF method. Mass spectra were acquired in scan mode from 40 to 650 amu, with detection threshold of 100 ion counts.

The raw GC-MS data were deconvoluted using the Automated Mass Spectral Deconvolution and Identification System (AMDIS) (online software distributed by the National Institute of Standards and Technology, USA – [http://www.amdis.net/](http://www.amdis.net/)). Data were normalised by internal standards, and batch-corrected using quality control samples. An in-house R-based software data mining and analysis-platform (open source, version 3.1.2 [https://www.r-project.org/](https://www.r-project.org/)) was used for metabolite identifications and peak height for relative quantification (Aggio et al, 2011).
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Statistical analysis

Statistical analysis was performed with IBM SPSS statistics version 22.0 and Metaboanalyst version 3.0 ([http://www.metaboanalyst.ca/](http://www.metaboanalyst.ca/)) (Xia et al., 2015). Independent \( t \) tests were performed to compare the concentration of fructosamine between vitamin D intervention group and the placebo group. There is a known association between BMI and 25-hydroxyvitamin D (Earthman et al., 2012) and BMI and fructosamine (Broussolle et al., 1991). Linear regression was performed to investigate whether 25-hydroxyvitamin D concentration at 34 weeks gestation was associated with fructosamine concentration after taking account of BMI and pregnancy weight gain.

Metabolite data were normalised by log transformation, mean centred and divided by the standard deviation of each variable prior to analysis. \( t \) tests and principal component analysis were performed to compare individual metabolites and the metabolome of the placebo and intervention groups. False discovery rates (FDR) were calculated to account for multiple comparisons. The FDR reports the ratio of significant differences found to the significant differences expected by chance for the number of tests performed. Principal Component Analysis (PCA) and Partial Least Square-Discriminant Analysis (PLS-DA) were performed to investigate metabolite profile separation between the vitamin D intervention and placebo group. Variable Importance in Projection (VIP) scores were calculated in the PLS-DA model to determine the most important metabolites differentiating the vitamin D supplementation and placebo groups (Xia et al., 2015).
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7.2.4 Results

Plasma samples collected at 34 weeks gestation were available for 455 participants taking placebo and 455 participants taking 1000 IU/day. The characteristics of the intervention and placebo groups at baseline are shown in Table 7.1.

| Table 7.1 MAVIDOS participant characteristics at baseline (14 weeks gestation) |
|---------------------------------|-----------------|-----------------|
|                                | 1000 IU daily    | Placebo group    |
|                                | \( n = 455 \)    | \( n = 455 \)    |
| Age (years, mean ± SD)         | 31.1 ± 5.0       | 30.8 ± 5.2       |
| BMI (kg/m², mean ± SD)         | 25.9 ± 5.1       | 26.4 ± 4.9       |
| 25-hydroxyvitamin D (nmol/L, mean ± SD) | 46.8 ± 17.5     | 45.7 ± 16.6     |
| Ethnicity                      |                  |                 |
| White (\( n \% \))             | 90.1             | 98.5             |

Table 7.2 shows that the mean 25-hydroxyvitamin D concentration in the vitamin D supplementation group was higher than the placebo group at 34 weeks gestation. There was however significant overlap between groups.

| Table 7.2 MAVIDOS Participant characteristics at follow-up (34 weeks gestation) |
|---------------------------------|-----------------|-----------------|
|                                | 1000 IU vitamin D daily | Placebo |
|                                | \( n = 455 \)    | \( n = 455 \)    |
| 25-hydroxyvitamin D (nmol/L mean ± SD) | 67.8 ± 22.1\(^a\) | 43.6 ± 22.4 |
| Weight Gain (kg ± mean SD)      | 9.5 ± 3.6        | 9.5 ± 3.7       |
| Season of 34 weeks gestation sample collection (%) |                  |                 |
| Summer                         | 109 (24.1)       | 113 (24.8)      |
| Autumn                         | 105 (23.2)       | 99 (21.9)       |
| Winter                         | 122 (27.7)       | 126 (27.7)      |
| Spring                         | 116 (25.7)       | 114 (25.2)      |
| Fructosamine (µmol/L)          | 194.7 ± 12.6     | 194.0 ± 11.7    |

\(^a\) \( p < 0.05 \) in comparison to placebo group

Fructosamine was analysed in 901 participants. Fructosamine concentrations were normally distributed (Shapiro-Wilk test \( p = 0.5 \)) and negatively correlated with BMI (-0.3 \( p < 0.001 \)) and pregnancy weight gain (-0.08 \( p = 0.03 \)). Mean fructosamine concentration was similar in the two groups (intervention group 194.7 (SD 12.6) µmol/L, placebo group (193.9 SD) 11.8 \( p = 0.5 \)). In multivariate linear regression analysis 25-hydroxyvitamin D concentration was
not associated with fructosamine concentration after adjustment for BMI and pregnancy weight gain ($\beta = 0.003$ 95% CI -0.03, 0.03 $p = 0.8$) Table 7.1.

Figure 7.1 The relationship between fructosamine and 25-hydroxyvitamin D concentration
A total of 125 metabolites were identified by MCF and 27 compounds by the TMS method. This included 23 amino acids, 34 fatty acids, four sugars, 26 organic acids and other organic compounds. No compounds were significantly different in relative abundance when comparing the intervention to placebo group.

Figure 7.2 presents a three dimensional PCA score plot of metabolite data. We found no separation between the intervention and placebo group. 49% of the total variance was determined by the first three principal components. The metabolites that contributed most to the variation between samples were oxalic acid, hexanoic acid and oxovaleric acid.

Analysis of Variable Importance of Projection scores from PLS-DA model indicated that fatty acids were uniformly lower in relative abundance in the vitamin D supplementation group compared to placebo (Figure 7.3). There was no such trend for other groups of compounds such as amino acids and other organic acids.
Figure 7.3 Variable Importance in Projection scores from Partial Least Squares - Discriminant Analysis (PLS-DA) model showing the relative abundance of all detected plasma free fatty acids are lower in the vitamin D supplementation group compared to placebo.
7.2.5 Discussion

In this study of the plasma metabolome following vitamin D supplementation in pregnancy there were no individual metabolites that differed between supplementation and placebo group and fructosamine concentration was similar in the intervention and placebo group. It appeared there was a global reduction in the relative abundance of fatty acids in the vitamin D supplementation group compared to placebo. The main strength of this study is samples used were from a large placebo controlled randomised controlled trial.

Lack of reduction in plasma fructosamine suggests that vitamin D supplementation does not improve glucose tolerance during pregnancy. Results from smaller randomised controlled trials in pregnancy suggest that a higher dose of vitamin D is unlikely to be more effective. Two studies, one of 159 women, one of 179 women comparing 400 IU to 5000 IU vitamin D daily from before 20 weeks gestation, found no difference in fasting and 2 hour post load plasma glucose concentration between groups at 26 – 28 weeks gestation (Yap et al, 2014, Yap et al, 2014). Two studies of 303 and 175 women comparing 400 IU vitamin D daily or calcium alone to 4000 IU daily did not find a reduction in GDM prevalence with high dose supplementation, although they did not report glucose concentrations (Wagner et al, 2013, Hossain et al, 2014).

As with the MAVIDOS study, the participants of the trials described above were recruited from a general obstetric population. Studies of women with hyperglycaemia in pregnancy have had more positive results. In a study of 54 women with GDM given either a single dose of placebo or 50,000 IU vitamin D at the time of diagnosis, the intervention group showed a reduction in fasting plasma glucose 6 weeks later (Asemi et al, 2013). In another study of 26 women with GDM, recruited within two years post-partum, vitamin D supplementation given for 6 months was associated with a lower HbA1c and lower plasma glucose 2 hours post glucose challenge (Yeow et al, 2015). It is possible that improvements in glycaemia could not be seen in the MAVIDOS participants because of their relatively good baseline pancreatic β-cell function and insulin sensitivity.

Consistent with finding no difference in plasma fructosamine concentration, we did not find individual metabolites that were significantly different between the vitamin D intervention and placebo groups. We are not aware of studies comparing the metabolome following vitamin D supplementation; however studies have identified metabolite profile phenotypes
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that respond favourably to vitamin D supplementation. The metabolome phenotype clearly separated healthy non-pregnant pre-menopausal women who responded, or did not respond, to vitamin D supplementation with improved insulin sensitivity as indicated by homeostatic model assessment scores and lower C-reactive protein (O'Sullivan et al, 2011).

An interesting finding was that the relative abundance of free plasma fatty acids was reduced in the group supplemented with vitamin D compared to placebo, perhaps due to an alteration in lipid metabolism. Free fatty acids are released into the circulation from the breakdown of triglycerides in adipocytes and are taken up by most cells (Zechn er et al, 2009). Fatty acids undergo beta oxidation within the mitochondria to generate acetyl CoA which is used in the citric acid cycle. We found that both endogenous and exogenous fatty acids were reduced in the vitamin D intervention group suggesting increased fatty acid utilisation.

Recent animal studies support this theory. In cultured mouse adipocytes treatment with 1,25-dihydroxyvitamin D decreased fat accumulation and increased fatty acid utilisation. mRNA expression of enzymes involved in beta-oxidation including CPT1α, PGC1α, PPARα, and UCP1 were also increased (Chang and Kim, 2016). These authors found a reduction in PPARγ which promotes lipid accumulation, consistent with other investigators who cultured cells from a diabetic rat model (Park et al, 2016) and a high fat diet rat model (Liu et al, 2015). Alternatively, lower plasma free fatty acids may be due to inhibition of lipolysis through the actions of vitamin D metabolites as demonstrated in cultured human adipocytes (SHI et al, 2001). Adipocytes incubated in 1,25 dihydroxyvitamin D showed an increase in lipogenesis together with an inhibition of lipolysis.

The main limitation of this study is fasting samples and glucose tolerance testing were not available. Given our finding of no relationship between 25-hydroxyvitamin D concentration and fructosamine, it is unlikely that any difference in fasting glucose and glucose tolerance would be of clinical significance. On the other hand, the plasma and serum metabolome is sensitive to changes after meals, and fasting samples would have allowed greater sensitivity to detect differences in the plasma metabolome (Carayol et al, 2015). The MAVIDOS trial was not designed to investigate the glucose outcomes in the participants. However, it is much larger than other randomised trials of vitamin D supplementation during pregnancy which were designed to investigate glycaemia (Asemi et al, 2013, Yap et al, 2014, Yeow et al, 2015).

Conclusion
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We did not find lower fructosamine concentrations in participants of the MAVIDOS trial supplemented with vitamin D. Although vitamin D supplementation did not affect the plasma metabolome significantly, free fatty acids were lower in relative abundance in the intervention group compared to placebo. This may represent an alteration in lipid and fatty acid metabolism, and warrants further investigation.
7.3 Additional Results and Discussion

In most populations there is a wide range of 25-hydroxyvitamin D concentration. This is most determined by the length of sun exposure, because for most individuals circulating 25-hydroxyvitamin D is obtained from synthesis in the skin.

While the 25-hydroxyvitamin D concentration was significantly different between the vitamin D intervention and placebo groups in the MAVIOS study, there was a considerable degree of overlap between the groups (Figure 7.4). Some of the participants in the intervention group have lower 25-hydroxyvitamin D concentrations than the placebo group, most noticeable in the late summer when natural 25-hydroxyvitamin D concentrations are at their highest.

The study reported a high level of compliance, with 72% of the placebo group and 71% of the vitamin D supplementation group self-reported as taking 75% or more of the study medication (Cooper et al, 2016).
Figure 7.4 Mean 25-hydroxyvitamin D concentration at 34 weeks gestation across the year in MAVIDOS participants.
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To explore the data further, participants in the intervention group with a 25-hydroxvitamin D above the 95\textsuperscript{th} centile of the members of the placebo group at 34 weeks gestation, measured in the same month, were analysed separately. This group were called ‘Good Responders’. 164 participants in the intervention group met this definition.

There were no differences in age and baseline BMI between Good Responders and the placebo group ($p = > 0.05$) (Table 7.3). Good Responders had higher 25-hydroxyvitamin D concentrations at baseline ($p = 0.007$) and were more likely to be identified in the spring and winter months when natural 25-hydroxyvitamin D concentrations are lowest ($p = 0.001$) Table 7.4.
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Table 7.3 Participant characteristics at baseline (14 weeks gestation): MAVIDOS trial vitamin D intervention (1000 IU daily), Good Responder and placebo groups

<table>
<thead>
<tr>
<th></th>
<th>1000 IU daily n = 455</th>
<th>Good Respondersa n = 164</th>
<th>Placebo group n = 455</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years, mean ± SD)</td>
<td>31.1 ± 5.0</td>
<td>31.6 ± 4.8</td>
<td>30.8 ± 5.2</td>
</tr>
<tr>
<td>BMI (kg/m², mean ± SD)</td>
<td>25.9 ± 5.1</td>
<td>26.0 ± 4.8</td>
<td>26.4 ± 4.9</td>
</tr>
<tr>
<td>25-hydroxyvitamin D (nmol/L, mean ± SD)</td>
<td>46.8 ± 17.5</td>
<td>50.1 ± 19.1b</td>
<td>45.7 ± 16.6</td>
</tr>
<tr>
<td>Ethnicity White (n %)</td>
<td>90.1</td>
<td>94.5</td>
<td>98.5</td>
</tr>
</tbody>
</table>

a Good Responders, subset of the intervention group, 25-hydroxyvitamin D 95th centile of placebo group in the same month as the assessment at follow-up  
b p = <0.05 in comparison to placebo group

Table 7.4 Participant characteristics at follow up (34 weeks gestation)

<table>
<thead>
<tr>
<th></th>
<th>1000 IU daily n = 455</th>
<th>Good Respondersa n = 164</th>
<th>Placebo n = 455</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-hydroxyvitamin D (nmol/L mean ± SD)</td>
<td>67.8 ± 22.1a</td>
<td>84.9 ± 16.8b</td>
<td>43.6 ± 22.4</td>
</tr>
<tr>
<td>Weight Gain (kg ± mean SD)</td>
<td>9.5 ± 3.6</td>
<td>9.2 ± 3.4</td>
<td>9.5 ± 3.7</td>
</tr>
<tr>
<td>Season of 34 weeks gestation sample collection (%)</td>
<td>109 (24.1)</td>
<td>28 (17.1)c</td>
<td>113 (24.8)</td>
</tr>
<tr>
<td>Summer</td>
<td>105 (23.2)</td>
<td>40 (24.4)</td>
<td>99 (21.9)</td>
</tr>
<tr>
<td>Autumn</td>
<td>122 (27.7)</td>
<td>38 (23.2)</td>
<td>126 (27.7)</td>
</tr>
<tr>
<td>Winter</td>
<td>116 (25.7)</td>
<td>58 (35.4)</td>
<td>114 (25.2)</td>
</tr>
<tr>
<td>Spring</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructosamine (μmol/L)</td>
<td>194.7 ± 12.6</td>
<td>193.9 ± 12.4</td>
<td>194.0 ± 11.7</td>
</tr>
</tbody>
</table>

a p <0.05 in comparison to placebo group
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Fructosamine concentration was similar in the Good Responder group and placebo, shown in Table 7.4. When plasma metabolite relative abundance was compared between the Good Responder and placebo group only four compounds were different. These were myristic acid (p = 0.01), myristoleic acid (p = 0.02), palmitoleic acid (p = 0.03), and caffeine (p = 0.04). The number of significant results was close to the number expected by chance (false discovery rate 0.98).

In PLS-DA modelling the vitamin D intervention and placebo group did not separate. Again fatty acids were lower in relative abundance, see Figure 7.5
Figure 7.5 Variable Importance in Projection scores from Partial Least Squares - Discriminant Analysis (PLS-DA) model showing the relative abundance of all detected plasma free fatty acids are lower in the Good Responders of the vitamin D supplementation group compared to placebo.
Chapter 8  Summary and Conclusion

8.1 Summary of findings

The aim of this thesis was to investigate whether optimal vitamin D status and vitamin D supplementation during pregnancy improved glycaemia, thereby preventing GDM and improving metabolic health in the offspring. Samples from two large studies were used to investigate these questions.

8.1.1 Findings from the SCOPE and Children of SCOPE studies

The Screening for Pregnancy Endpoints (SCOPE) study is a large international prospective cohort study following 5,558 women through pregnancy. Participants were first interviewed and had biological samples collected at 15 weeks gestation. Interview data and biological samples were again collected at 20 weeks gestation and soon after delivery.

25-hydroxyvitamin D concentrations were analysed on 15 week serum samples from 1710 women from Auckland who participated in SCOPE. Of those 1710, 1544 underwent a polycose screening test, and 32 were diagnosed with GDM. While 25-hydroxyvitamin D concentrations were lower in women who developed GDM, when adjustments were made for confounders the association was no longer significant. Nor was there an association between vitamin D status and other pregnancy outcomes including pre-eclampsia, preterm birth and small for gestational age (SGA) infants. There was significant variation in GDM, pre-eclampsia and SGA according to season.

Children of SCOPE is a large prospective cohort study following children from birth to 6 years of age. At 6 years of age, indices of adiposity were measured and common chronic illnesses including eczema and asthma were recorded. Maternal 25-hydroxyvitamin D concentrations at 15 weeks gestation were negatively associated with child percentage body fat (PBF) at 6 years of age, after adjustment for confounders. There was no association between maternal vitamin D status, and child lean body mass, BMI z-score or the presence of asthma or eczema at 6 years of age.

Women were also invited to participate in follow-up 6 years post-partum. Maternal 25-hydroxyvitamin D concentrations in pregnancy were negatively associated with maternal BMI and maternal BMI increase at 6 years follow-up. Skin fold thickness over triceps, biceps...
and subscapular regions were also negatively associated. There was no association between vitamin D status in pregnancy and blood pressure. Few women met the criteria for metabolic syndrome \((n = 46, 7.3\%)\). No association was detected between developing metabolic syndrome over the intervening 6 years and 25-hydroxyvitamin D concentrations in pregnancy.

### 8.1.2 Findings from the MAVIDOS study

The MAVIDOS study was a large randomised control trial of vitamin D supplementation from 15 weeks’ gestation. It was larger than any study of vitamin D supplementation in pregnancy reporting on glycaemia. The study, in this thesis, of the plasma metabolome of MAVIDOS participants was the largest study investigating the plasma metabolome following vitamin D supplementation and the only one of pregnant women.

Fructosamine was chosen as the measure of mean plasma glucose and was analysed on 34 week plasma samples from 910 participants (455 in the placebo and 455 in the intervention group). After 19 weeks of supplementation, mean plasma fructosamine was not significantly different between the intervention and placebo groups. There was no linear relationship between 25-hydroxyvitamin D and plasma fructosamine concentrations.

An investigation of the plasma metabolome was performed using a gas chromatography mass spectrometry platform. Using two sample preparation methods, 130 compounds were detected in plasma collected at 34 weeks gestation. There were no significantly different metabolites between the vitamin D supplementation and placebo groups. There was no significant separation between the two groups on principal component analysis and partial least squares – discriminant analysis. However, although not significantly different, all fatty acids were lower in the vitamin D supplementation group. There was no such pattern with amino acids and other organic compounds.

Findings from the Auckland SCOPE and MAVIDOS studies do not support vitamin D supplementation for the improvement of maternal glycaemia and the prevention of GDM. Vitamin D status at 15 weeks gestation was not associated with the development of GDM in the SCOPE study and vitamin D supplementation from 15 weeks gestation was not associated with a reduction in fructosamine at 34 weeks gestation after 19 weeks of supplementation. However, evidence from the Children of SCOPE study suggests there are potential benefits long-term benefits, through reduced adiposity, for women and their children associated with optimal 25-hydroxyvitamin D concentrations during pregnancy.
8.2 Discussion

An association between vitamin D status and many health outcomes has been found including cardiovascular disease (Wang et al., 2012), respiratory infections (Ginde et al., 2009), and malignancy (Ma et al., 2011). Randomised trials of vitamin D supplementation in many cases have failed to demonstrate the same benefit (Autier et al., 2014, Theodoratou et al., 2014). Vitamin D supplementation has not even been shown to reduce fall and fracture risk in multiple meta-analyses of randomised trials (Avenell et al., 2014). This has led some to suggest that vitamin D status is a marker of health, rather than a determinant of health (Anonymous, 2014).

The finding of an overall reduction in plasma fatty acids in the MAVIDOS participants supplemented with vitamin D, and the favourable relationship between vitamin D status and adiposity in the SCOPE and Children of SCOPE participants at 6 years follow-up suggests that vitamin D may still play an active role in metabolism.

The significance of a reduction in plasma free fatty acids in the intervention group MAVIDOS participants is not clear. Elevated free fatty acids have been found to be associated with increased all-cause mortality (Miedema et al., 2014). Acute elevation of plasma free fatty, by intravenous infusion of lipids, in healthy individuals has been shown to worsen insulin resistance (Boden et al., 2001) and induce an inflammatory response (Tripathy et al., 2003). However, a corresponding reduction in plasma fructosamine with vitamin D supplementation was not seen, therefore no evidence of improved insulin sensitivity. Ideally other measures of insulin sensitivity would have been performed to determine this. The gold standard to determine insulin resistance measure is the hyperinsulinaemic euglycamic clamp (DeFronzo et al., 1979). More commonly for convenience, the Homeostasis Model Assessment for Insulin Resistance or Quantitative Insulin Sensitivity Check Index are calculated from fasting plasma glucose and insulin (Venkatesan et al., 2016).

25-hydroxyvitamin D concentrations have been associated with plasma glucose in response to glucose load in other observational studies without an association between vitamin D status and GDM (Burris et al., 2012). In a randomised controlled trial vitamin D supplementation was associated with a reduction 2 hour plasma glucose, without a reduction in GDM prevalence or fasting plasma glucose (Asemi et al., 2014).
Any beneficial metabolic effect leading to a reduction in adiposity is at best small and may not have a noticeable impact to the individual. However, a small reduction in adiposity across many at the individual level may have significant benefits at the population level.

It should also not be forgotten that vitamin D is vital for normal bone health. Osteomalacia and rickets due to vitamin D deficiency can still be found in populations around the world (Munns et al., 2012) (Uush, 2013). Advice on the recommended daily intake of vitamin D and target 25-hydroxyvitamin D concentration based on bone health will always be relevant. The United States, National Academy of Medicine (formally the Institute of Medicine) guidelines recommend a daily dietary intake of 600 IU vitamin D for pregnant women and recommend a 25-hydroxyvitamin D target of 50 nmol/L (Institute of Medicine, 2011). These recommendations have been endorsed by other groups for the prevention of nutritional rickets (Munns et al., 2016).

Some have proposed that a higher concentration of 25-hydroxyvitamin D than that proposed by the National Academy of Medicine is required to achieve all the extra-skeletal benefits from vitamin D (Heaney and Holick, 2011). In the SCOPE study most participants had concentrations above the 50 nmol/L measure, and a relationship between adiposity at 6 year follow-up in mothers and children was found suggesting there may be benefits at greater concentrations. To the contrary, evidence from observational studies of non-pregnant adults has shown that the relationship between vitamin D status and incident cardiovascular disease is non-linear. A reduction of incident cardiovascular events with increasing 25-hydroxyvitamin D concentrations up to 50 nmol/L has been observed, but concentrations above 50 nmol/L conveys no additional benefit (Wang et al., 2008).
8.3 Limitations

The SCOPE study was limited by the low number of participants developing GDM and the low number of participants with vitamin D deficiency. As discussed in Chapter 4, the prevalence of GDM in the Auckland SCOPE participants was lower than expected probably due to the study selection criteria: healthy women in their first pregnancy. This may mean that a true effect was not detected. When the IADPSG criteria for GDM was used more than twice as many women met the criteria for GDM compared to the New Zealand GDM guidelines, yet the association between GDM and 25-hydroxyvitamin D concentration at 15 weeks weakened. This suggests it is unlikely, that an association would be found in a larger study population.

Participants of the SCOPE study underwent routine screening for GDM consisting of a non-fasting glucose response test, followed by an oral glucose tolerance test only on those with a positive screening test. An oral glucose tolerance test on all participants would have provided information on whether vitamin D status is associated with either fasting plasma glucose or response to glucose load even within the ‘normal’ glucose range.

Vitamin D was measured once for each woman at 15 weeks gestation in the SCOPE study. While it is possible that vitamin D status has an effect at critical time points during pregnancy, it is more likely that any effects relate to ongoing exposure. This raises the possibility of confounding by the known seasonal variation of vitamin D due changes in sun exposure. Repeated measure of 25-hydroxyvitamin D would have been helpful to estimate vitamin D exposure through the seasons. Our best alternative was to use a seasonal adjustment and a measure standardised within the month of 25-hydroxyvitamin D analysis.

As discussed in Chapter 5, bioimpedence has been validated as a reasonable alternative for measuring percentage body fat in children (Boeke et al., 2013). Nevertheless DEXA is considered a better reference standard for determining adiposity. This was not used in the Children of SCOPE because of concerns over exposing children to radiation when an alternative was available, together with the practicality of performing DEXA on so many children. The choice of bioimpedence over DEXA is not likely to have interfered with the relationship between maternal vitamin D status and the outcome.

Also discussed in Chapters 5 and 6 is the limited information for some important confounders particularly physical activity. Exercising more is associated with higher 25-hydroxyvitamin
D concentrations, likely due to greater sun exposure and lower percentage body fat. Exercise data were not available on children, and the exercise data available for mothers were self-reported frequency and intensity. Data on sun exposure and outdoor activity were not collected. There may have been an interaction between activity, adiposity and vitamin D status that could not be accounted for. Dietary data were also limited; this is less likely as diet has very little influence on vitamin D status.

The MAVIDOS study has the advantage of being a large placebo controlled randomised controlled trial. It was not, however, designed to investigate GDM or glycaemia in pregnancy so that the only measure of glycaemia was fructosamine. As discussed in Chapter 8, fructosamine has been validated as a measure of glycaemia in pregnancy, however other measures such as an oral glucose tolerance test would have allowed a more direct comparison with other studies.

Like the Auckland SCOPE participants, the MAVIDOS participants were at low risk for developing GDM, however they were more vitamin D deficient. At 15 weeks gestation 21.5% of SCOPE participants had a 25-hydroxyvitamin D concentration < 50 nmol/L compared to 60.5% in the MAVIDOS study. There was, however, significant overlap in 25-hydroxyvitamin D concentration between the intervention and placebo groups of the MAVIDOS study (Figure 7.4). Any effect of vitamin D is likely to be small. Therefore the similarity between groups may have hidden the genuine effects of vitamin D supplementation.
8.4 Recommendations for future research

The finding of a relationship between vitamin D status in pregnancy and a reduction in adiposity at 6 years post-partum in the SCOPE and Children of SCOPE participants warrants further investigation.

Increased adiposity is a global problem, and vitamin D supplementation in pregnancy is a simple and cost-effective measure that is acceptable to many in the target population.

Long term follow-up of both mothers and children who participated in randomised trials of supplementation in pregnancy including measurements of adiposity is required to draw any definitive conclusion. The effects on adiposity in the SCOPE and Children of SCOPE studies were small. A 25-hydroxyvitamin D increase of 26.4 nmol/L was associated with a 0.5 kg weight decrease for a woman 164 cm tall. For comparison, an increase of one unit in BMI (2.7 kg for a 164 cm tall women) is associated with an increased risk of developing type 2 diabetes mellitus (relative risk 1.24, 95% CI 1.20, 1.27) (Schienkiewitz et al, 2006).

Future studies of vitamin D supplementation should focus on populations most likely to benefit, particularly those at risk for vitamin D deficiency. This might increase time and costs of recruitment.

There are some populations where a benefit of vitamin D supplementation on adiposity is of particular interest. Many of the most obese populations also have a high prevalence of vitamin D deficiency. Excluding the United States, in the most recent International Food Policy Research Institute, Global Nutrition Report, the top 20 most overweight nations are all either Arab or Pacific Island nations (International Food Policy Research Institute, 2016). Vitamin D deficiency is well-known to be highly prevalent amongst Arab women (Al-Faris, 2016, Pirdehghan et al, 2016). Already the studies showing metabolic benefits with vitamin D supplementation in pregnancy have come from Iran (Asemi et al, 2013, Soheilykhah et al, 2013, Asemi et al, 2015, Mojibian et al, 2015). In a study of pregnant women residing in South Auckland, New Zealand, 80% of Pacific women were vitamin D deficient (< 50nmol/L) compared to 43% of NZ European women (Ekeroma et al, 2015). The prevalence of obesity amongst Pacific adults is higher than other ethnic groups in New Zealand (Metcalf et al, 2000). Childhood obesity is also common amongst Pacific children living in New Zealand, with 70% of children overweight and 50% obese in one study (Rush et al, 2013).
8.5 Conclusion

Vitamin D status or supplementation in pregnancy was not associated with glycaemia later in pregnancy. Vitamin D status was negatively associated with measures of adiposity at 6 years follow-up in both mother and child and subtle changes in the plasma metabolome suggest vitamin D may have a small but real effect on metabolism. The possibility that vitamin D supplementation may have a beneficial effect on adiposity in the long-term is potentially important, particularly in high risk populations.
Appendix I. Observational studies investigating the relationship between vitamin D and GDM

Appendix table 1 Cohort studies investigating the relationship between vitamin D status and GDM

<table>
<thead>
<tr>
<th>Author</th>
<th>Country</th>
<th>n</th>
<th>Gestation of 25-hydroxyvitamin D analysis</th>
<th>GDM diagnostic criteria</th>
<th>Prevalence of GDM</th>
<th>Mean 25-hydroxyvitamin D (nmol/L)²</th>
<th>Association with GDM</th>
<th>Included in meta-analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Maghbooli et al, 2008)</td>
<td>Iran</td>
<td>741</td>
<td>24 – 28 weeks’</td>
<td>Carpenter &amp; Coustan</td>
<td>7%</td>
<td>16.5 ± 10.4 23.0 ± 18.3</td>
<td>Yes</td>
<td>Poel, Aghajafari, Wei</td>
</tr>
<tr>
<td>(Clifton-Bligh et al, 2008)</td>
<td>Australia</td>
<td>244</td>
<td>24 – 28 weeks’</td>
<td>ADIPS</td>
<td>32%</td>
<td>48.6 ± 24.9 55.3 ± 23.3</td>
<td>No</td>
<td>Poel, Aghajafari</td>
</tr>
<tr>
<td>(Farrant et al, 2009)</td>
<td>Indian</td>
<td>559</td>
<td>30 weeks’</td>
<td>Carpenter &amp; Coustan</td>
<td>7%</td>
<td>median 38.8 median 37.8</td>
<td>No</td>
<td>Poel, Aghajafari, Wei</td>
</tr>
<tr>
<td>(Walsh et al, 2013)</td>
<td>Ireland</td>
<td>60</td>
<td>12 – 16 weeks’</td>
<td>Not given</td>
<td></td>
<td>45.8 ± 22.5</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>28 weeks</td>
<td></td>
<td></td>
<td>54.4 ± 33.4</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>(Fernández-Alonso et al, 2012)</td>
<td>Spain</td>
<td>466</td>
<td>11 – 14 weeks’</td>
<td>ADA</td>
<td>7.7%</td>
<td></td>
<td>No</td>
<td>Aghajafari, Wei</td>
</tr>
</tbody>
</table>
A summary of diagnostic criteria is given in Table 1.3

<table>
<thead>
<tr>
<th>Author</th>
<th>Country</th>
<th>n</th>
<th>Gestation of 25-hydroxyvitamin D analysis</th>
<th>GDM diagnostic criteria$^1$</th>
<th>Prevalence of GDM</th>
<th>Mean 25-hydroxyvitamin D (nmol/L)$^2$</th>
<th>Association with GDM</th>
<th>Included in meta-analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Burris et al, 2012)</td>
<td>USA</td>
<td>1314</td>
<td>26 – 28 weeks'</td>
<td>ADA</td>
<td>5.2%</td>
<td>132.0 ± 39.9</td>
<td>133.0 ± 44.3</td>
<td>No</td>
</tr>
<tr>
<td>(McLeod et al, 2012)</td>
<td>Australia</td>
<td>399</td>
<td>28 weeks’</td>
<td>IADPSG</td>
<td>12%</td>
<td>39.1 ± 17.9</td>
<td>42.9 ± 24.3</td>
<td>No</td>
</tr>
<tr>
<td>(Tomedi et al, 2013)</td>
<td>USA</td>
<td>429</td>
<td>&lt; 16 weeks'</td>
<td>Carpenter &amp; Coustan</td>
<td>11%</td>
<td>39.1 ± 17.9</td>
<td>42.9 ± 24.3</td>
<td>Yes</td>
</tr>
<tr>
<td>(Bener et al, 2013)</td>
<td>Qatar</td>
<td>1873</td>
<td>Third trimester</td>
<td>FPG ≥ 7.0</td>
<td>13.9%</td>
<td>17.8 ± 7.7</td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>(Whitelaw et al, 2014)</td>
<td>England</td>
<td>1491</td>
<td>26 weeks’</td>
<td>WHO</td>
<td>9.3%</td>
<td>Median 23.3 (IQR 13.0 – 42.3)</td>
<td></td>
<td>No</td>
</tr>
<tr>
<td>(Lacroix et al, 2014)</td>
<td>Canada</td>
<td>644</td>
<td>6 – 13 weeks’</td>
<td>IADPSG</td>
<td>8.2%</td>
<td>57.5 ± 17.2</td>
<td>63.5 ± 18.9</td>
<td>Yes</td>
</tr>
<tr>
<td>(Park et al, 2014)</td>
<td>Korea</td>
<td>523</td>
<td>12 – 14 weeks’</td>
<td>Carpenter &amp; Coustan</td>
<td>4.4%</td>
<td>35.3 ± 16.5</td>
<td>32.0 ± 14.5</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>22 – 24 weeks’</td>
<td></td>
<td></td>
<td>46.7 ± 18.4</td>
<td>46.5 ± 23.6</td>
<td>No</td>
</tr>
</tbody>
</table>
A summary of diagnostic criteria is given in Table 1.3

Mean ± standard deviation unless otherwise stated
Appendix table 2 Case control studies investigating the relationship between vitamin D status and GDM

<table>
<thead>
<tr>
<th>Author</th>
<th>Country</th>
<th>GDM Case n</th>
<th>Control n</th>
<th>Gestation of 25-hydroxyvitamin D analysis</th>
<th>GDM diagnostic criteria</th>
<th>Mean 25-hydroxyvitamin D (nmol/L)¹</th>
<th>Association with GDM</th>
<th>Included in meta-analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Zhang et al, 2008)</td>
<td>USA</td>
<td>57</td>
<td>114</td>
<td>16 weeks’</td>
<td>ADA</td>
<td>60.5 ± 21.3</td>
<td>75.3 ± 24.3</td>
<td>Yes</td>
</tr>
<tr>
<td>(Soheilykhah et al, 2010)</td>
<td>Iran</td>
<td>54</td>
<td>111</td>
<td>24 – 28 weeks’</td>
<td>Carpenter &amp; Coustan</td>
<td>Median 24.1</td>
<td>Median 32.3</td>
<td>Yes</td>
</tr>
<tr>
<td>(Makgoba et al, 2011)</td>
<td>England</td>
<td>90</td>
<td>154</td>
<td>11 – 13 weeks’</td>
<td>WHO</td>
<td>47.2 ± 26.7</td>
<td>47.6 ± 26.7</td>
<td>No</td>
</tr>
<tr>
<td>(Savvidou et al, 2011)</td>
<td>England</td>
<td>100</td>
<td>1000</td>
<td>11 – 14 weeks’</td>
<td>WHO</td>
<td>Population median 46.8</td>
<td></td>
<td>No</td>
</tr>
<tr>
<td>(Baker et al, 2012)</td>
<td>USA</td>
<td>60</td>
<td>120</td>
<td>11 – 14 weeks’</td>
<td>NDDG</td>
<td>97 ± 29</td>
<td>86 ± 22</td>
<td>No</td>
</tr>
<tr>
<td>(Parlea et al, 2012)</td>
<td>Canada</td>
<td>116</td>
<td>219</td>
<td>15 – 18 weeks’</td>
<td>Carpenter &amp; Coustan</td>
<td>56.3 ± 19.4</td>
<td>62.0 ± 21.6</td>
<td>Yes</td>
</tr>
<tr>
<td>(Parildar et al, 2013)</td>
<td>Turkey</td>
<td>44</td>
<td>78</td>
<td>26 – 28 weeks’</td>
<td>IADPSG</td>
<td>48.8 ± 23.3</td>
<td>57.3 ± 25.0</td>
<td>Yes</td>
</tr>
<tr>
<td>(Zuhur et al, 2013)</td>
<td>Turkey</td>
<td>168</td>
<td>234</td>
<td>24 – 26 weeks’</td>
<td>IADPSG</td>
<td>30.8 ± 16.3</td>
<td>36.0 ± 16.2</td>
<td>No</td>
</tr>
<tr>
<td>(Cho et al, 2013)</td>
<td>Korea</td>
<td>20</td>
<td>40</td>
<td>At delivery</td>
<td>Carpenter &amp; Coustan</td>
<td>29.1 ± 22.8</td>
<td>86.2 ± 48.1</td>
<td>Yes</td>
</tr>
<tr>
<td>(El Lithy et al, 2014)</td>
<td>Egypt</td>
<td>80</td>
<td>80</td>
<td>Not given</td>
<td>Not given</td>
<td>47.3 ± 10.3</td>
<td>46.6 ± 6.1</td>
<td>No</td>
</tr>
</tbody>
</table>

¹Mean ± standard deviation unless otherwise stated
Appendix II. GC-MS Standard Mix

A standard mixture is included in the sequence for GC-MS. The standard mixture contains a known quantity of known compounds. The purpose is to provide an additional quality control particularly of the derivitization step, because poor recovery indicates that derivitazation has not been adequate. It also improves metabolite identification particularly for compounds, such as sugars that overlap on the chromatogram and have the same mass/charge ratio.

The standard mixture is made in a batch and frozen. The compounds are dissolved individually to make stock solutions. A volume of each stock solution is added to a 15mL falcon tube and mixed together to make the standard mix stock solution. The standard mix stock solution is frozen in 90μL aliquots. When required for analysis an aliquot is defrosted and a mixed with a solvent. The volumes required to make each standar mix is given in Appendix table 3, Appendix table 4, Appendix table 5 and Appendix table 6.
### Appendix II

#### Appendix table 3 MCF standard mix

<table>
<thead>
<tr>
<th></th>
<th>Weight$^1$ (g)</th>
<th>Solvent</th>
<th>Solvent volume (mL)</th>
<th>Stock solution (mM)</th>
<th>Volume added to mixture (μL)</th>
<th>Standard mix conc (mM)</th>
<th>Final conc (mM)$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-alanine</td>
<td>0.1783</td>
<td>Water</td>
<td>50</td>
<td>40.0</td>
<td>1000</td>
<td>4.4</td>
<td>1.00</td>
</tr>
<tr>
<td>L-leucine</td>
<td>0.1317</td>
<td>Water</td>
<td>25</td>
<td>40.2</td>
<td>1000</td>
<td>4.5</td>
<td>1.00</td>
</tr>
<tr>
<td>L-lysine</td>
<td>0.1828</td>
<td>Water</td>
<td>25</td>
<td>40.0</td>
<td>1000</td>
<td>4.4</td>
<td>1.00</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.133</td>
<td>0.1 M NaOH</td>
<td>25</td>
<td>40.0</td>
<td>1000</td>
<td>4.4</td>
<td>1.00</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>0.2039</td>
<td>0.1 M NaOH</td>
<td>25</td>
<td>39.9</td>
<td>1000</td>
<td>4.4</td>
<td>1.00</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>0.1215</td>
<td></td>
<td>25</td>
<td>20.1</td>
<td>1000</td>
<td>4.5</td>
<td>1.00</td>
</tr>
<tr>
<td>2-hydroxybutyrate</td>
<td>0.1048</td>
<td>Water</td>
<td>25</td>
<td>40.3</td>
<td>1000</td>
<td>4.5</td>
<td>1.00</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.1928</td>
<td>Water</td>
<td>25</td>
<td>40.1</td>
<td>1000</td>
<td>4.5</td>
<td>1.01</td>
</tr>
<tr>
<td>3-methyl-2-oxovaleric acid</td>
<td>0.1306</td>
<td>Water</td>
<td>25</td>
<td>34.3</td>
<td>1000</td>
<td>3.8</td>
<td>0.86</td>
</tr>
</tbody>
</table>

$^1$ Weight of compound in powder form to be added to solvent

$^2$ Final concentration after addition of 400 μL of chloroform to 90 μL of the standard mixture
## Appendix II

### Appendix table 4 TMS standard mix 1

<table>
<thead>
<tr>
<th>Weight(^1)</th>
<th>Solvent</th>
<th>Solvent volume (mL)</th>
<th>Stock solution (mM)</th>
<th>Volume added to mixture (μL)</th>
<th>Standard mix conc (mM)</th>
<th>Final conc (mM)(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribitol</td>
<td>0.1524</td>
<td>water</td>
<td>100</td>
<td>10.02</td>
<td>3200</td>
<td>4.01</td>
</tr>
<tr>
<td>Ribose</td>
<td>0.3753</td>
<td>water</td>
<td>50</td>
<td>50.00</td>
<td>1600</td>
<td>10.00</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.4504</td>
<td>water</td>
<td>50</td>
<td>50.00</td>
<td>1600</td>
<td>10.00</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.4504</td>
<td>water</td>
<td>50</td>
<td>50.00</td>
<td>1600</td>
<td>10.00</td>
</tr>
</tbody>
</table>

\(^1\) Final concentration after addition of 160 μL of derivatizing solution

### Appendix table 5 TMS standard mix 2

<table>
<thead>
<tr>
<th>Weight(^1)</th>
<th>Solvent</th>
<th>Solvent volume (mL)</th>
<th>Stock solution (mM)</th>
<th>Volume added to mixture (μL)</th>
<th>Standard mix conc (mM)</th>
<th>Final conc (mM)(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribitol</td>
<td>0.1524</td>
<td>water</td>
<td>100</td>
<td>10.02</td>
<td>3200</td>
<td>4.01</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.3753</td>
<td>water</td>
<td>50</td>
<td>50.00</td>
<td>1600</td>
<td>10.00</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.4504</td>
<td>water</td>
<td>50</td>
<td>50.00</td>
<td>1600</td>
<td>10.00</td>
</tr>
<tr>
<td>Mannitol</td>
<td>0.4554</td>
<td>water</td>
<td>50</td>
<td>50.00</td>
<td>1600</td>
<td>10.00</td>
</tr>
</tbody>
</table>

\(^1\) Final concentration after addition of 160 μL of derivatizing solution

### Appendix table 6 TMS standard mix 3

<table>
<thead>
<tr>
<th>Weight(^1)</th>
<th>Solvent</th>
<th>Solvent volume (mL)</th>
<th>Stock solution (mM)</th>
<th>Volume added to mixture (μL)</th>
<th>Standard mix conc (mM)</th>
<th>Final conc (mM)(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribitol</td>
<td>0.1524</td>
<td>water</td>
<td>100</td>
<td>10.02</td>
<td>3200</td>
<td>4.01</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.3753</td>
<td>water</td>
<td>50</td>
<td>50.00</td>
<td>1600</td>
<td>10.00</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.4504</td>
<td>water</td>
<td>50</td>
<td>50.00</td>
<td>1600</td>
<td>10.00</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.1712</td>
<td>water</td>
<td>100</td>
<td>50.01</td>
<td>1600</td>
<td>10.00</td>
</tr>
</tbody>
</table>

\(^1\) Final concentration after addition of 160 μL of derivatizing solution
Appendix III. Metabolites identified in plasma by GCMS

Appendix table 7 Metabolites detected in MCF derivatized samples

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mean relative abundance in plasma samples ± standard deviation ( n = 269 )</th>
<th>Average coefficient of variation ( n = 269 ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fatty acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Saturated fatty acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arachidic acid</td>
<td>0.0036 ± 0.0010</td>
<td>28.1</td>
</tr>
<tr>
<td>Decanoic acid</td>
<td>0.0036 ± 0.0005</td>
<td>14.2</td>
</tr>
<tr>
<td>Dodecanoic acid</td>
<td>0.0079 ± 0.0017</td>
<td>22.0</td>
</tr>
<tr>
<td>Hexanoic acid</td>
<td>0.0074 ± 0.0027</td>
<td>36.9</td>
</tr>
<tr>
<td>Margaric acid</td>
<td>0.0555 ± 0.0147</td>
<td>26.4</td>
</tr>
<tr>
<td>Myristic acid</td>
<td>0.0618 ± 0.0145</td>
<td>23.4</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>1.8155 ± 0.5494</td>
<td>30.3</td>
</tr>
<tr>
<td>Pentadecanoic acid</td>
<td>0.0216 ± 0.0055</td>
<td>25.6</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>1.4103 ± 0.384</td>
<td>27.2</td>
</tr>
<tr>
<td><strong>Monounsaturated fatty acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-Heptadecenoic acid</td>
<td>0.0030 ± 0.0008</td>
<td>27.1</td>
</tr>
<tr>
<td>Gondoic acid</td>
<td>0.0528 ± 0.0141</td>
<td>26.6</td>
</tr>
<tr>
<td>Myristoleic acid</td>
<td>0.0136 ± 0.0033</td>
<td>24.3</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>0.6310 ± 0.1836</td>
<td>29.1</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>0.0137 ± 0.0033</td>
<td>24.2</td>
</tr>
<tr>
<td><strong>Polyunsaturated fatty acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10,12-Octadecadienoic acid</td>
<td>0.0542 ± 0.0150</td>
<td>27.7</td>
</tr>
<tr>
<td>11,14,17-Eicosatrienoic acid</td>
<td>0.0791 ± 0.0205</td>
<td>26.0</td>
</tr>
<tr>
<td>11,14-Eicosadienoic</td>
<td>0.0134 ± 0.0038</td>
<td>27.9</td>
</tr>
<tr>
<td>Adrenic acid</td>
<td>0.0047 ± 0.0013</td>
<td>27.6</td>
</tr>
<tr>
<td>Alpha-linolenic acid</td>
<td>0.4332 ± 0.1243</td>
<td>28.7</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>0.2371 ± 0.0613</td>
<td>25.8</td>
</tr>
<tr>
<td>Conjugated linoleic acid</td>
<td>0.9013 ± 0.2571</td>
<td>28.5</td>
</tr>
<tr>
<td>Docosahexaenoic acid</td>
<td>0.0567 ± 0.0139</td>
<td>24.6</td>
</tr>
<tr>
<td>Docosapentaenoic acid</td>
<td>0.0209 ± 0.0057</td>
<td>27.0</td>
</tr>
<tr>
<td>Eicosapentaenoic acid</td>
<td>0.0419 ± 0.0111</td>
<td>26.4</td>
</tr>
<tr>
<td>Gamma-linolenic acid</td>
<td>0.0217 ± 0.0054</td>
<td>24.7</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>1.0162 ± 0.2942</td>
<td>28.9</td>
</tr>
<tr>
<td>Linolelaidic acid</td>
<td>0.3748 ± 0.1453</td>
<td>38.8</td>
</tr>
<tr>
<td><strong>Branched chain fatty acid</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Appendix III

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mean relative abundance in plasma samples ± standard deviation ( n = 269 )</th>
<th>Coefficient of variation ( n = 269 ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Itaconic acid</strong></td>
<td>0.0006 ± 0.0001</td>
<td>23.7</td>
</tr>
<tr>
<td><strong>Fatty acid esters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octanoic acid</td>
<td>0.0098 ± 0.0031</td>
<td>31.2</td>
</tr>
<tr>
<td><strong>Amino acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha-aminobutyric acid</td>
<td>0.0157 ± 0.0014</td>
<td>8.9</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.3098 ± 0.0251</td>
<td>8.1</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.0133 ± 0.0018</td>
<td>13.9</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.0247 ± 0.0038</td>
<td>15.6</td>
</tr>
<tr>
<td>4-Hydroxyproline</td>
<td>0.0018 ± 0.0003</td>
<td>17.3</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.0127 ± 0.0029</td>
<td>23.1</td>
</tr>
<tr>
<td>Dimethyl-aminomalonic acid</td>
<td>0.0038 ± 0.0013</td>
<td>34.0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.0153 ± 0.0024</td>
<td>15.8</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.0031 ± 0.0073</td>
<td>23.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.0187 ± 0.0065</td>
<td>34.7</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.0140 ± 0.0021</td>
<td>14.9</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.0451 ± 0.0070</td>
<td>15.4</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.1393 ± 0.0199</td>
<td>14.3</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.1736 ± 0.0232</td>
<td>13.5</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.0120 ± 0.0019</td>
<td>16.0</td>
</tr>
<tr>
<td>Ornithine</td>
<td>0.0638 ± 0.0094</td>
<td>14.7</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.0843 ± 0.0154</td>
<td>18.2</td>
</tr>
<tr>
<td>Proline</td>
<td>0.3406 ± 0.0458</td>
<td>13.6</td>
</tr>
<tr>
<td>Pyroglutamic acid</td>
<td>0.0041 ± 0.0008</td>
<td>19.2</td>
</tr>
<tr>
<td>Serine</td>
<td>0.0087 ± 0.0013</td>
<td>14.9</td>
</tr>
<tr>
<td>tert-Leucine</td>
<td>0.0303 ± 0.0036</td>
<td>11.9</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.0302 ± 0.0034</td>
<td>11.3</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.0833 ± 0.0145</td>
<td>17.4</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.0904 ± 0.0157</td>
<td>17.4</td>
</tr>
<tr>
<td>Valine</td>
<td>0.2668 ± 0.0298</td>
<td>11.1</td>
</tr>
<tr>
<td><strong>Peptide</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione</td>
<td>0.0026 ± 0.0005</td>
<td>17.6</td>
</tr>
<tr>
<td><strong>Organic acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Keto-acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Oxovaleric acid</td>
<td>0.0038 ± 0.0012</td>
<td>30.8</td>
</tr>
<tr>
<td>3-Methyl-2-oxovaleric acid</td>
<td>0.0135 ± 0.0042</td>
<td>31.3</td>
</tr>
<tr>
<td>4-Methyl-2-oxopentanoic acid</td>
<td>0.0227 ± 0.0070</td>
<td>30.7</td>
</tr>
</tbody>
</table>
### Appendix III

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mean relative abundance in plasma samples ± standard deviation</th>
<th>Coefficient of variation n = 269 (%)</th>
</tr>
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<tbody>
<tr>
<td><strong>Organic acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D_{4}-Methyl-2-oxopentanoic acid</td>
<td>0.0231 ± 0.0073</td>
<td>31.4</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>0.0080 ± 0.0022</td>
<td>27.4</td>
</tr>
<tr>
<td>Other organic acids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha-hydroxyisobutyric acid</td>
<td>0.0135 ± 0.0019</td>
<td>13.7</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>0.0016 ± 0.0005</td>
<td>32.8</td>
</tr>
<tr>
<td><em>cis</em>-Aconitic acid</td>
<td>0.0038 ± 0.0006</td>
<td>15.3</td>
</tr>
<tr>
<td>Citraconic acid</td>
<td>0.0007 ± 0.0002</td>
<td>22.6</td>
</tr>
<tr>
<td><strong>Organic acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citramalic acid</td>
<td>0.0128 ± 0.0018</td>
<td>13.9</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.1055 ± 0.0130</td>
<td>12.3</td>
</tr>
<tr>
<td>Citric acid (secondary peak)</td>
<td>0.0015 ± 0.0003</td>
<td>21.1</td>
</tr>
<tr>
<td>Fumaric acid</td>
<td>0.0006 ± 0.0002</td>
<td>31.8</td>
</tr>
<tr>
<td>Glyoxylic acid</td>
<td>0.0024 ± 0.0007</td>
<td>28.2</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>0.3813 ± 0.0648</td>
<td>14.0</td>
</tr>
<tr>
<td>Malic acid</td>
<td>0.0009 ± 0.0002</td>
<td>22.3</td>
</tr>
<tr>
<td>Malonic acid</td>
<td>0.0007 ± 0.0003</td>
<td>40.5</td>
</tr>
<tr>
<td>Methylthioacetic acid</td>
<td>0.0006 ± 0.0002</td>
<td>33.8</td>
</tr>
<tr>
<td>Oxalic acid</td>
<td>0.0045 ± 0.0013</td>
<td>28.1</td>
</tr>
<tr>
<td>para-Toluic acid</td>
<td>0.0008 ± 0.0002</td>
<td>30.6</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>0.0039 ± 0.0011</td>
<td>27.2</td>
</tr>
<tr>
<td>Tartaric acid</td>
<td>0.0011 ± 0.0004</td>
<td>33.7</td>
</tr>
<tr>
<td>10,13-dimethyltetradecanoic acid</td>
<td>1.8893 ± 0.5426</td>
<td>28.7</td>
</tr>
<tr>
<td><strong>Other organic compounds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,5-Diiodo-L-tyrosine</td>
<td>0.0021 ± 0.0021</td>
<td>98.7</td>
</tr>
<tr>
<td>Caffeine</td>
<td>0.0090 ± 0.0014</td>
<td>15.8</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.0086 ± 0.0022</td>
<td>25.5</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>0.0156 ± 0.0059</td>
<td>37.8</td>
</tr>
<tr>
<td>Dodecane</td>
<td>0.0131 ± 0.0047</td>
<td>35.9</td>
</tr>
<tr>
<td>Pentadecane</td>
<td>0.0088 ± 0.0024</td>
<td>27.9</td>
</tr>
<tr>
<td>Tridecane</td>
<td>0.0087 ± 0.0025</td>
<td>28.3</td>
</tr>
</tbody>
</table>
## Appendix III

### Appendix table 8 Metabolites detected in TMS derivatized samples

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Average relative abundance in plasma samples n = 114</th>
<th>Coefficient of variation n = 114 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fatty acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stearic acid</td>
<td>0.001 ± 0.0005</td>
<td>51</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>0.004 ± 0.0016</td>
<td>42</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>0.007 ± 0.0031</td>
<td>43</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>0.012 ± 0.0064</td>
<td>56</td>
</tr>
<tr>
<td><strong>Sugars and sugar alcohols</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>0.011 ± 0.002</td>
<td>14</td>
</tr>
<tr>
<td>Glucose peak 1</td>
<td>1.368 ± 0.134</td>
<td>10</td>
</tr>
<tr>
<td>Glucose peak 2</td>
<td>0.204 ± 0.030</td>
<td>15</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.038 ± 0.011</td>
<td>28</td>
</tr>
<tr>
<td>Meso inositol</td>
<td>0.010 ± 0.001</td>
<td>12</td>
</tr>
<tr>
<td><strong>Amino acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>0.076 ± 0.018</td>
<td>23</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.108 ± 0.027</td>
<td>25</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.012 ± 0.002</td>
<td>20</td>
</tr>
<tr>
<td>Valine</td>
<td>0.015 ± 0.004</td>
<td>29</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.011 ± 0.003</td>
<td>29</td>
</tr>
<tr>
<td>Valine</td>
<td>0.062 ± 0.016</td>
<td>26</td>
</tr>
<tr>
<td>Proline</td>
<td>0.079 ± 0.022</td>
<td>27</td>
</tr>
<tr>
<td>Serine</td>
<td>0.006 ± 0.001</td>
<td>20</td>
</tr>
<tr>
<td><strong>Organic acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isocitric acid</td>
<td>0.028 ± 0.004</td>
<td>14</td>
</tr>
<tr>
<td>Beta hydroxybutyric acid</td>
<td>0.018 ± 0.004</td>
<td>24</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>0.701 ± 0.099</td>
<td>14</td>
</tr>
<tr>
<td><strong>Other organic compounds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.869 ± 0.122</td>
<td>14</td>
</tr>
<tr>
<td>Urea</td>
<td>0.887 ± 0.121</td>
<td>14</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.076 ± 0.036</td>
<td>47</td>
</tr>
</tbody>
</table>


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Vitamin D Supplementation and the Effects on Glucose Metabolism During

Yap, C, Cheung, NW, Gunton, JE, Athayde, N, Munns, CF, Duke, A, and McLean, M,
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