Intake and status of iron and fatty acids during pregnancy in association with birth outcomes in women residing in urban South Africa

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"If I have seen further, it is by standing on the shoulders of giants."

– Isaac Newton

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PREFACE

This thesis is presented in article format and consists of six chapters as approved by the North-West University guidelines for postgraduate studies. This PhD study is a sub-study nested within a larger research project, which was implemented and executed as part of this PhD project. Background and detail about the methods of the larger project is supplied in Chapter 3. The layout of the thesis is as follows:

Chapter 1 introduces the topic under investigation and supplies the motivation for conducting the research. The study aims and objectives are provided in chapter 1. It also supplies the details of the research team involved in the larger research project.

Chapter 2 provides an overview of literature relevant to this research topic, focusing on the role iron and polyunsaturated fatty acids play in health and in pregnancy and early development; current knowledge on dietary intake of these nutrients as well as the iron and fatty acid status of women residing in South Africa. This is followed by discussions on associations between these nutrients and its effects on the birth outcomes under investigation.

Chapter 3 is a published article on the protocol of the larger project titled: *Nutrition during pregnancy and early development (NuPED) in urban South Africa: protocol for a prospective cohort study*. This article has been published in the journal *BMC Pregnancy and Childbirth* in 2018. The questionnaires and data collection sheets used for obtaining the data on the exposure and outcome variables are attached as Annexure 11.

Chapter 4 is in manuscript format and presents the results on iron intake and status with the title: *Antenatal iron-deficiency in iron supplemented pregnant women is associated with premature birth and higher birth weight: the NuPED prospective study*. This manuscript was submitted to the journal *PLOS One* for consideration for publication in the special edition on “Maternal and Child Health & Nutrition”. The manuscript was accepted for publication during thesis examination. The accepted manuscript is now attached as Annexure 10.

Chapter 5 is in manuscript format and provides the results on fatty acid intake and status with the title: *Associations of maternal PUFA intake and status in early pregnancy with birth weight and gestational age in Johannesburg, South Africa: the NuPED prospective study* This manuscript is prepared for submission to the journal *Prostaglandins, Leukotrienes and Essential Fatty Acids.*

Chapter 6 is the final chapter supplying a summary of the main findings, conclusions and recommendations.

AUTHOR CONTRIBUTIONS

The following sections will describe the role of the PhD student in this project and give an overview of the roles of the promoters and other researchers:

Ms EA Symington

- Conceptualised the study with promoters and Dr L Malan.
- Identified the study sites and initiated meetings with the relevant external institutions.
- Identified and appointed fieldworkers and co-ordinated training.
- Drafted and finalised ethical clearance applications for both the PhD and the larger study at two institutions.
- Drafted and finalised applications to governmental structures for permission to conduct the study at a public hospital.
- Principal investigator for a successful National Research Foundation Thuthuka grant (Grant No. 99374).
- Regularly visited Florida, Sophiatown, Bosmont and Zandspruit primary healthcare clinics for participant recruitment between March 2016 and April 2017.
- Co-ordinated on-site data collection activities between March 2016 and August 2017.
- Assisted in data cleaning and creating a database in preparation for statistical analyses. Statistical analyses were conducted under supervision of the promoters.
- Planning, drafting and submitting manuscripts for publication.
- Planning, drafting and compiling the thesis under the required supervision.

Prof CM Smuts (promoter), Prof J Baumgartner (co-promoter)

Supplied important guidance in the conceptualisation of the project. The promoters supervised and guided the implementation and execution of the project within the context of the research team. Prof Baumgartner played an important guiding role in the data preparations and statistical analyses. The promoters made recommendations for the framework, writing and composition of the manuscripts (chapters 3, 4 and 5). They were primary applicants for funding of the larger research project.

Dr L Malan

Dr Malan took part in the study conceptualisation. As part of the research team, she played a critical role in the implementation and execution of the study, specifically for the larger postnatal part. She co-ordinated the biochemical sample analyses. She was highly involved in co-ordinating

data capturing and cleaning. She contributed to specialist content of the manuscripts (chapters 3 and 4).

Dr AJ Wise

Dr Wise made recommendations on the study framework for implementation at the study site. She acted as supervisory gynaecologist and were responsible for co-ordinating and executing on-site medical assessments. She provided specialist feedback on manuscript content (chapter 4).

Dr L Zandberg

Dr Zandberg were highly involved in the project execution and were specifically involved in execution of the postnatal section of the larger study. She co-ordinated the biological sample handling and genetic analyses for the larger project. She provided specialist input to the manuscripts (chapters 3 and 4).

Dr C Ricci

Dr Ricci was responsible for preparation electronic formats for data capturing and overseeing statistical plans for data analyses. He contributed specialist biostatistical content to the manuscripts (chapters 3 and 4).

Hereby, I declare that I approved the manuscripts aforementioned and that my role in this study as stated above is representative of my actual contribution. I also give my consent that these manuscripts may be used as part of the PhD thesis of Ms EA Symington.

Prof CM Smuts	Prof J Baumgartner
Dr L Malan	Dr L Zandberg
Dr AJ Wise	Dr C Ricci

ABSTRACT

Background

Nutrient requirements increase during pregnancy and women are vulnerable to inadequate nutritional status during this period of rapid foetal growth. Poor dietary intake and nutritional status during pregnancy have been associated with poor birth outcomes and increased risks for non-communicable diseases in the offspring's adult life. Low birth weight (LBW) and prematurity are risk factors for disease in later life. It would therefore benefit population health to identify and address predictors of LBW and preterm birth, particularly in low- and middle-income populations. In South Africa, approximately 15% of babies are born LBW and approximately 12% are born preterm. Maternal iron deficiency (ID) has been associated with increased risk for LBW, while poor omega-3 (n-3) polyunsaturated fatty acid (PUFA) intake and status have been associated with premature birth. Iron is important for erythropoiesis, growth and neurodevelopment. PUFAs form an important part of the structural component of cell membranes and play a critical role in cell signalling and are therefore considered essential for optimal foetal growth and development. There are limited data available on the diet as well as the nutritional status of pregnant women in South Africa. A national study on non-pregnant women of child-bearing age has indicated that 15% of women were ID and between 23% and 33% of women were anaemic. No national data exists for fatty acid status, however regional studies on non-pregnant women have indicated that fatty acid status were reflective of dietary intake and that n-3 PUFA intakes are low and n-6 PUFA high. It is probable that the South African iron and PUFA status may contribute to complications during pregnancy, specifically related to length of gestation and birth weight.

Aim

Therefore, the aim of this research was to assess dietary intake and status of iron and long-chain polyunsaturated fatty acids (LCPUFA) during pregnancy; and to determine associations of maternal iron and LCPUFA status during pregnancy with birth outcomes, in particular gestational age and birth weight, in pregnant women giving birth at Rahima Moosa Mother and Child Hospital (RMMCH) in Johannesburg.

Design and methodology

A prospective, longitudinal study design was applied. Generally healthy pregnant women (<18 weeks of gestation) were recruited from four primary healthcare clinics in Johannesburg and n=250 were enrolled and followed-up at a tertiary hospital. Participants' socio-economic data, obstetric history and dietary intake data were collected at study entry. Maternal venous blood was

drawn at <18, 22 and 36 weeks of gestation. Additionally, foetal sonography assessments and maternal anthropometrical measurements were performed. Haemoglobin concentrations were determined from whole blood and adjusted for altitude. Iron parameters, serum ferritin and soluble transferrin receptor (sTfR), as well as inflammatory markers were determined using quantitative chemiluminescent multiplex assays. Serum ferritin concentrations were adjusted for inflammation. Red blood cell (RBC) total phospholipid fatty acid composition was determined using gas chromatography. Oral glucose tolerance tests were conducted between 24 and 28 weeks of gestation. At birth, maternal and neonatal health were assessed. Birth weight was obtained using calibrated infant scales and gestational period calculated from first visit sonography data. Logistic and multivariate regression models were used to determine associations between the independent variables (nutrient status) and the outcome variables, namely birth weight and gestational age. Models were adjusted for confounders.

Results

Most of the women were of black-African descent (88%) with a median age of 27 (24-32) years. At enrolment the median gestation was 14 (12-16) weeks and the median BMI (26.3 [23.0-30.6] kg/m^2) was above a healthy range with 33% of women being overweight and 28% obese. At enrolment, the mean reported iron intake was 19.1 (4.6-46.1) mg per day from foods, which included fortified foods. There was no significant difference in dietary iron intake between the anaemic and non-anaemic women (p=0.45) nor between the ID and non-ID women (p=0.24). Most women (62%) consumed less iron than the Estimated Average Requirement (EAR) (22 mg/day) for women during pregnancy, and two women (1%) had a dietary iron intake above the upper limit (UL) (45 mg) from foods.

At enrolment, the prevalence of anaemia, ID and iron deficiency erythropoiesis (IDE) was 28%, 15% and 15%, respectively, which increased significantly with pregnancy progression. There was a high prevalence (60%) of inflammation (CRP >5 mg/L) at enrolment. Multivariable regression analyses showed that anaemia and ID at 22 weeks, as well as IDE at 36 weeks of gestation were associated with higher birth weight (β=168.1; 95% CI: 19.5, 316.7 and β=227.6; 95% CI: 58.4, 396.8 and β=168.7; 95% CI: 27.9, 309.5, respectively). Women in the lowest ferritin quartile at 22 weeks gave birth to babies weighing 312 g (95% CI: 94.8, 528.8) more than those in the highest quartile. In contrast, IDE at 22 weeks was associated with a higher risk for premature birth (OR: 4.62, 95% CI: 1.56, 13.68) and women in lower haemoglobin quartiles at <18 weeks had a shorter gestation by 7 days (β=-6.9, 95% CI: -13.3, -0.6) compared to those in the highest quartile.

In terms of the fatty acid intake and status, we found that dietary consumption of n-6 linoleic acid (LA) was high, while n-3 α-linoleic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic

acid (DHA) intake were low. This resulted in a high LA:ALA ratio of 39:1 (28:1-51:1). RBC fatty acid composition changed significantly during pregnancy, however DHA did not decrease in late pregnancy as expected. In fully adjusted models, higher dietary n-6 PUFA and higher RBC arachidonic acid (AA) to EPA ratio were associated with an increased risk for premature birth (OR: 5.03, 95% CI: 1.16, 21.82, *p*=0.031 and OR: 4.51, 95% CI: 1.29, 15.79). By contrast, higher dietary n-6 PUFA and LA were associated with a lower risk for LBW (OR: 0.05, 95% CI: 0.00, 0.97, *p*=0.047 and OR: 0.02, 95% CI: 0.00, 0.60, *p*=0.025).

The median birth weight was 3050 (2324-3380) grams and 14% (n=29) of the babies were born with LBW (<2500 g). The median gestational age at birth was 274 (266-282) days and 11% (n=26) of babies were born preterm (<259 days).

Conclusion

At early pregnancy, the ID and anaemia prevalence in our sample were comparable to the reported prevalence in non-pregnant women from national surveys. However, anaemia and iron deficiency increased with pregnancy progression, regardless of routine iron supplementation. We suspect that the inflammatory status affected iron bioavailability through the mechanism of increased hepcidin. Noteworthy is that iron deficiency at mid-pregnancy was associated with higher birth weight but increased risk for preterm birth. Similar results were found for women who consumed higher amounts of n-6 PUFA at early pregnancy, namely increased risk for preterm birth and decreased risk for low birth weight. This may be reflective of the interplay between micronutrient status and fatty acid metabolism. Women consumed higher than the recommended n-6 LA and lower than the recommended n-3 ALA and DHA. The change in RBC fatty acid composition with pregnancy progression did not follow the same pattern as shown in literature from other populations.

In the context of an urban environment, this sample of generally healthy pregnant women presented with a high prevalence of overweight, obesity and inflammation as well as anaemia and iron-deficiency by late pregnancy. It is important to identify and address nutritional factors that may lower the risk for low birth weight and premature birth to improve the health of future generations in South Africa.

Keywords

Iron, anaemia, fatty acids, pregnancy, birth weight, premature birth

LIST OF ABREVIATIONS

24-HR	24-hour recall
AGP	α1-acid glycoprotein
ALA	α-linoleic acid
ANC	antenatal care
AA	arachidonic acid
BDNF	brain-derived neurotrophic factor
BMI	body mass index
CHL	crown-heel length
CHO	carbohydrates
CI	confidence interval
CRP	C-reactive protein
DGLA	dihomo-γ-linolenic acid
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
DOHaD	developmental origins of health and disease
EAR	estimated average requirements
ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid
ESRU	Empilweni Services and Research Unit
FAO	Food and Agricultural Organisation
FAME	fatty acid methyl esters
Fe	iron
GLA	γ-linolenic acid
GC-MS/MS	gas chromatography-mass spectrometry
Hb	haemoglobin
HC	head circumference
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
HRP2	histidine-rich protein 2
HREC	human research ethics committee
ID	iron deficiency
IDA	iron deficiency anaemia
IOM	Institute of Medicine
IQR	interquartile range

IUFD	intrauterine foetal death
IUGR	intrauterine growth restriction
LA	linolenic acid
LBW	low birth weight
LCPUFA	long-chain polyunsaturated fatty acid
MAC	midarm circumference
MAF	multidimensional assessment of fatigue
MUAC	mid-upper arm circumference
MUFA	monounsaturated fatty acid
n-3	omega-3
n-6	omega-6
NCD	non-communicable disease
NuPED	Nutrition during Pregnancy and Early Development
NWU	North-West University
OGTT	oral glucose tolerance test
OR	odds ratio
PUFA	polyunsaturated fatty acid
QFFQ	quantified food frequency questionnaire
RBC	red blood cell
RDA	recommended dietary allowances
RMMCH	Rahima Moosa Mother and Child Hospital
ROS	reactive oxygen species
RPR	rapid plasma reagin
SD	standard deviation
SFA	saturated fatty acid
SGA	small-for-gestational age
sTfR	soluble transferrin receptor
TC	thoracic circumference
Tf	transferrin
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION, BACKGROUND AND RATIONALE

NuPED

Nutrition during Pregnancy and Early Development

1.1 Introduction

The importance of maternal nutrition has been recognised for many decades. The role of maternal nutrition in foetal growth, placental development and maternal health seems obvious since nutrients are required for complex biological processes, such as cell division and differentiation, in a relatively short period of time. Research has shown that poor nutritional conditions before and during pregnancy influence placental and foetal development, and may result in intrauterine growth restriction (IUGR), neural tube defects, cretinism, preterm birth and poor maternal health (Wu *et al.*, 2012). However, in recent decades, evidence on the consequences of poor maternal nutrition on long-term health of the adult offspring became available. Barker and colleagues (Barker, 1986) suggested that the cardiovascular diseases they observed in an adult population from England and Wales were at least partly associated with poor early nutrition, and specifically undernutrition *in utero* (Barker, 2007). Further epidemiological studies in natural history cohorts found associations between lower birth weight and hypertension, glucose intolerance and hyperlipidaemia in later life (Kunz & King, 2007). Apart from undernutrition, maternal obesity and gestational diabetes are associated with an increased risk for offspring obesity and metabolic syndrome (Catalano & Ehrenberg, 2006). This relationship between early nutrition and long-term health was coined the *Developmental Origins of Health and Disease* (DOHaD) theory and is also the theme of this study.

The DOHaD theory served as motivation to investigate the relationship between maternal nutrition during pregnancy and birth outcomes in South Africa due to a rapidly increasing trend of non-communicable diseases (NCDs) as well as obesity in the adult population (Nojilana *et al.*, 2016). On average 12% of South African babies are born low birth weight (OECD, 2011) and an estimated 12% born preterm (Blencowe *et al.*, 2012), which may, according to the DOHaD theory, impact the future health status of South Africans. Very little is known about the nutritional status of pregnant women in South Africa. With this in mind, the *Nutrition during Pregnancy and Early Development: the NuPED study* was initiated to assess dietary intake and nutritional status of urban pregnant women in Johannesburg, South Africa, and to determine associations with birth outcomes, measures of maternal health, as well as measures of offspring health and development. This PhD study is a sub-study nested within this larger research project, which was implemented and executed as part of this PhD project. Background and detail about the methods of the larger NuPED study is provided in Chapter 3, which is in the format of a protocol article. Methodology relevant to the particular objectives of this PhD study is provided in Chapters 4 and 5.

1.2 Background and rationale for the project

Adverse birth outcomes, such as low birth weight (LBW) (<2500 g) and preterm birth (<37 completed weeks' gestation), are associated with short and long-term health outcomes. LBW is a risk factor for non-communicable diseases in later life, such as hypertension (Ediriweera *et al.*, 2017), coronary heart disease (Barker, 1995) and obesity (Oken & Gillman, 2003). LBW could be the result of IUGR, preterm birth or other constituent factors. Small-for-gestational (SGA), a common proxy for IUGR, is similarly associated with increased risk for neonatal and infant mortality (Katz *et al.*, 2013), as well as long-term health (Osmond & Barker, 2000). It would therefore benefit public health to address risk factors for LBW, SGA and preterm birth.

Risk factors for these adverse events could be genetic, but it is estimated that environmental factors can be attributed to 60% of these cases. These include socio-demographic factors such as heavy alcohol consumption (Patra *et al.*, 2011), cigarette smoking, ethnicity, primiparity, short birth intervals, low educational level and extremes of maternal age (Valero de Bernabé *et al.*, 2004). Furthermore, maternal health related factors are short maternal stature (Kozuki *et al.*, 2015), low pre-pregnancy body mass index, anaemia, undernutrition (Wu *et al.*, 2012), overnutrition (Wu *et al.*, 2004), infections such as HIV (Valero de Bernabé *et al.*, 2004) and malaria (Rijken *et al.*, 2014), chronic hypertension, renal disease, glucose metabolism disorders and multiple pregnancies (Valero de Bernabé *et al.*, 2004).

Several nutritional factors *per se* have been investigated in terms of adverse birth outcomes, however, for this PhD study, the emphasis was on maternal iron and long-chain polyunsaturated fatty acids (LCPUFA) intake and status. The rationale for focusing on these nutrients is clarified in the following paragraphs.

Firstly, both these nutrients are essential and have possible interactions. Although the human body can synthesize saturated and monounsaturated fatty acids, both parent n-3 and n-6 polyunsaturated fatty acids (PUFAs) are essential and should be obtained from the diet. The main source of PUFAs in the South African diet is vegetable oils (in particular sunflower oil/margarines) (MacIntyre *et al.*, 2002; Mchiza *et al.*, 2015). Sunflower oil supplies mostly linolenic acid (LA;C18:2 n-6) and only trace amounts of alpha-linoleic acid (ALA;C18:3 n-3) (Orsavova *et al.*, 2015). Oily fish and seafood are considered the major sources of the n-3 LCPUFA eicosapentaenoic acid (EPA;C20:5 n-3) and docosahexaenoic acid (DHA;C22:6 n-3), whereas meat, poultry and eggs are major sources of the n-6 LCPUFA arachidonic acid (AA;C20:4 n-6) (Meyer *et al.*, 2003). Thus, the general South African diet is considered to be high in n-6 PUFAs and inadequate in n-3 PUFAs and n-3 LCPUFAs. Human metabolism allows for some LCPUFA synthesis by converting the essential n-3 fatty acid ALA to EPA and DHA, however, this conversion is limited in humans,

especially to DHA, and is affected by diet and gender (Arterburn *et al.*, 2006). This conversion is nevertheless essential. It involves the iron-containing hepatic desaturases enzymes, which introduce a double bond in the fatty acid carbon chain (Nakamura & Nara, 2004). Being dependent on iron for its functionality, limited iron availability may thus influence the effectiveness of the enzyme and inhibit conversion of ALA to the longer chain n-3 PUFAs.

Iron is a trace element found in nearly all living organisms. The normal adult diet should contain 13-18 mg of iron/day of which only 1 mg is absorbed (Miret *et al.*, 2003). Foods of animal origin are major sources of haem iron, which is two to three times better absorbed than non-haem iron (found more so in plant-based foods and dairy products) (Qiu *et al.*, 2011). Even though South Africans consume more non-haem iron (Faber & Wenhold, 2009), its bioavailability is negatively influenced by other dietary factors such as calcium, polyphenols, certain vegetable proteins and phytates (Gibson *et al.*, 2014; Scheers, 2013). Micronutrient fortification (including iron) of two staple foods is mandatory in South Africa since implementing legislation in 2003. In addition, pregnant women are supplied with iron supplements during pregnancy (National Department of Health, 2015). Even so, from available data, iron intakes and status remain low, especially among women and children (Harika *et al.*, 2017; Steyn *et al.*, 2007; van Stuijvenberg *et al.*, 2008).

Iron and fatty acids share the liver as an important site of metabolism and a main possible site for interaction between these pathways (Ahmed *et al.*, 2012). Three decades ago, Cunnane and McAdoo (1987) indicated that moderate iron deficiency in rats resulted in mild impairment of essential fatty acid metabolism (Cunnane & McAdoo, 1987), which has later been ascribed to impaired desaturation by the desaturases enzymes (Stangl & Kirchgessner, 1998). Desaturase enzymes are considered non-haem iron containing proteins (Okayasu *et al.*, 1981), which explains the impaired enzyme function in an iron deficient environment. When iron supplementation was provided to iron deficient schoolchildren with low fish consumption, it resulted in increased morbidity (mostly respiratory). However, these negative effects were attenuated when iron and n-3 LCPUFA were provided in combination, further demonstrating interactions between iron and n-3 LCPUFAs status (Malan *et al.*, 2015). Considering the essential role of iron in fatty acid desaturation and the interaction between iron and fatty acid status, we hypothesise that iron deficiency during pregnancy compromises n-3 and n-6 LCPUFA status. And therefore, we expect differences in the fatty acid profile of iron deficient and iron replete pregnant mothers. We hypothesise that mothers with this altered fatty acid profile, specifically in early pregnancy, will deliver new-borns with a lower birthweight and shorter gestational age. Investigating these interactions in future work would result in novel data. However, for the purposes of this thesis, these nutrients were investigated individually in order to fill the gap of knowledge specifically in the South African context.

Secondly, both iron and LCPUFAs have been shown to be critical in early development. It is well known that essential fatty acids are required for growth and development of all organ systems, including the brain and retina (Briend *et al.*, 2011). Global observational studies on maternal LCPUFA status imply that the maternal fatty acid profile in early pregnancy predicts foetal growth, specifically, that a higher n-3:n-6 ratio is associated with a significantly longer gestational period, and that EPA levels are positively associated with birth weight (Grandjean *et al.*, 2001; Van Eijsden *et al.*, 2008). The mechanisms for these associations are not fully understood. It is, however, understood that the foetus requires LCPUFA supply from maternal circulation. Nevertheless, studies have shown that foetal concentrations of DHA and AA are 300- to 400-fold higher compared with maternal plasma phospholipids, while the precursors of these are lower (i.e. LA and ALA) (Innis, 2003). Dutta-Roy (2000) identified a placental plasma membrane fatty acid binding protein that favours LCPUFA uptake over non-essential fatty acids (Dutta-Roy, 2000), which therefore benefits the foetal LCPUFA status.

Globally, maternal iron status has been extensively studied. It is understood that early maternal iron deficiency anaemia (IDA) is associated with an increased risk of low birth weight, preterm delivery and delayed offspring neurological development (Alwan *et al.*, 2011; Scholl, 2011). Furthermore, normal birth weight infants born to anaemic mothers have an increased risk for low haemoglobin levels and low iron stores when compared to infants born of non-anaemic mothers (Alwan *et al.*, 2011; de Pee *et al.*, 2002). The placental iron transfer system regulates iron transport to the foetus. Low maternal iron status results in increased placental transferrin receptor expression, which allows the placenta to take up more iron. However, the effectiveness of increased placental transferrin receptors is limited by maternal iron status and with maternal iron deficiency, iron transfer would be limited (Allen, 2000).

Thirdly, there are limited data available on maternal nutritional status in South Africa, specifically during pregnancy. Recent national data on women of reproductive age indicated that 15% had low serum ferritin levels (<15 ng/mL) and 10% had IDA (Shisana *et al.*, 2013). Overall, anaemia was prevalent in 22% of all women, while 24% of women between the ages of 26 and 35 were anaemic (Shisana *et al.*, 2013). During pregnancy, blood volume expansion, as well as the growth of the foetus and other maternal tissues increase the demand for iron three-fold (Umbreit, 2005; Vricella, 2017). It can therefore be expected that a proportion of the South African pregnant population may be iron deficient since iron stores are likely inadequate to cover the increased demand with pregnancy progression. The South African Comparative Risk Assessment group estimated that in the year 2000, 9 – 12% of South African pregnant women had IDA, which contributed to 7.3% of perinatal deaths and 4.9% of maternal deaths (Nojilana *et al.*, 2007). Even though the burden from IDA is estimated to be less serious in South Africa

compared to other low- and middle-income countries, the consequences remain the same and it is preventable. Appropriate strategies should be in place to assess and address the situation. The WHO recommends daily oral iron and folic acid supplementation as part of antenatal care. Daily elemental iron is recommended at 30 – 60 mg per day to all pregnant women (World Health Organization, 2012, 2017). According to the South African guidelines for maternity care, routine antenatal care includes 200 mg ferrous sulphate tablets daily (supplying approximately 65 mg elemental iron) (National Department of Health, 2015). Potential risks exist with iron supplementation in iron sufficient women during pregnancy. The possible association between iron supplementation and oxidative stress has been suggested to increase susceptibility to infections, including malaria, as well as increasing the risk of developing gestational diabetes (Mwangi *et al.*, 2017; Oppenheimer, 2001; Scholl, 2005). Furthermore, a risk of haemoconcentration during pregnancy with daily iron supplementation has been reported, however, the causality with supplementation remains uncertain (Peña-Rosas & Viteri, 2009).

South African dietary intake data on LCPUFA are limited. A summary of findings reported from various cross-sectional dietary intake studies indicates that women from rural areas typically consumed less total fat compared to women from urban areas. Mean PUFA intakes were less than 10% of total energy and were also compromised if the total fat intake was low. The ratio of polyunsaturated to saturated fatty acids was lower in urban settings due to higher consumption of animal-derived foods (Smuts & Wolmarans, 2013). The ratio of n-6 to n-3 fatty acid intake was high confirming the suspected low levels of EPA and DHA on average in South Africa (Richter, 2010). More recently, reported intakes from three areas in South Africa confirmed the higher fat intake of women in urban vs women in rural areas (32% and 37% in urban vs 16% in rural). The ratio of reported LA:ALA intake, however, did not differ among urban and rural areas and were between 34:1 and 42:1 (Ford *et al.*, 2016). Furthermore, reported EPA and DHA intakes did not reach the adequate intake (AI) levels. It is therefore important to have more insight into the LCPUFA status of pregnant women.

Due to the limited information on these nutrients during pregnancy in South Africa, it is important to investigate the intake and the status of these nutrients in pregnant women. The role of these nutrients may also be better understood when prospectively assessing the associations between maternal intake and status with birth outcomes. This may further provide a basis for studying the interactions between nutrient and for nutritional interventions which can improve birth outcomes and long-term quality of life.

1.3 Aim and objectives

The aim of this research is to assess dietary intake and status of iron and LCPUFA during pregnancy; and to determine associations of maternal iron and LCPUFA status throughout pregnancy with birth outcomes, in particular gestational age and birth weight, in pregnant women giving birth at Rahima Moosa Mother and Child Hospital (RMMCH) in Johannesburg.

The objectives are to:

1. conceptualise, implement and execute the larger NUPED prospective cohort in which the PhD study is nested,
2. describe the dietary iron intake at early pregnancy, as well as the iron status at early, mid- and late pregnancy and to determine associations of iron status with both birth weight and gestational age, and
3. describe the dietary LCPUFA intake at early pregnancy, as well as the LCPUFA status at early, mid- and late pregnancy and to determine associations of LCPUFA intake and status with birth weight and gestational age.

1.4 Ethical approval and other ethical considerations

The North-West University Human Research Ethics Committees (NWU-00186-15-A1) and the University of Witwatersrand, Johannesburg (M150968) provided ethical approval for the study (Annexures 1 and 2). Permission to conduct the research in the relevant clinical settings was obtained from the Gauteng Health Department (Annexure 3), City of Johannesburg District Research Committee (Annexure 4), the Clinical Manager of RMMCH (Annexure 5) as well as the Head of Department: Obstetrics and Gynaecology (Annexure 6). All participants signed written informed consent before data collection (Annexure 7). All participants received refreshments and reimbursement for travel at each visit. At the third study visit (or study exit with adverse pregnancy outcome) participants received a token of appreciation. The women enrolled into the study probably received better medical care due to the study location at the hospital with a referral doctor instead of standard of care at primary healthcare facilities.

1.5 Research team and contributions

The team members and their roles in this PhD projected are indicated in Table 1.1. Team members and their contributions in the larger project are indicated in Table 1.2.

Table 1.1: PhD study promotors and student details

Affiliation	Name	Role	Relevant Expertise
North-West University	Prof. Marius Smuts	Principal Investigator Supervisor Initiated project	Essential fatty acid and micronutrient nutrition; Nutrition during pregnancy and infancy; Community-based clinical trials
North-West University	Prof. Jeannine Baumgartner	Co-Principal Investigator Co-supervisor Implementation and execution of project	Essential fatty acid and micronutrient nutrition; Role of essential fatty acids and iron in neurodevelopment; Community-based clinical trials
North-West University/ Unisa	Elize Symington	PhD student Project conceptualisation Implementation and execution of project On-site project coordination	Dietetics; nutrition during pregnancy

Table 1.2: Additional team members of the larger NuPED study

North-West University	Dr. Linda Malan	Investigator/Researcher Implementation and execution of project Coordination of sample analyses	Essential fatty acid and micronutrient nutrition; Role of essential fatty acids and iron in immune development and functioning; Community-based clinical trials, Laboratory specialist
RMMCH; University of Witwatersrand	Dr. Amy Wise	Investigator and Supervisory Gynaecologist On site medical assessments	Gynaecology
RMMCH; University of Witwatersrand	Prof. Ashraf Coovadia	Investigator and Supervisory Paediatrician Head of Department of Paediatrics and Child Health	Paediatrics and Child Health
North-West University	Dr. Cristian Ricci	Biostatistician and post-doctoral fellow Statistical coordination	Statistics and data analysis
North-West University	Dr. Lizelle Zandberg	Post-doctoral fellow Biological sample coordination and genetic analyses	Molecular nutrition; Nutrigenetics; Epigenetics
Unisa	Dr. Olusola Sotunde	Post-doctoral fellow On site coordination (2016)	Body composition; Nutrition during pregnancy
North-West University	Dr. Marinel Hoffman	Post-doctoral fellow On site coordination (2017)	Infant nutrition and development

1.6 Thesis outline

This study is presented in article format and consists of six chapters. Following this introduction, **Chapter 2** provides an overview of literature relevant to this research topic, focusing on the role these nutrients play in health and in pregnancy and early development; current knowledge on dietary intake of these nutrients as well as the iron and n-3 fatty acid status of women living in South Africa. This is followed by discussions on associations between these nutrients and its effects on the birth outcomes under investigation.

Chapter 3 is an article on the protocol of the larger project titled: *Nutrition during pregnancy and early development (NuPED) in urban South Africa: protocol for a prospective cohort study*. This article has been published in the journal *BMC Pregnancy and Childbirth*. See Annexure 8 for the published format. It describes the background and methods of the larger project and therefore supplies comprehensive context in which the PhD study is nested.

Chapter 4 is in manuscript format and presents the results on iron intake and status with the title: *Antenatal iron-deficiency in iron supplemented pregnant women is associated with premature birth and higher birth weight: the NuPED prospective study*. This manuscript was submitted to the journal *PLOS One* for consideration for publication in the special edition on "Maternal and Child Health & Nutrition". The manuscript was accepted during thesis examination and the accepted manuscript is attached as Annexure 10.

Chapter 5 is the third article with the title *Associations of maternal PUFA intake and status in early pregnancy with birth weight and gestational age in Johannesburg, South Africa: the NuPED prospective study.* This manuscript is prepared for submission to the journal *Prostaglandins, Leukotrienes and Essential Fatty Acids.*

Chapter 6 is the final chapter supplying a summary of the main findings, conclusions and recommendations.

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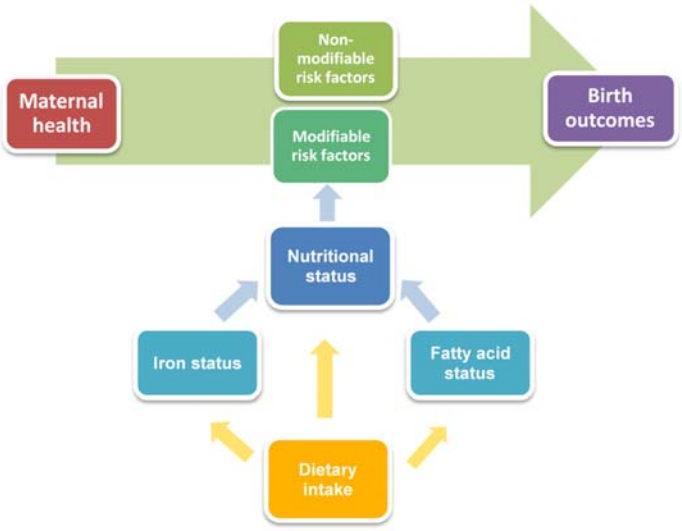
CHAPTER 2
LITERATURE REVIEW



2.1 Introduction

Both iron and long-chain polyunsaturated fatty acids (LCPUFAs) are essential nutrients that are critical in early development and utilise the liver as an important site of metabolism. Iron and LCPUFA intake and status have also been associated with maternal health and birth outcomes. Figure 2.1 below depicts a simplistic illustration of the topic under investigation and provides the context for this literature review.

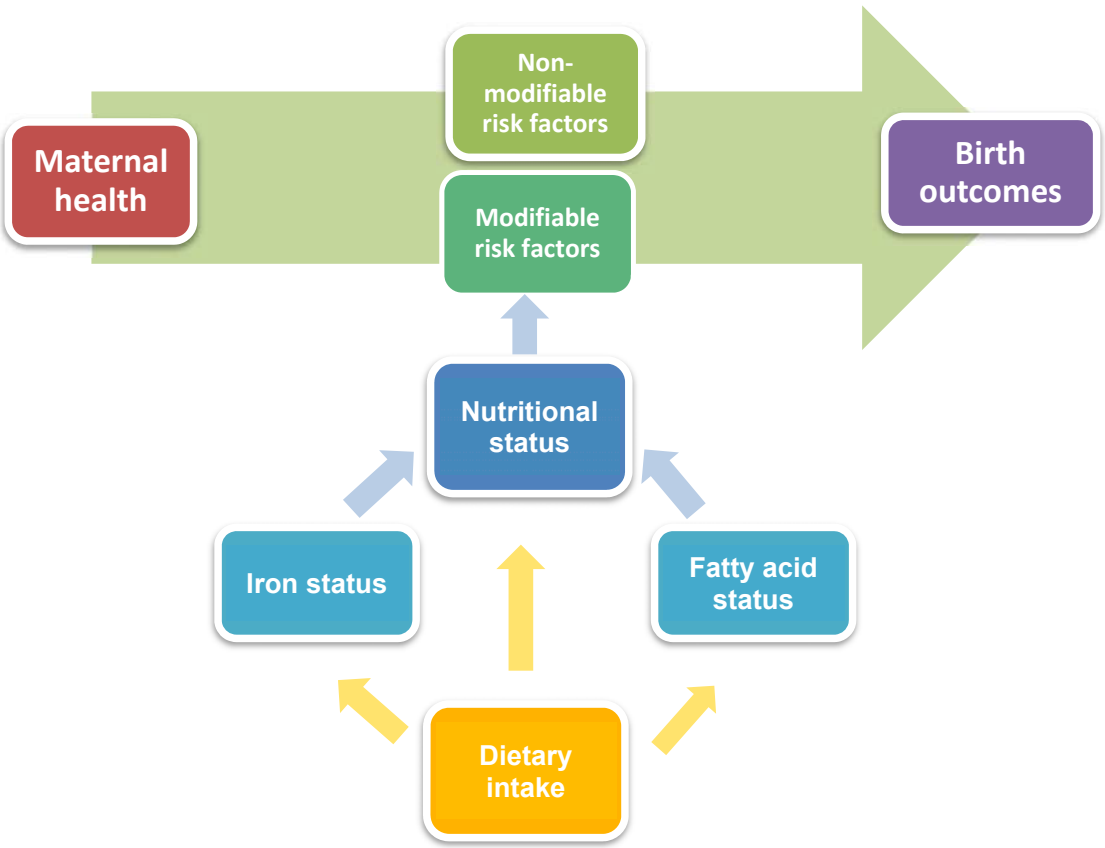


Figure 2.1: Simplistic conceptual framework of the topic under investigation

Firstly, each nutrient will be discussed by supplying background in terms of its characteristics, functions, metabolism, requirements and consequences of deficiencies and possibly overdoses. These discussions will be in general as well as in the context of pregnancy and foetal development. This will be followed by deliberations on associations between these nutrients and the birth outcomes under investigation.

2.2 Iron – an essential nutrient

Iron is a metal element and abundantly available on earth. Iron mostly exists in one of two oxidation states, namely Fe^{+2} (ferrous) or Fe^{+3} (ferric) and serves as an electron donor. This trait explains iron's important role as a cofactor to enzymes involved in oxidation-reduction reactions, including lipid metabolism (Cairo *et al.*, 2006; Chipperfield, 2003). Iron is therefore also normally bound to proteins (Chipperfield, 2003). For instance, iron binds with a protoporphyrin ring to form haem proteins in body cells. It is also this haem protein found in the flesh of animals, such as meats, poultry and fish, that supplies dietary haem iron in a highly bioavailable form. Non-haem iron, on the other hand, is not bound to proteins and is found in plant- and animal-derived dietary sources and is less bioavailable (Rolfes *et al.*, 2018a).

The reactive ability of iron can lead to the generation of reactive oxygen species (ROS) (Gozzelino & Arosio, 2016). ROS are created during normal metabolism and play a role in cell signalling and regulation of cellular functions (Gamaley & Klyubin, 1999). However, at high levels, ROS triggers oxidative stress, lipid peroxidation and DNA damage (Gozzelino & Arosio, 2016). Therefore, systemic and cellular iron homeostasis is required to prevent these deleterious effects. Iron homeostasis is discussed in the following sections.

2.2.1 Iron in human physiology

Iron is an essential trace element, thus required in small amounts from the diet for normal physiological functioning (Goldhaber, 2003). The human body contains on average about 4.5 grams of iron (Klawe, 2003). Approximately 75% of body iron is found in haemoglobin (within circulating erythrocytes), while 25% is in readily available iron storage, such as bound to ferritin and hemosiderin (within the spleen, liver and bone marrow), or is bound to myoglobin (within muscle tissue) and enzymes (Chitturi *et al.*, 2015).

Iron is required as a structural component of oxygen transport and storage proteins, specifically haemoglobin and myoglobin, as well as for iron-containing enzymes involved in electron transfer (Abbaspour *et al.*, 2014). In addition, iron plays an important role in neurodevelopment. Iron-containing enzymes are involved in central nervous system myelination, neurotransmitter synthesis and brain-energy metabolism (Chang *et al.*, 2013). Each day, approximately 25 mg of iron is required for erythropoiesis, incorporation into enzymes and other important functions. Only 1 to 2 mg iron are obtained from intestinal absorption, thus various mechanisms play a role in systemic iron homeostasis (Mackenzie *et al.*, 2008). Humans have no active excretory mechanisms for iron. Only small amounts are lost in bile and urine as well as exfoliation of skin

and gastrointestinal cells (Gulec *et al.*, 2014). Therefore, a balance is required between iron uptake, transport, storage and utilisation to maintain iron homeostasis. The major iron regulatory site is therefore at absorption.

Absorption

One of the mechanisms of iron homeostasis involves modulation of iron absorption. Both haem and non-haem iron, as obtained from the diet, are absorbed mainly in the duodenum and proximal jejunum – an acidic environment due to gastric acid. Iron is more soluble in an acidic environment and the soluble form is better absorbed. As stated previously, haem iron is more bioavailable than non-haem iron. Intact haem iron can be transported across the duodenal brush border into enterocytes – without iron and the porphyrin ring being split, unlike non-haem iron, which will be discussed later. The direct transport mechanism of haem iron is not well understood (Abbaspour *et al.*, 2014; Miret *et al.*, 2003). Since haem iron is bound to porphyrin during direct absorption, reduction to ferrous iron is not required and other dietary constituents have little effect on its absorption. Only upon entering the enterocyte, the iron is split from the porphyrin by haem oxygenase 1 and released into the cytoplasmic pool within the enterocytes as Fe^{+2}. Non-haem iron (the inorganic form), on the other hand, is highly insoluble and mainly available in chelate complexes. Its absorption is influenced by other dietary constituents. Some dietary constituents enhance iron absorption, namely ascorbic acid (in citrus fruits, bell peppers etc.) and a “meat enhancing factor” in meat, fish and poultry. However, other factors inhibit non-haem iron absorption, such as phytates (in legumes, unrefined cereals, etc.), polyphenol compounds (in tea and coffee) (Gibson *et al.*, 2014), and high amounts of calcium intake (Scheers, 2013). For non-haem iron to be absorbed, it needs to be reduced from ferric (Fe^{+3}) to ferrous (Fe^{+2}) iron and is then absorbed through facilitated transport by means of a divalent metal ion transporter 1 (DMT1) (see Figure 2.2.2) (Gulec *et al.*, 2014). The absorbed iron is pooled in the cytoplasma of enterocytes.

Upon being required elsewhere, enterocyte iron is exported into circulation by means of an iron exporter protein on the enterocyte membrane, namely ferroportin. This process includes reoxidation of Fe^{+2} to Fe^{+3} (Abbaspour *et al.*, 2014; Gulec *et al.*, 2014). With high concentrations of circulating iron, hepcidin (a liver-derived hormone) binds the iron exporter protein (ferroportin), inducing internalisation and degradation of this protein to suppress iron release and ultimately limiting iron export from enterocytes (Abbaspour *et al.*, 2014). Therefore, hepcidin is considered to play a major role in iron homeostasis (Fisher & Nemeth, 2017; Gozzelino & Arosio, 2016).

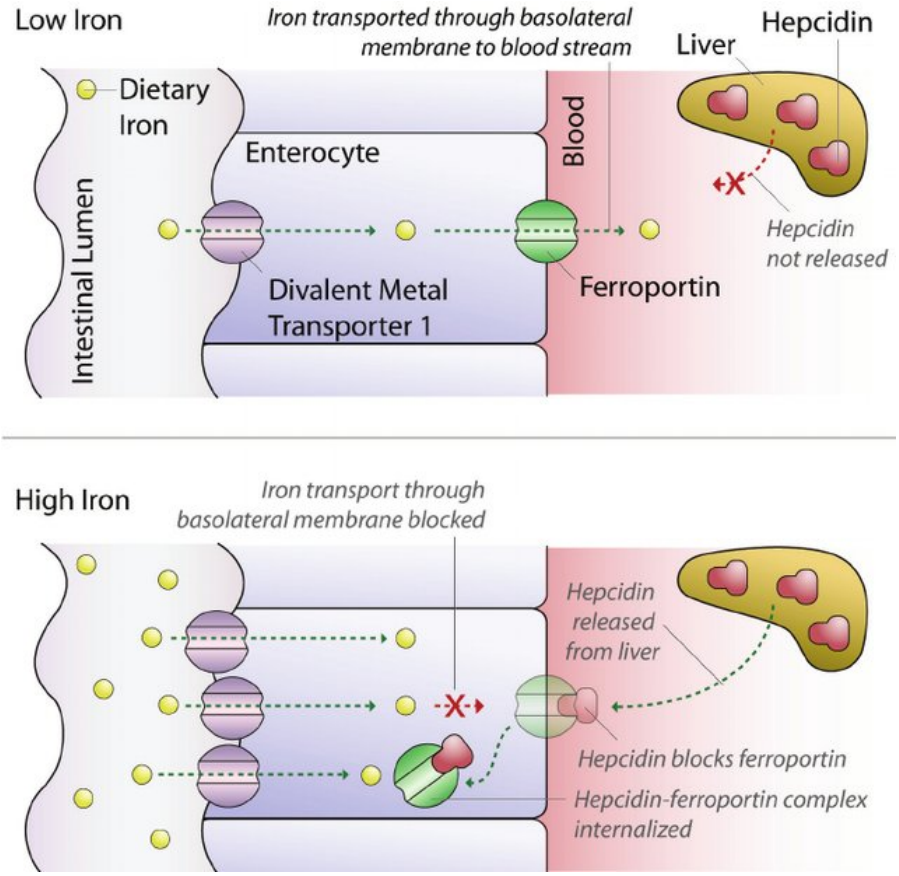


Figure 2.2: Intestinal iron absorption in low and high iron status
(obtained from: Naigamwalla et al*., 2012)*

Transport

Circulating Fe^{+3} is scavenged by apo-transferrin (once bound to iron, called transferrin [Tf]) and transported to where required (Abbaspour *et al.*, 2014). In humans, most iron is transported to bone marrow for haemoglobin (Hb) synthesis as part of developing erythroid cells (erythropoiesis) (Ponka & Lok, 1999). Iron status can thus directly influence Hb levels. With iron delivery to the cells, Tf binds to a membrane glycoprotein receptor, namely transferrin receptor (TfR). The receptor-Tf-complex is then internalised into the cell and iron is released after which the complex is returned to the cell surface and the apo-Tf released again. TfRs are present on all cells in concentrations relevant to its iron requirements. TfRs are present in high concentrations on reticulocytes and are shed from the membrane which is detectable as soluble TfR (sTfR) in serum (Koulaouzidis *et al.*, 2009). With tissue iron deficiency, there is a proportional increase in the number of TfR through a negative feedback system (Ponka & Lok, 1999). sTfR is therefore one

biomarker used in the clinical diagnosis of iron deficiency anaemia (IDA) (Koulaouzidis *et al.*, 2009).

Storage

Iron is stored within the iron storage protein, ferritin, mainly in the liver. Low cellular iron levels suppress the synthesis of ferritin to increase cellular use of iron instead of storing it. Under stable conditions, serum ferritin concentrations correlate well with iron stores. Therefore, serum ferritin is the most convenient biochemical marker to estimate iron stores (Abbaspour *et al.*, 2014). However, serum ferritin concentrations increase during inflammation, infection, cancer, liver diseases and responses to oxidative stress (Arosio *et al.*, 2009). High levels of serum ferritin during inflammation should therefore not be interpreted without adjusting ferritin levels for inflammation (Thurnham *et al.*, 2015). In addition, hepatocytes produce more hepcidin with inflammation. Therefore, less iron is delivered to plasma Tf and plasma iron status becomes depleted (Nemeth & Ganz, 2014). Thus, even when there is sufficient dietary iron, inflammation results in less iron reaching circulation.

2.2.2 Iron metabolism and status during pregnancy

Maternal enterocyte iron absorption is increased during pregnancy mainly due to suppression of hepcidin, specifically during the second and third trimesters of healthy pregnancies. Hepcidin is decreased even in iron-replete pregnant women which indicates that hepcidin is actively decreased specifically during pregnancy (Fisher & Nemeth, 2017). Animal-origin haem iron seems to be preferentially transported from maternal enterocytes to the foetus during the third trimester of pregnancy, while non-haem iron to a lesser extent. There is a correlation between placental iron transfer and neonatal Hb status (Young *et al.*, 2012) indicating the foetal dependency on maternal iron concentrations.

During pregnancy, iron stores are also increasingly mobilised from the maternal liver and are passed to the foetus via the placenta, of which the mechanisms are still unclear. It is understood that nutrient transport across the placenta occurs through the epithelial layer covering the placental villi, known as the syncytiotrophoblasts (Sangkhae & Nemeth, 2019). These epithelial cells express nutrient transporters including TfR facing maternal circulation. Maternal Tf binds the placental TfR and is released into the syncytiotrophoblasts in the same fashion as explained earlier. When required, iron is released from the cells through ferroportin (located on the foetal facing side of the cells). Ferroportin expression increases with pregnancy progression supporting

the foetal demand. After release from ferroportin, iron binds with foetal circulating Tf for delivery to the foetal liver (Gambling *et al.*, 2011; Sangkhae & Nemeth, 2019).

It is understood that the transfer from placental cell to foetal vasculature is also regulated by hepcidin (Ganz, 2005). The amount of iron transferred from maternal stores to foetal stores rises as gestation progresses (Gambling *et al.*, 2011). Therefore, maternal iron requirements differ between trimesters of pregnancy (Bothwell, 2000). In the first trimester, less iron is required than pre-pregnancy due to cessation of menses (Bothwell, 2000). The foetus obtains the majority of its iron stores during the third trimester (Young *et al.*, 2012), which also explains the risk for iron deficiency (ID) in prematurely born infants and the need for optimal maternal iron status going into the last two trimesters.

Although it is not well understood or confirmed, maternal iron homeostasis seems to favour foetal supply. As indicated earlier, the number of TfR increases with tissue iron deficiency. Similarly, the number of placental TfR increases as the amount in maternal tissues decreases. This results in a larger proportion of the transported iron to be taken up by the placenta and foetus. Maternal haematocrit has second priority for receiving iron and maternal iron stores last priority (Gambling *et al.*, 2011). These changes are depicted in reported iron biomarker concentrations in the course of pregnancy which is not only explained by the plasma volume expansion or haemodilution (Vricella, 2017). Haemodilution is responsible for physiological anaemia measured as a sudden decrease in Hb concentration in the second trimester up to early third trimester (Scholl, 2011; Vricella, 2017). Similarly, there is a rise in ID as gestation progresses. In a prospective cohort (without supplementation) in the United States, low total body iron was more prevalent in the second and third trimesters, compared to the first (Mei *et al.*, 2011). A longitudinal trajectory of iron biomarkers with pregnancy progression in a group of women (mostly receiving supplementation) also from the United States, indicated a decline in ferritin concentration through mid-pregnancy with a plateau towards the end of pregnancy. Conversely, even in a diluted medium, sTfR concentrations increased with pregnancy progression (Rawal *et al.*, 2017; Scholl, 2011) indicative of increased iron requirements with low circulating iron. This underlines the importance of optimal iron stores prior to pregnancy for maternal health during pregnancy, lactation and following pregnancies.

2.2.3 Iron requirements of women

The male human body comprises on average approximately 4.0g of iron and the female an average of 2.5g (Gleason & Scrimshaw, 2007). Iron is typically conserved and there are very few

losses apart from menstruation, childbirth or bleeding due to other causes. Therefore, adult men need to absorb approximately 1 mg/day to maintain iron balance, while menstruating women should absorb about 1.5 mg/day on average. During pregnancy, due to expansion of the maternal erythroid mass and the iron needed by the developing foetus, 4 to 5 mg/day of iron should be absorbed to preserve iron balance (Institute of Medicine, 2001). With sufficient iron absorption, the iron is stored which supplies iron when requirements increase, such as with pregnancy. Iron stores that have been utilised are then gradually replaced with increased absorption (Lynch, 2007). Hepcidin decreases with increased erythropoietin demand (such as with blood volume expansion during pregnancy) and therefore increases iron absorption during pregnancy (Gulec *et al.*, 2014). However, the presence of inflammation will increase hepcidin levels and reduce iron release into the circulation (Nemeth & Ganz, 2014).

The recommended dietary allowance (RDA) for women of child-bearing age has been determined by modelling the components of iron requirements (estimated to be absorbed at the 97.5th centile), and an upper limit of 18% iron absorption (Institute of Medicine, 2001). However, absorption in persons from industrialised countries have shown to range from as low as 0.7% to as much as 22.9% (Collings *et al.*, 2013). The RDA for iron in women between the ages of 19 and 50 years is 18 mg/day, while the RDA for postmenopausal women is 8 mg/day (the same as for men).

The RDA modelling for pregnancy has been calculated on an upper limit of 25% iron absorption. The RDA for iron in pregnant women between the ages of 14 and 50 years is 27 mg/day, which is the target or goal for intake by an individual (Institute of Medicine, 2001). The Estimated Average Requirement is used for assessing population usual intake. The EAR is the average amount that is mostly likely sufficient for half of the population (Institute of Medicine, 2000). The EAR for iron for pregnant women is 22 mg/day (Institute of Medicine, 2001). The upper limit (UL) for iron during pregnancy is 45 mg/day. Few women's diets supply 27 mg iron per day and the risk of IDA during pregnancy is high (Gleason & Scrimshaw, 2007). Therefore, the WHO recommends, as a public health measure, daily oral iron (and folic acid) supplementation of 30 to 60 mg/day to all pregnant women. In settings where anaemia in pregnant women is higher than 40%, a daily dose of 60 mg elemental iron is recommended (World Health Organization, 2012).

2.2.4 Aetiology and functional consequences of iron deficiency

Iron deficiency in non-pregnant women

Iron status fluctuates continuously. However, with a long-term negative iron balance and when the body's iron storage levels are reduced, ID develops. ID is identified by reduced serum ferritin

(in the absence of infection or inflammation). Continuous iron depletion can lead to a compromised supply of iron to apotransferrin for delivery where needed, thus reducing saturation of Tf, and increasing expression of TfR. Consequently, little iron reaches the bone marrow for erythropoiesis. This stage is known as iron-deficient erythropoiesis (IDE). Hb synthesis usually remains unaffected at this stage. However, once iron supply is insufficient to sustain normal Hb concentration, IDA will develop and is known as the extreme lower end of the range of ID (Koulaouzidis *et al.*, 2009; UNICEF *et al.*, 2001; Zimmermann, 2008).

The World Health Organization (WHO) estimates that approximately 2 billion people are anaemic, as characterised by low Hb levels alone. Anaemia *per se* could be caused by a number of factors including ID (approximately 50% of all cases); infectious diseases (such as hookworm and malaria); other micronutrient deficiencies (such as vitamin B_{12}; folate and vitamin A); or hereditary conditions affecting red blood cells (Scholl, 2011; WHO & CDC, 2007).

Causes of ID, on the other hand, include increased iron requirements during specific life stages (such as rapid growth in infancy and during pregnancy with increased red blood cell mass and total blood volume expansion); inadequate dietary intake; increased loss (e.g. with menses or gastro-intestinal disorders) and decreased absorption (e.g. with coeliac disease or *Helicobacter pylori* infection, chronic or acute inflammation) (Lopez *et al.*, 2016). Even though ID can occur in all stages of life, young children and pregnant women are more at risk (Abbaspour *et al.*, 2014).

The consequences of ID depend on the degree of deficiency. Functional changes may occur with ID, but the most functional deficits seem to occur with the development of IDA. IDA has been associated with impaired cognitive development, immunity and work capacity (Abbaspour *et al.*, 2014).

ID in pregnancy

Maternal iron intake and status at different stages during pregnancy, or even preconception, are associated with variable birth outcomes (Darnton-Hill & Mkparu, 2015). In general, maternal ID is associated with increased risk of maternal mortality, perinatal mortality and low birth weight (Abbaspour *et al.*, 2014). Preconception IDA has been associated with reduced infant growth and increased risk of adverse pregnancy outcomes (Cetin *et al.*, 2009). Total iron intake early in pregnancy has shown a positive association with birth weight (Alwan *et al.*, 2011). Maternal IDA in the third trimester has been associated with mental development of the child (Chang *et al.*, 2013). The causes of poor maternal iron status are multifactorial and not only influenced by total iron intake. Other contributing factors include early onset of childbearing, high number of

pregnancies, short interval between pregnancies, poor ANC access, HIV infection, obesity and inflammatory status (Balarajan *et al.*, 2011; Finkelstein *et al.*, 2012; Garcia-Valdes *et al.*, 2015).

Since Hb is more often assessed in population studies, anaemia is more widely researched in larger populations. Anaemia during pregnancy has been associated with poor maternal and birth outcomes (World Health Organization, 2017). However, more recently, the U-shaped associated between Hb concentration and birth outcomes has been identified whereby not only low but also high Hb concentrations (>13 g/dL) have been associated with adverse pregnancy and birth outcomes, especially when assessed early in pregnancy (Aghamohammadi *et al.*, 2011; Dewey & Oaks, 2017; Scanlon *et al.*, 2000). Elevated Hb concentrations may lead to suboptimal placental and foetal oxygen and nutrient supply (Gaillard *et al.*, 2014). These high concentrations may be due to insufficient blood volume expansion, acute dehydration and cigarette smoking (Scanlon *et al.*, 2000; Yip, 2000).

2.2.5 Iron overload

Generally, women consume less than the RDA (Institute of Medicine, 2006a) for iron during pregnancy (27 mg/day) from food sources alone (Hwang *et al.*, 2013; Mostert *et al.*, 2005; Savard *et al.*, 2018). Since the consequences of inadequate iron have been shown consistently, iron supplementation during pregnancy is generally recommended. The WHO recommends routine daily iron supplementation (30-60 mg elemental iron) to all pregnant women and a daily dose of at least 60 mg of elemental iron in settings where anaemia prevalence exceeds 40% (World Health Organization, 2012). In South Africa, public healthcare provides daily 60 mg elemental iron to all pregnant women (National Department of Health, 2015). The tolerable upper intake level (UL) for iron is 45 mg/day (Institute of Medicine, 2006a). The risk of iron overload from food sources is therefore less likely than from supplementation.

As previously stated, there is evidence for adverse pregnancy outcomes with high iron and/or Hb concentrations. There is a U-shape relationship between iron status and foetal growth and/or birth weight (Brannon & Taylor, 2017; Dewey & Oaks, 2017). The most recent Cochrane review (Peña-Rosas *et al.*, 2015) on effects of daily oral iron supplements for pregnant women indicates that the effects on maternal and infant outcomes are less clear. From moderate quality evidence, women who more frequently used iron supplementation gave birth to slightly heavier babies. The outcome of the review, however, could be compounded by the fact that routine iron supplementation has different effects in pregnant women depending on their iron status.

In a South Korean prospective cohort (Hwang *et al.*, 2013) where iron supplementation is not routine, foetal growth at mid-pregnancy was lower among the mothers in the third tertile of iron intake (>17.04 mg/day) when compared to mothers in the second tertile (11.49 – 17.04 mg/day). In the third tertile, almost 90% of the women were iron supplement users. Dietary iron intake from food was low in the total cohort (12.5 mg ± 3.9). The researchers therefore considered foetal growth according to the dietary iron intake from food alone, adjusting for iron supplementation. Foetal growth was better in supplement users than in non-supplement users. The authors concluded that iron supplementation is necessary for improved foetal growth, but as their results indicated, excessive iron intake from supplementation may negatively influence foetal growth at mid-pregnancy. The limitation of this study was that the women's iron status was not assessed to determine the association of iron supplementation on foetal growth in non-anaemic and non-ID women.

Overall, a number of authors have expressed concern regarding iron supplementation in non-anaemic and/or non-ID pregnant women (Brannon & Taylor, 2017; Daru *et al.*, 2017; Scholl, 2011) and supplied evidence of adverse outcomes including LBW, preterm birth as well as gestational diabetes.

2.2.6 Assessment of iron status

Anaemia

Anaemia can be classified according to different approaches: 1) based on *the cause* of anaemia (i.e. nutritional, haemorrhagic, hypochromic, haemolytic, aplastic and hypoplastic anaemia); and 2) based on the *erythrocyte morphologic characteristics* (e.g. microcytic, macrocytic, normocytic, megaloblastic and hypochromic anaemia) (Mosby's Medical Dictionary, 2018).

Clinical signs may indicate severe anaemia, however, blood analysis is required to confirm diagnosis. Further investigation should be done to determine the cause of anaemia – whether it is iron deficiency or not. Clinical signs may include significant pallor of the eyelids, tongue, palms and nail beds (UNICEF *et al.*, 2001).

Anaemia is identified as reductions in Hb concentrations, red blood cell (RBC) count or packed cell volume (haematocrit) (WHO & CDC, 2007). Anaemia manifests due to either RBC loss or insufficient RBC production (see Table 2.1). RBC loss occurs due to bleeding, such as with menses, childbirth, gastrointestinal lesions, or due to haemolysis. Insufficient RBC production occurs as a result of insufficient availability of nutrients required for Hb and erythrocyte synthesis

which include iron, vitamin B_{12} and folic acid (Balarajan *et al.*, 2011). The cause of anaemia determines the type of anaemia. Thus, measuring Hb alone would not indicate the prevalence of iron-deficiency. It is acknowledged that Hb measurements have low specificity and sensitivity for iron-deficiency (Zimmermann, 2008).

Anaemia during pregnancy can be physiological or pathological. In physiological anaemia, haemodilution is caused by a greater plasma volume expansion than the erythrocyte volume. The plasma volume expansion is essential for healthy pregnancy outcomes. It is normal for haemoglobin to reduce by 0.5 g/dL, specifically in the second trimester (Vricella, 2017). Due to these reductions, several researchers make use of a more conservative cut-off in the second and third trimesters of pregnancy to classify anaemia (i.e. 10.5 g/dL instead of 11 g/dL) (Pavord *et al.*, 2012; South Australian Maternal & Neonatal Community, 2016).

A full blood count and microscopic blood smear allows assessment of RBC size, shape and colour in combination with Hb and haematocrit. Based on these assessments and analysis a diagnosis can be made and the type of anaemia be differentiated (Alli *et al.*, 2016). Mean corpuscular volume (MCV) measures the average size of the RBC and decreases with iron deficiency. MCV is a late indicator of iron deficiency (Biesalski & Erhard, 2007). Mean corpuscular Hb (MCH) is an indication of the average amount of Hb in the RBC.

Table 2.1: Causes and general groups of anaemia
(Alli *et al.*, 2016; Kumar & Clark, 2016; Mosby's Medical Dictionary, 2009; Pavord *et al.*, 2012; South Australian Maternal & Neonatal Community, 2016; World Health Organization, 2017)

	Causes		**General groups of anaemia**	**Haematological markers**
Non-anaemic			Healthy (no anaemia)	MCV 82-98 fl MCHC 31-37 g/dl MCH 26-34 pg Hb: ≥11 g/dL in 1st trimester ≥10.5 g/dL in 2nd & 3rd trimesters ≥12 g/dL for non-pregnant females
Anaemic*	RBC loss	Acute blood loss Haemolysis Bone marrow failure Anaemia of chronic disease Renal failure	Normocytic, normochromic	↓ Hb Normal MCV Normal MCH
	↓ RBC production	Vitamin B_{12} deficiency Folic acid deficiency	Megaloblastic (macrocytic, normochromic)	↓ Hb ↑ MCV Normal MCH
		Hereditary defects, such as Thalassemia **Iron-deficiency** Sickle cell anaemia Other hemoglobinopathies	Microcytic, hypochromic	↓ Hb ↓ MCV ↓ MCH

fl – femtoliters; pg – pictograms; RBC – Red blood cell; MCV - Mean corpuscular volume; MCH - Mean corpuscular haemoglobin; Hb – Haemoglobin.
*Anaemia diagnosed at Hb <12g/dL in non-pregnant women; Hb <11 g/dL in pregnant women (WHO & CDC, 2007)

Iron status

Analyses of biomarkers additional to Hb are required to describe iron status, especially since only advanced iron deficiency affects Hb (Biesalski & Erhard, 2007) (See Table 2.2). Bone marrow aspiration is considered the gold standard assessment of iron status, but it is a very invasive procedure (Daru *et al.*, 2017) and not suitable for population surveys. Biochemical markers are clinically useful in such settings. Hb, serum ferritin, serum iron, transferrin (total iron-binding capacity) as well as the sTfR/serum ferritin ratio will allow for a detailed iron status description (Umbreit, 2005; UNICEF *et al.*, 2001). These are not assessed universally during antenatal care (ANC) in South Africa. Hb is assessed by finger prick and hand-held mobile devices at first ANC visit and repeated at 32 weeks of gestation. Only when Hb <7.9 g/dL will a full blood count be ordered from the laboratory (National Department of Health, 2015), and iron status would still not be confirmed. Diagnosing iron deficiency is rather complex and analysis of a combination of the above mentioned biomarkers seems to provide the best assessment of iron status (Lopez *et al.*, 2016).

Serum ferritin is the best indicator of iron stores (in the absence of inflammation) since ferritin decreases already in the first stage of iron deficiency. However, several factors increase ferritin, mainly infection and inflammation, therefore, high ferritin values cannot solely be used as an indication of good iron stores (Biesalski & Erhard, 2007). In a setting with high prevalence of obesity (and thus low grade inflammation) and HIV infection such as South Africa, it is necessary to correct ferritin values according to levels of inflammation (Nel *et al.*, 2015; Turgeon O'Brien *et al.*, 2016). Thurnham *et al.* developed correction factors based on inflammatory markers C-reactive protein (CRP) and α_1-acid glycoprotein (AGP) levels (Thurnham *et al.*, 2015). Furthermore, there is debate about the ferritin threshold value to be used in pregnancy. A 2017 systematic review (considering published literature, national guidelines and randomised controlled trials) showed variation in the definition of the ferritin cut-off used for iron deficiency in pregnancy. The authors concluded that an internationally unified definition on this cut-off value based on published evidence does not exist (Daru *et al.*, 2017). The WHO defines serum ferritin ≤15 µg/L in pregnancy as depleted iron stores (World Health Organization, 2017).

Due to the uncertainty with inflammation, sTfR levels should be assessed additionally (Beguin, 2003; Fatima & Demmouche, 2015). As the supply of iron declines, the expression of sTfR increases. It is an indicator of the balance between cellular iron requirements and iron supply and indicates the severity of insufficiency or inadequate supply. sTfR levels are less affected by inflammation (WHO, 2014). To improve certainty of diagnosis, a combination of measurements of iron stores and functional tissue iron can be used, namely the sTfR/ferritin ratio (Turgeon O'Brien *et al.*, 2016). The ratio of sTfR/ferritin is a quantitative estimate of total body iron. In iron

replete persons, the logarithm of this ratio is directly proportional to the amount of iron stored; while in iron deficiency it is proportional to the tissue iron deficit (Zimmermann & Hurrell, 2007). However, since ferritin increases with inflammation, this ratio is not useful in women with increased inflammatory markers (Zimmermann, 2008).

Transferrin saturation is also a useful screening assessment for iron deficiency. It is determined as the ratio of plasma iron to the total iron-binding capacity (Zimmermann, 2008). Receptor saturation decreases with iron deficiency. Persons with IDA typically have a transferrin saturation of <16% (UNICEF *et al.*, 2001).

An additional assessment, which can be used to confirm iron status, is the concentration of zinc protoporphyrin (ZnPP) in erythrocytes. ZnPP increases with ID since zinc is built into the protoporphyrin ring in erythrocytes due to the unavailability of iron (Zimmermann, 2008). ZnPP also increases with other conditions such as haemolytic anaemia, lead poisoning, infection, inflammation etc. (Lopez *et al.*, 2016). A combination of indices should therefore be interpreted to confirm iron status (see **Table 2.2**).

Table 2.2: Biochemical indicators of iron status and respective thresholds in non-pregnant and pregnant women

(Goodnough *et al.*, 2010; Pavord *et al.*, 2012; UNICEF *et al.*, 2001; WHO, 2011; Zimmermann, 2008; Zimmermann & Hurrell, 2007)

	Parameter	**Iron depletion (ID)**	**Iron deficient erythropoiesis (IDE)**	**Iron deficiency anaemia (IDA)**
ID or IDA diagnosis with *no inflammation* (low CRP)	**Hb** - Non-pregnant women - Pregnant women	Normal	Hb <12 g/dL Hb <11 g/dL	Hb <12 g/dL Hb <11 g/dL
	Ferritin	<15 µg/L	Normal	<15 µg/L
Additional assessments *with inflammation* (increased CRP)	**sTfR**	Increased[1]	Increased[1]	Increased[1]
	ZnPP	>40 mmol/mol[2] >80 mmol/mol[3]	>40 mmol/mol[2] >80 mmol/mol[3]	>40 mmol/mol[2] >80 mmol/mol[3]
	Tf saturation	<16 %	<16 %	<16 %

[1] Using assay cut-off values; [2] Washed erythrocytes; [3] Unwashed erythrocytes
Hb – haemoglobin; sTfR – soluble transferrin receptor; ZnPP – zinc protoporphyrin; Tf - transferrin

2.2.7 Iron status of women in the South African context

Dietary iron intake data of women in South Africa are limited. Regional data on an apparently healthy African population in the North-West Province indicated that the usual mean iron intake ranged from 7.5 to 10.4 mg/day with the urban upper class consuming higher amounts of iron (MacIntyre *et al.*, 2002). These dietary intake assessments were conducted before the food fortification programme was implemented. During the same time period, pregnant women (n=315) in the Vaal Triangle (Gauteng province) in South Africa, reported a mean iron intake of 9.7 mg iron per day (Kesa & Oldewage-Theron, 2005). In both these cases, the mean intakes were comparable with the Estimated Average Requirements for pregnant women (8.1 mg/day) (Institute of Medicine, 2001). Even so, poor dietary iron intake contributes to poor iron status.

Data on anaemia in pregnant women in South Africa is sparse. Global estimates were made based on the 1994 Vitamin A Consultation Group (SAVACG) survey and the 2005 National Food Consumption Survey (NFCS), and calculated that approximately 30% of pregnant women are anaemic and less than 0.5% severely anaemic (Stevens *et al.*, 2013). The 2012 South African National Health and Nutrition Examination Survey (SANHANES-1) indicated an anaemia prevalence (Hb ≤12 g/dL) of 23% in non-pregnant women between the ages of 15 and 35 years. In a sub-sample, 6% presented with ID (ferritin <15 µg/L and Hb ≥12 g/dL) and 10.8% with IDA (ferritin <15 µg/L and Hb ≤12 g/dL). The more recent South Africa Demographic and Health Survey (SADHS) conducted in 2016, indicated that 33% were anaemic (Hb <12 g/dL for non-pregnant women; Hb <11 g/dL for pregnant women; 15 to 49 years) (National Department of Health (NDoH) *et al.*, 2017).

Smaller regional studies in South Africa reported varying levels of prevalence. An older study in the rural Limpopo Province reported a low anaemia prevalence of 16.4% (unadjusted Hb <10.5 g/dL) and iron depletion of 26.4% (unadjusted ferritin <12 µg/L) in 276 pregnant women in their third trimester (Mamabolo *et al.*, 2004). At the same time period (data collected in 2003) in clinics from both rural and urban areas in the KwaZulu-Natal Province, a much higher prevalence was reported. Most women (n=1214) were in the second and third trimesters and 30.1% were anaemic (Hb <10 g/dL) while 57.7% were considered anaemic if the WHO cut-off was used (Hb <11 g/dL) (Hoque *et al.*, 2007). At the district level hospital in KwaZulu-Natal, the same authors reported 15.7% anaemia (Hb <10 g/dL) and 39.9% anaemia (Hb <11 g/dL) among pregnant women in their second and third trimesters (Hoque *et al.*, 2006). However, more recently in an urban setting in KwaZulu-Natal, the anaemia prevalence was 42.7% (Hb <11 g/dL) among 2000 women in their second trimester (Tunkyi & Moodley, 2015). Anaemia prevalence at early pregnancy is not clear since women attend ANC late in pregnancy. This impacts the ability of the healthcare system for early detection and treatment of anaemia and other risk factors of suboptimal pregnancy and birth

outcomes. In Cape Town only 52% of women attended ANC before 20 weeks of gestation (Smith, 2016) while in Durban a mere 23% of pregnant women attended before 20 weeks of gestation (Sibeko & Moodley, 2006). This poses a challenge also for studies assessing maternal health and nutrition status early in pregnancy.

From the above information, it can be said that anaemia is prevalent among women of childbearing age. South Africa does not have national data on the anaemia status of pregnant women which should serve as the basis to determine if South Africa is a “setting where anaemia is a severe public health problem amongst pregnant women (40% or higher)” (World Health Organization, 2017). The South African guidelines for maternity care indicate that all pregnant women (Hb >10 g/dL) should receive 200 mg ferrous sulphate daily (supplying ~65 mg elemental iron) to *prevent* anaemia. More aggressive interventions are recommended in women with mild and severe anaemia (National Department of Health, 2015). Thus, all pregnant women in South Africa receive ~65 mg elemental iron daily, irrespective of iron status.

In addition, the South African government implemented the mandatory fortification of maize meal and wheat flour with eight micronutrients, including iron (35 mg electrolytic iron per kg), in October of 2003 (Steyn *et al.*, 2008). Therefore, the general population consuming these staple foods are obtaining additional iron.

Iron deficiency typically contributes to half of the anaemia prevalence (World Health Organization, 2017). It is important that the other factors contributing to anaemia receive attention.

2.2.8 Concluding remarks on iron in pregnancy

Iron homeostasis is mainly managed at absorption (and release from stores) since there is no system for excreting additional iron. Several factors affect iron release from enterocytes and stores. These factors include inflammation, dietary constituents (tannins, phytates, high calcium intakes, ascorbic acid) and other nutrient statuses (copper and zinc).

Many factors contribute to the complexity of assessing and addressing iron deficiency in pregnancy. Firstly, Hb may be the more practical to assess at population level, but it lacks specificity and sensitivity to assess iron status. Secondly, to assess additional haematological markers (ferritin and sTfR) are expensive and more time consuming than finger-prick assessments (such as with determining Hb). Furthermore, ferritin is influenced by inflammation and most studies only recently adjusted for inflammation when reporting results. Thirdly, iron status during pregnancy changes with physiological adjustments and universal cut-offs for some

haematological markers are debated. It is important that ID women receive iron supplementation to support a healthy pregnancy and birth outcomes. However, in the attempt to universally prevent anaemia and iron deficiency in pregnancy, we may be exposing non-anaemic and/or non-ID women to large amounts of iron in the form of supplementation and fortification which may have adverse effects on pregnancy and birth outcomes. Little data on iron intake and status with pregnancy progression, in the context of iron supplementation, exist in South Africa.

2.3 Long-chain polyunsaturated fatty acids (LCPUFAs)

LCPUFAs are part of the lipid family. All fatty acids are composed of chains of carbon, hydrogen and oxygen with a methyl group (CH_3) at one end of the chain and a carboxyl group (COOH) at the other end. When each carbon atom in the fatty acid chain fully bound to hydrogen atoms (therefore single bonds between carbon atoms), the fatty acid is classified as saturated. One or more double bonds between carbon atoms present monounsaturated (MUFAs) and polyunsaturated fatty acids (PUFAs), respectively (Rolfes *et al.*, 2018b). Further classification of PUFAs can be based on the position of the first double bond counted from the methyl end of the chain (Abedi & Sahari, 2014). PUFAs with the first double bond located between the third and fourth carbon atoms are classified as n-3 PUFAs, while those with the first double bond between the sixth and seventh carbon atoms are classified as n-6 PUFAs. See Figure 2.3 below for an illustration of the polyunsaturated fatty acid structures. For the purpose of this thesis, the interest is in the long-chain PUFAs (LCPUFA) consisting of 18 or more carbons, which are summarised in Table 2.3. It should be noted that the FAO/WHO recommends the nomenclature of LCPUFA be applied to fatty acids with twenty to twenty four carbon atoms (FAO & WHO, 2010), however, due to the conversion of the ALA and LA being essential and being the precursors for the longer chain fatty acids, these are important to investigate and will be included when referred to LCPUFA for the purposes of this study.

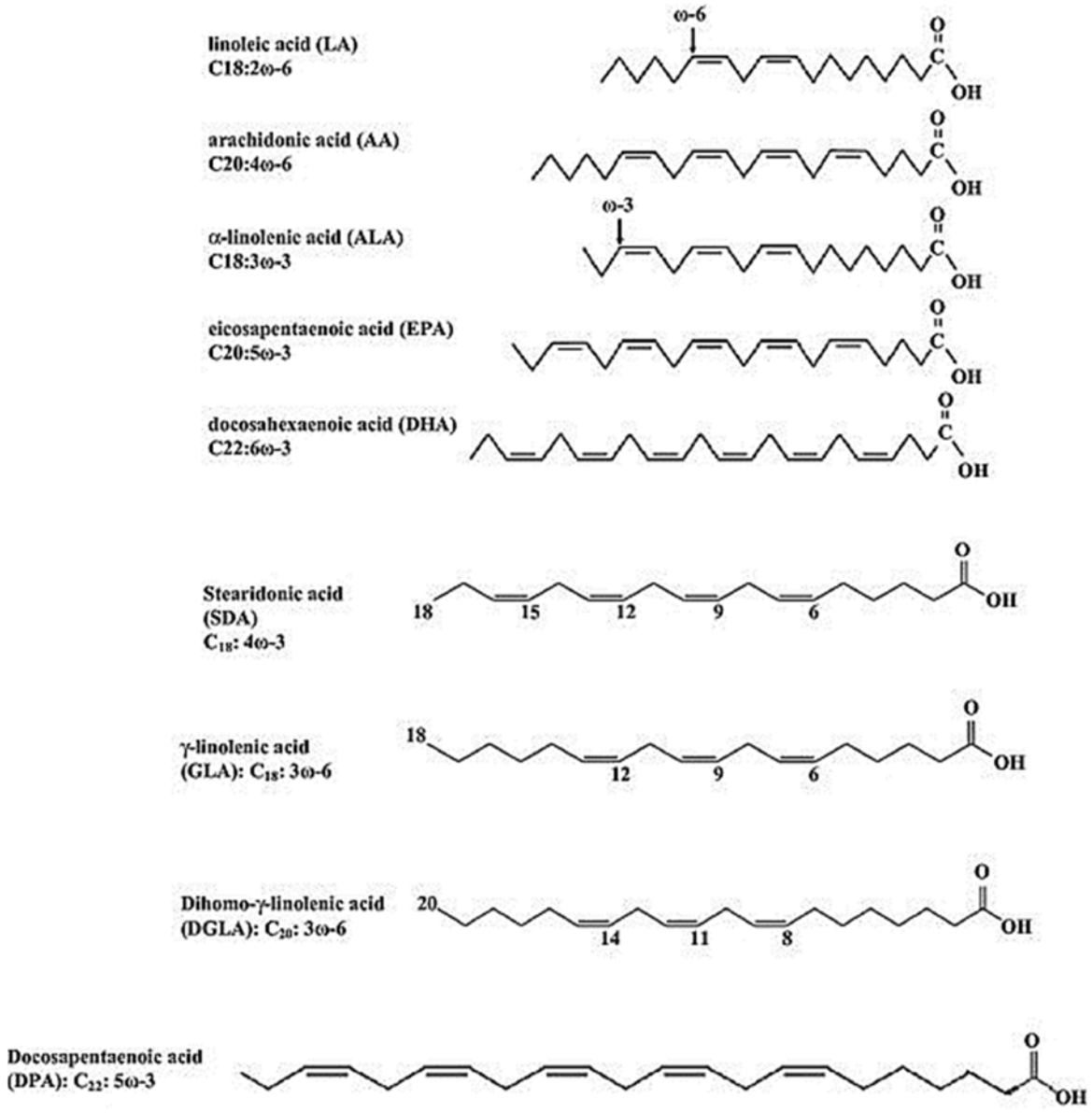


Figure 2.3: Illustration of some polyunsaturated fatty acid structures
(obtained from *Abedi & Sahari, 2014)*

Table 2.3: Summary of long-chain polyunsaturated fatty acids under investigation

(Abedi & Sahari, 2014; Calder, 2016; Drouin *et al.*, 2019; Enser *et al.*, 1996; FAO & UNICEF, 1994; Vorster *et al.*, 2004; Whelan, 2009; Wood *et al.*, 2008)

Abbreviation†	Common name	Major dietary sources
18:2n-6*	Linoleic acid (LA)	Vegetable oils: sunflower, corn, safflower, also smaller amounts from beef, lamb and pork
18:3n-3*	α-linolenic acid (ALA)	Green leafy vegetables, algae, plankton, soya beans, walnuts, chia seeds, flaxseeds and -oil, canola oil
18:3n-6	γ-linolenic acid (GLA)	Nuts, seeds, vegetable and seed oils, evening primrose oil
18:4n-3	Stearidonic acid	Fish, fish oil, echium oil, blackcurrant oil, modified canola and soybean oils
20:2n-6	Eicosadienoic acid	Human milk, pork meat
20:3n-6#	Dihomo-γ-linolenic acid (n-6 DGLA)	Organ meats, beef, lamb and pork meat, human milk
20:4n-6	Arachidonic acid (AA)	Animal derived foods, mostly meats and eggs
20:5n-3	Eicosapentaenoic acid (EPA)	Oily fish and marine oils from mackerel, pilchards, salmon, herring, sardines
22:4n-6	Adrenic acid	Beef and pork meat
22:5n-3#	Docosapentaenoic acid (n-3 DPA) or clupanodonic acid	Oily fish and marine oils from mackerel, pilchards, salmon, herring, sardines. Seal meat and fats. Variable amounts in meat and poultry.
22:5n-6#	Docosapentaenoic acid (n-6 DPA)	Oily fish and marine oils from mackerel, pilchards, salmon, herring, sardines. Seal meat and fats. Variable amounts in meat and poultry.
22:6n-3	Docosahexaenoic acid (DHA)	Oily fish and marine oils from mackerel, pilchards, salmon, herring, sardines; smaller amounts from beef, lamb and pork

*Essential fatty acids

#Isomers

†Abbreviations are based on the number of carbon atoms: number of unsaturated bonds and the number of first atom of which has a double bond counted from the methyl end. E.g. linoleic acid has 18 carbon atoms: 2 unsaturated points and the first double bond is at the 6th carbon atom, therefore 18:2n-6.

The 18-carbon α-linolenic acid (ALA; 18:3n-3) and linoleic acid (LA; 18:2n-6) act as parent fatty acids for the synthesis of respective LCPUFAs. Mammalian cells cannot synthesize either ALA or LA. These fatty acids should be obtained from dietary sources and are therefore considered essential fatty acids. In the case of ALA or LA deficiencies, there is a high risk of developing LCPUFAs deficiencies (Rolfes *et al.*, 2018b). In mammalian cells, ALA and LA can be converted to LCPUFA to a limited extent by means of desaturase and elongase enzymes (Lee *et al.*, 2016) (see Figure 2.4). In addition, the n-3 and n-6 fatty acids compete for the enzymes responsible for the LCPUFA synthesis and therefore influence the balance of fatty acid derivatives. Eicosapentaenoic acid (EPA; 20:5n-3), docosapentaenoic acid (DPA; 22:5n-3), and docosahexaenoic acid (DHA; 22:6n-3) derive from ALA while arachidonic acid (AA; 20:4n-6) derives from LA. Thus, the carbon chain can be elongated, and the number of double bonds can increase, however the position of the double bond remains the same. These fatty acids are considered major LCPUFAs which have been shown to play an important role in human health (Baumgartner, 2016; Saini & Keum, 2018).

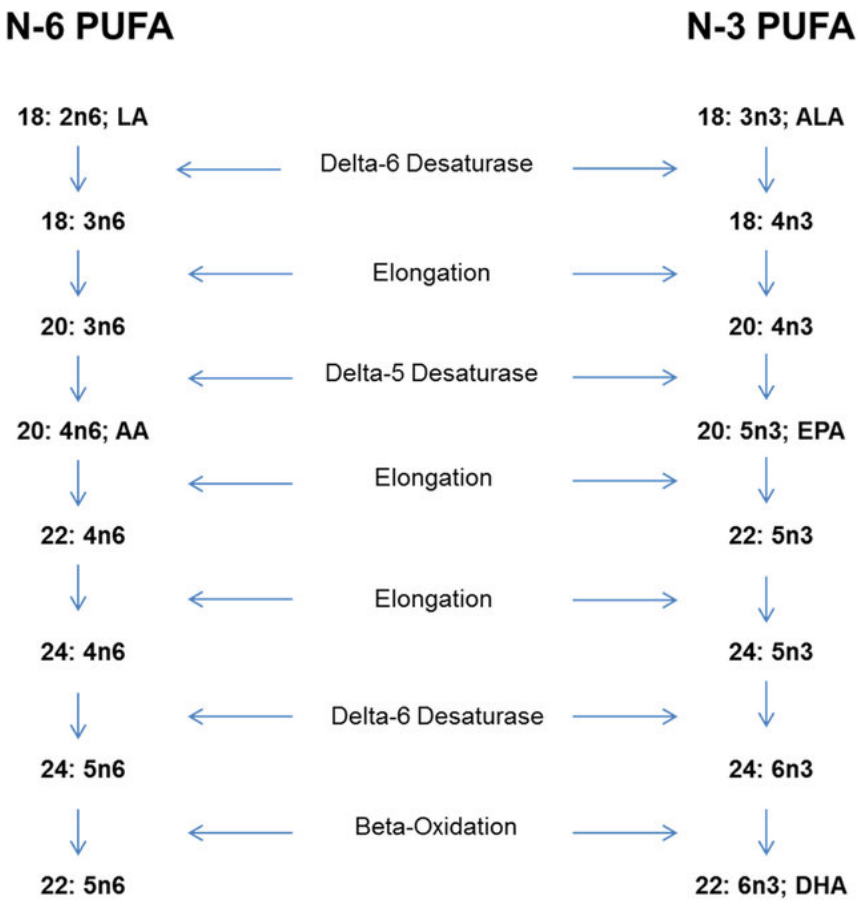


Figure 2.4: Desaturation and elongation of polyunsaturated fatty acids
(Obtained from Akerele & Cheema, 2016)
AA: Arachidonic acid; ALA: Alpha linolenic acid; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; LA: Linoleic acid; PUFA: Polyunsaturated fatty acid

Crop seeds and vegetable oils are major dietary sources of n-6 LA and to a lesser extent n-3 ALA. Flaxseeds and oils as well as canola oil are considered good sources of ALA. Beef and other red meat are good sources of longer chain n-6 AA while fish and seafood are good sources of n-3 EPA and DHA (Meyer *et al.*, 2003; Russo, 2009; Saini & Keum, 2018) (see Table 2.3).

The following sections will further discuss LCPUFAs in human health and the consequences of deficiencies or excesses. Methods to assess LCPUFA status will be addressed. The discussion will then also be applied to the pregnant population and the current situation in South Africa.

2.3.1 Role of LCPUFA in human health

Dietary fats, consisting mainly of triacylglycerol and small amounts of phospholipids and sterols, are mostly hydrolysed in the small intestine by means of pancreatic and intestinal lipases (Institute of Medicine, 2006a; Rolfes *et al.*, 2018b). Small molecules (short- and medium-chain fatty acids and glycerol) diffuse easily into intestinal cells, while larger monoglycerides and long-chain fatty acids form micelles, which can diffuse into enterocytes. These molecules are repackaged with cholesterol, phospholipids and apoproteins to form chylomicrons (a type of lipoprotein) which are then released into the lymphatic system and transported via the thoracic duct until release into the venous system (Institute of Medicine, 2006a; Rolfes *et al.*, 2018b). These plasma lipids therefore reflect dietary intake. Cells take up triglycerides from the chylomicrons as needed for energy or for storage in adipocytes (Rolfes *et al.*, 2018b).

Apart from dietary supply, all fatty acids can be synthesised endogenously, except for ALA and LA as explained earlier (Arab, 2003). The synthesis mainly occurs in hepatocytes. Lipoproteins, mainly very low density and low density lipoproteins, are responsible for transporting the absorbed and synthesised lipids in the venous system (Rolfes *et al.*, 2018b).

Apart from supplying energy and carrying fat-soluble vitamins (Institute of Medicine, 2006a), fatty acids are major structural components of all cell membranes and are the precursors of bioactive lipid mediators. Therefore, fatty acids can be found in serum/plasma, cell membranes and adipocytes (Arab, 2003).

Fatty acids can be incorporated into triglycerides (consisting of a glycerol backbone esterified with three fatty acids) or phospholipids (consisting of one glycerol, two fatty acids, a phosphate and a molecule of choline) (Rolfes *et al.*, 2018b). In the body, phospholipids are mainly situated in cell membranes (Arab, 2003). These membranes are composed of phospholipid bilayers and therefore may fluctuate in ratio of various fatty acids, which may exert variable effects. Fatty acids

contribute to the membranes' physical-chemical properties including the functions of membrane-bound enzymes, transporters and receptors (Muskiet *et al.*, 2006). Fatty acid activities influence the function and responsiveness of cell membranes and tissue metabolism. These can be described broadly as the regulation of membrane structure and function, intracellular signalling pathways and the production of bioactive lipid mediators (Zárate *et al.*, 2017). These actions have variable health effects and PUFAs specifically are considered to be important bioactive nutrients (Lee *et al.*, 2016). For example, as part of the cell structure, AA plays a major role in synaptic signal transduction and is therefore important in neural function. DHA forms part of the structural component of retinal photoreceptor and is therefore known for its role in retinal health, especially during foetal development. Both AA and DHA play a role in cardiovascular endothelial health (Russo, 2009).

LCPUFAs are the precursors for bioactive lipid mediators. These are produced when fatty acids are desaturated and elongated by means of enzymes. The metabolism of the 20-carbon chain PUFAs (AA and EPA) results in specific modulators, namely eicosanoids, which are cell-signalling hormone-like compounds (Kaur *et al.*, 2014). There are different classes of eicosanoids depending on the enzyme involved, each class having a number of series: 1) prostaglandins, 2) prostacyclins, 3) thromboxanes (all due to cyclooxygenase activity), 4) leukotrienes, 5) lipoxins (both due to lipoxygenase activity), 6) hydroxyeicosatetranoic acids (HETEs), and 7) epoxyeicosatrienoic acids (EETs) due to CYP-450 activity (Zárate *et al.*, 2017). Recently, additional lipid mediators have been discovered, namely resolvins, protectins and maresins (Zárate *et al.*, 2017). The eicosanoids produced from AA in the n-6 fatty acid pathway are the 2-series prostanoids (thromboxane A_2, prostaglandin E_2 and prostacyclin$_2$) and the 4-series leukotrienes. On the other hand, the eicosanoids from the n-3 fatty acid pathway include the 3-series prostanoids and 5-series leukotrienes. The 5-series leukotrienes derived from EPA cause less of an inflammatory response than the 4-series from AA. Prostanoids have a pro-inflammatory effect and the 2-series prostanoids synthesised from the n-6 pathway are more active than the 3-series from the n-3 pathway (Saini & Keum, 2018; Zárate *et al.*, 2017). Therefore, a ratio of leukotrienes in favour of the 5-series and prostanoids of the 3-series (all derived from the n-3 fatty acid pathway) will result in a less inflammatory state compared to the n-6 series modulators. Therefore, when considering fatty acid intake or status, the ratio between n-6 and n-3 is an important part of the investigation.

LCPUFAs have been demonstrated to have a range of physiological roles and potential benefits. n-3 PUFAs, specifically due to the modulators, play a role in the regulation of blood pressure, platelet function, vascular function, inflammation, immune function; insulin sensitivity, tumor cell growth and visual signalling (Calder, 2012; Mozaffarian *et al.*, 2010; Riediger *et al.*, 2009).

LCPUFA are also known to play an essential role in growth and development, which is the focus of the following section.

2.3.2 LCPUFA during pregnancy: the dynamics and requirements for foetal development

There are many complex physiological changes during pregnancy, including altered lipid metabolism (Misra *et al.*, 2011). Literature confirms there is a general change in fatty acids in normal pregnancy progression which is a direct consequence of hyperlipidaemia (Bartels & O'Donoghue, 2011; Larqué *et al.*, 2012). Total absolute amounts of all fatty acids in maternal plasma phospholipids increase throughout gestation (Al *et al.*, 1995; Matorras *et al.*, 2001; Otto *et al.*, 1997; Rump *et al.*, 2001; Zhao *et al.*, 2016). The results of the altered lipid metabolism are increased fat stores in the first half of pregnancy and greater fat mobilisation in the second half (Bartels & O'Donoghue, 2011).

However, the change in relative composition of various fatty acids differs with pregnancy progression. In plasma phospholipids, saturated and monounsaturated fatty acid relative composition increase while PUFAs decrease (Al *et al.*, 1995; Matorras *et al.*, 2001; Meher *et al.*, 2016; Rump *et al.*, 2001), although Meher *et al.* (2016) only found a decrease in n-6 PUFA and no change in n-3. Relative composition changes were different when measured in erythrocyte phospholipids compared to plasma phospholipids in a small cohort in Spain. N-6 DGLA and DPA relative composition increased with pregnancy progression, while the n-3 EPA relative composition decreased and DHA increased (Matorras *et al.*, 2001). Even so, DHA absolute amounts have been shown to increase the most among the PUFAs and has been ascribed to the increased mobilisation from fat stores to supply foetal requirements (Larqué *et al.*, 2012). In the third trimester, foetal requirements for AA and DHA specifically are high because of rapid synthesis of brain tissue during the start of the growth spurt, which continues through the first year of life (Carlson, 2009; Koletzko *et al.*, 2008). Neural membranes consist of a phospholipid bilayer with DHA and AA as the major components (Janssen & Kiliaan, 2014). These are important for cell membrane fluidity, permeability and conformation and therefore determine important cell functions (Wadhwani *et al.*, 2018). DHA in particular forms part of the membranes of neuronal synapses and photoreceptors (Al *et al.*, 2000; Koletzko *et al.*, 2008). Since the foetus has limited ability to synthesise DHA, it is dependent on maternal supply of these fatty acids (Al *et al.*, 2000; Herrera, 2002; Larqué *et al.*, 2012). The LCPUFAs are therefore essential for normal foetal development and growth. The placenta is permeable to fatty acids (Wadhwani *et al.*, 2018). Since fatty acids are not soluble in an aqueous solutions, it requires carrier proteins (lipoproteins

or placental transport proteins) to cross the placenta (Wadhwani *et al.*, 2018), however these mechanisms are not clearly understood.

It is understandable that maternal plasma phospholipid fatty acid relative composition changes due to mobilisation and supply of essential fatty acids for placental transfer to the foetus. Adequate maternal dietary intake of the essential fatty acids becomes critical for optimal foetal development. Due to limited conversion of C18 to C20 and longer-chain fatty acids (such as DHA), these are also considered conditionally essential in early development (Uauy, 2009).

The general recommendation is that essential PUFA should contribute 6% to 10% of total daily energy intake (Uauy, 2009), with n-6 PUFA providing 5% to 8% and n-3 PUFA 1% to 2% of energy (Smuts & Wolmarans, 2013). The Dietary Reference Intakes (DRI) supplies Adequate Intake (AI) levels instead of Estimated Average Requirements (EAR) due to insufficient data available with the last revision of DRIs in 2006. Adequate Intake (AI) levels are specified for LA and ALA, but not for the longer-chain PUFAs. Women of childbearing age should consume 12000 mg LA and 1100 mg ALA daily. It is recommended that during pregnancy, this be increased to 13000 mg/day and 1400 mg/day, respectively (Institute of Medicine, 2006b). The Joint FAO/WHO Expert Consultation on Fats and Fatty Acids in Human Nutrition recommended for adult pregnant women a minimum intake of 300 mg/day EPA+DHA, of which DHA should be 200 mg/day. AA is not essential for a healthy adult whose LA intake is >2.5% of energy intake. The upper acceptable macronutrient range for EPA+DHA is set at 2000 mg/day (FAO & WHO, 2010).

2.3.3 Effects of low LCPUFA intake and status in pregnancy

When maternal intake and supply of LCPUFA is inadequate, there are expected detrimental effects. Lower maternal LCPUFA levels have been associated with a number of pregnancy complications such as preeclampsia, intra-uterine growth restriction, preterm birth and low birth weight (Dhobale *et al.*, 2011; Meher *et al.*, 2016; Wadhwani *et al.*, 2018). Long term consequences have also been demonstrated. Lower n-3 LCPUFA availability in diabetic pregnant women was associated with insulin resistance in their offspring (Zhao *et al.*, 2014), while higher maternal n-3 PUFA dietary intake and concentrations in umbilical cord plasma phospholipids were associated with lower adiposity in the offspring at 3 years of age (Donahue *et al.*, 2011). Higher maternal DHA status during pregnancy has been associated with a lower risk for emotional symptoms in the child at 5 years of age (Loomans *et al.*, 2014).

Meta-analyses of n-3 LCPUFA supplementation trials indicate that there is a lower risk for preterm and early preterm birth with supplementation and that the supplemented women gave birth to

modestly heavier babies compared to the placebo groups (reduced risk of LBW) (Imhoff-Kunsch *et al.*, 2012; Middleton *et al.*, 2018; Smuts *et al.*, 2003). In the same sense, the risk of prolonged gestation (>42 weeks) was moderately increased (from 1.6% to 2.6%) in women who received n-3 LCPUFA supplementation (Middleton *et al.*, 2018). Even though daily supplementation of 1200 mg EPA and 800 mg DHA increased maternal n-3 LCPUFA concentrations in women at risk for repeat preterm, there was no effect on rate of repeat preterm (Harper *et al.*, 2010), which supports the fact that the aetiology of preterm delivery is complex. For infants, there is possibly a reduced risk of perinatal death in supplemented vs placebo groups during pregnancy (Middleton *et al.*, 2018).

Interestingly enough, even though supplement trials indicated benefits in terms of risk of preterm birth and, to a lesser extent birth weight, these did not show benefits for other outcomes including pre-eclampsia, maternal blood pressure, stillbirth, infant death or infant growth (measured as new-born length or head circumference) (Imhoff-Kunsch *et al.*, 2012; Middleton *et al.*, 2018). It should be noted that many of the LCPUFA supplement trials were done in middle- or high-income countries and the effect in low-income countries is mostly unknown.

There are limited reports on the side-effects of high dose LCPUFA supplementation. However, it seems that n-3 LCPUFA supplementation is well tolerated in perinatal women, even at high doses of 1.9 g/day of EPA and DHA combined (Freeman & Sinha, 2007). Belching and bad taste has been reported more often in supplemented groups (Imhoff-Kunsch *et al.*, 2012). It should be noted that the effects of n-3 LCPUFAs on neurodevelopment may reach saturation and higher doses may not have additional benefit (Larqué *et al.*, 2012).

When supplementation is not an option, it is imperative to encourage the consumption of marine foods twice a week in order to obtain the LCPUFAs. Foods rich in the essential fatty acids should also be encouraged (see Table 2.3). There are concerns regarding contaminant content of fatty fish, such as heavy metals, specifically during pregnancy due to its detrimental effects on foetal development (Starling *et al.*, 2015). However, moderate fish consumption in European cohorts have shown associations with lower risk of preterm birth and small significant increases in birth weight (Leventakou *et al.*, 2014). Dietary recommendation is therefore still to consume moderate amounts of fatty fish during pregnancy, taking cognisance of country specific fish species known to have high pollutant concentrations.

It is necessary for public health purposes to understand dietary behaviour of pregnant women and assess LCPUFA status in order to address nutritional status optimally. The following section will describe the LCPUFA status assessment methods.

2.3.4 Assessment of LCUPFA status

Fatty acid assessments are challenging. Sample collection, preparation and storage can influence fatty acid stability and methods should be appropriately applied. Determining individual fatty acids requires separation, identification and quantification phases as part of chromatography, which is time consuming and requires laboratory expertise. The method of separation also influences the precision of quantitative analyses (Arab, 2003).

Several circulating fatty acid pools can be sampled to determine fatty acid status for research purposes. Globally three types of pools are mostly used: 1) plasma total lipids, 2) plasma phospholipids, or 3) erythrocyte phospholipids. There are different reasons for choosing different pools. When using plasma total lipids, it is important to quantify a comprehensive range of fatty acids for proper interpretation, since lipids from complexes other than membranes may vary greatly within and across individuals. When using the plasma phospholipids as pool, there is more stability and it better represents the cell membrane fatty acid composition. However, phospholipids are more concentrated in the lipid monolayer of lipoproteins resulting in a biased view on the fatty acid composition of lipid bilayers. On the other hand, erythrocytes have a more complete array of fatty acid composition and therefore better reflect the cell membranes of the biological system (Brenna *et al.*, 2018). However, the sample collection, storage and preparation of erythrocytes can pose challenges due to possible oxidation of PUFAs and cell lysis. Preventative steps should be part of the sample preparation methods. Erythrocyte fatty acid profiles are considered a good marker for habitual dietary fatty acid intake since it reflects aggregated fatty acids of the lifespan of the cell (an estimated 120 days) and does not require the participant to be fasting (Arab, 2003).

Fatty acid results can be reported as absolute concentrations or as relative to other fatty acids. Reporting the absolute concentration of a fatty acid is appropriate when it is required to determine the total amount of the specific fatty acid available. High levels of a single fatty acid may be due to a greater total lipid pool, but it’s not possible to report this proportion if the total lipid pool is not determined. Therefore, the fatty acids are typically relatively reported, expressed as percentage of total fatty acids. It is also known as fatty acid profiling. This reporting method does not capture changes in the total fatty acid pool (Brenna *et al.*, 2018).

2.3.5 LCPUFA in the South African context

Little is known about fatty acid distribution in total fat intake in low-income countries (Shim *et al.*, 2014). There is also no national data available on the type of fats consumed by South African

adults. The National Food Consumption Survey of 1999 reported food intake of children only (Labadarios *et al.*, 2005). The following survey in 2004 included data of women of childbearing age, however dietary fat intake or fatty acid status was not determined (Labadarios *et al.*, 2008). Smuts and Wolmarans summarised a number of cross-sectional studies reporting dietary fat intake of South Africans (Smuts & Wolmarans, 2013). Women from rural areas typically consumed less total fat compared to those in urban areas. Mean PUFA intakes were less than 10% of total energy and were also compromised if the total fat intake was low. It should be noted that the summary mainly represented South Africans who did not follow a Western-type diet (Smuts & Wolmarans, 2013). Richter *et al.* confirmed that n-3 PUFA intakes were below recommendations for people from urban and rural areas for one of these cross sectional studies of more than 2000 participants (Richter *et al.*, 2014). In the meantime, Ford *et al*. reported on the dietary fat intake and the fatty acid status of children and their primary female caregivers from three different areas in South Africa (Ford *et al.*, 2016). The areas have distinct differences in eating patterns. The women from the two urban areas consumed significantly more total and saturated fat compared to the women from the rural area. The mean total fat intake of these three groups of women were 32%, 37% and 16% of total energy, respectively and the mean PUFA 6%, 9% and 3%. Their LA:ALA ratios were 34:1 and 42:1 for the urban women and 36:1 for the rural women, indicative of poor n-3 fatty acid intake from all areas. Mean absolute DHA consumption was reported to be much lower than recommended intakes, i.e. 54, 67 and 24 mg/day, respectively. Margarine was reported as a commonly consumed food and in these three groups brick margarine was mostly used. Brick margarine typically contains more saturated fatty acids compared to unsaturated and have a high ratio of n-6/n-3 fatty acids (Albrecht, 2010). Their red blood cell membrane total phospholipid fatty acid profiles were reflective of their dietary intakes (Ford *et al.*, 2016).

From this, the assumption is that women in South Africa are consuming less than the recommended n-3 fatty acids. Total PUFA intake may be sufficient, however, this is attributed to high n-6 intake, mainly LA. Furthermore, women's DHA intake is also expected to be less than the recommended 200 mg/day. This seems to be the case in many countries (Imhoff-Kunsch *et al.*, 2012). It is probable that the South African LCPUFA status may contribute to complications during pregnancy, specifically length of gestation and birth weight, which will be the focus of discussion in the following sections.

2.4 Birth outcomes under investigation

Adverse birth outcomes are a group of identifiable events functioning as measures of maternal and/or offspring health, morbidity or mortality. Pregnancy complications at delivery or during the neonatal period indicates maternal or infant morbidity (Kramer, 2003). These measures may include preterm birth, low birth weight, low placental weight, intrauterine growth restriction, still births, miscarriages, congenital anomalies, gestational diabetes mellitus and preeclampsia (Abu-Saad & Fraser, 2010; Alwan *et al.*, 2011; Barger, 2010; Dhobale *et al.*, 2011; Kramer, 2003). The foetal programming hypothesis suggests that infants born prematurely or small for gestational age may have altered physiological processes and an increased risk of early mortality and chronic diseases of lifestyle in adulthood (Barker, 2001). It is thus obvious that adverse birth outcomes have severe health implications and should be addressed and prevented as best possible. The birth outcomes of interest in this study are gestational age at birth and birth weight.

2.4.1 Gestational age at birth

The normal length of pregnancy is between 38 and 42 of completed weeks measured from the first day of last menstrual period. Births are classified as term (≥37 weeks) or preterm (<37 weeks or <259 days of gestation from first day of last menstrual period) (Spong, 2013). This is considered the normal range since births prior or post these periods are associated with maternal and foetal health risks. Prematurity is considered the most important cause of death in the first month of life (March of Dimes *et al.*, 2012). In South Africa, complications of prematurity contribute to 48% of neonatal deaths (Rhoda *et al.*, 2018). Preterm infants are at increased risk of death, short- and long-term pulmonary, ophthalmologic and neurologic morbidity; and delayed psychomotor development (Kramer, 2003). Preterm birth may follow spontaneous parturition or as an obstetric intervention when risk of continuing pregnancy is greater than the risks associated with preterm birth (Platt, 2014).

In 2010, globally 14.9 million babies were born preterm, which was 11.1% of all live births. Sub-Saharan Africa's estimated mean preterm birth rate was 12.3% of live births (Blencowe *et al.*, 2012). The aetiology of preterm birth is complex and poorly understood. Spontaneous preterm labour of singletons is thought to be a syndrome initiated by multiple mechanisms, including infection or inflammation, uteroplacental ischaemia or haemorrhage, uterine overdistension, stress, and other immunologically mediated processes (Goldenberg *et al.*, 2008; Quinn *et al.*, 2016). However, modifiable factors such as low pre-pregnancy body mass index, obesity, maternal age <17 or >35 years, cigarette smoking, low energy intake, maternal malnutrition in the

pre-pregnancy and periconceptional periods etc. have been identified risk factors for preterm birth and intrauterine growth restriction (Bloomfield, 2011; Kramer, 2003; Platt, 2014; Quinn *et al.*, 2016).

What is important, is that the molecular signal for the onset of parturition, whether at term or preterm, is not well understood. There is some evidence indicating that spontaneous parturition includes characteristics of inflammatory processes to initiate uterine contractions. Prostaglandins play a role in the induction of labour (Agrawal & Hirsch, 2012). As explained earlier, prostaglandins have a pro-inflammatory effect. The 2-series prostaglandins synthesised from the n-6 pathway are more active than the 3-series from the n-3 pathway (Saini & Keum, 2018). This may thus be one pathway to explain why n-3 PUFAs are associated with longer gestation and possibly why maternal n-3 fatty acid supplementation reduces the risk of preterm birth (Salvig & Lamont, 2011). Others hypothesise that altered maternal micronutrients, n-3 fatty acids and the consequent oxidative stress results in altered epigenetic mechanisms influencing the expressions of matrix metalloproteinases (involved in placental development as well as parturition) during pregnancy (Sundrani *et al.*, 2011). Nevertheless, the aetiology is complex and identified risk factors should be addressed to reduce risk of adverse outcomes.

2.4.2 Birth weight

Neonates born with a body weight <2500 g are classified as low-birth weight (LBW) (United Nations Children's Fund & World Health Organization, 2004), while neonates born with a body weight ≥4000 g are classified as macrosomic (Chauhan *et al.*, 2005). LBW could be a result of premature birth, intrauterine growth restriction (IUGR) or other constituent factors. The concern is mostly in full term neonates with LBW indicating that there was *in utero* growth faltering (i.e. IUGR) (Valero de Bernabé *et al.*, 2004). Therefore, LBW is categorised as follows: 1) preterm LBW babies; 2) term LBW babies; and 3) post term LBW babies (Valero de Bernabé *et al.*, 2004).

LBW is associated with increased infant morbidity and mortality. Severe growth-restricted foetuses are at increased risk of stillbirth and the live births have an increased risk of neonatal death, significant short-term morbidity and permanent deficits in growth and neurocognitive development (Kramer, 2003). LBW infants also have increased risk of diseases later in life (Kunz & King, 2007).

Between 2009 and 2013, the global average of LBW new-borns was 16% (data, however, excludes China), with East and Southern Africa at 11% (UNICEF, 2014). From low-income and middle-income countries, it was estimated that 18 million babies were born LBW in 2010. Of

these, 59% were small-for-gestational-age and 41% preterm (Lee *et al.*, 2013). In 2012, an estimated 14.7% of babies were born LBW in South Africa (Pattinson & Rhoda, 2014).

Many risk factors for LBW have been identified and have been summarised in a narrative review by Valero de Bernabé *et al.* (2004). The authors classified the risks into socio-demographic risks, medical risks before pregnancy, risks of current pregnancy, health care and environmental and behavioural risk factors. Sociodemographic risk factors include genetics (which is estimated to contribute to 40% of birth weight), maternal age, ethnicity, educational level and socio-economic level. Medical risks before pregnancy include chronic hypertension, renal disease, glucose metabolism disorders, cardiorespiratory disease, genitourinary anomalies, autoimmune disease and obstetric history. Risks within the current pregnancy include gestational hypertension, gestational diabetes, maternal weight gain, maternal nutrition, birth intervals, multiple pregnancies, placental health, vaginal bleeding, increased α-fetoprotein, haemoglobin concentration, infections and foetal congenital anomalies. The quality of prenatal care may also influence the risk of LBW. Environmental and behavioural risks for LBW include maternal stress, smoking, alcohol consumption, caffeine consumption, illicit drug use, exposure to toxic and other environmental substances (Mumbare *et al.*, 2012; Suhag & Berghella, 2013; Valero de Bernabé *et al.*, 2004).

The aetiology of IUGR resulting in LBW is complex and not well understood. Hypotheses are mainly around uteroplacental blood flow, oxygen-carrying capacity and nutrition to the foetus (Suhag & Berghella, 2013) and therefore conditions that affect these may affect foetal growth. Oxidative stress has also been identified to affect foetal growth (Stein *et al.*, 2008). In normal pregnancy counteraction of oxidative stress is possible, however, pregnancy is also a state in which the equilibrium is easily disrupted (Al-Gubory *et al.*, 2010). Oxidative stress contributes to premature ageing of the placenta which results in placental insufficiency in transfer of substances to and from the foetus (Sultana *et al.*, 2017). Reactive oxygen species are formed during inflammation and lipid peroxidation. Iron also plays a role in the production of reactive oxygen species increasing the oxidative state. Lipid peroxidation is the process in which oxidants attach lipids containing double bonds, especially PUFAs, in cell membranes and therefore results in cell damage (Al-Gubory *et al.*, 2010; Ayala *et al.*, 2014). Iron supplementation (100 mg/day as fumarate) in non-anaemic iron replete women resulted in uncontrolled lipid peroxidation (Lachili *et al.*, 2001). Factors contributing to an oxidative state may ultimately influence placental function and therefore foetal growth.

2.5 Interactions between iron and LCPUFA specific to early development

It is evident that nutritional exposures *in utero* have both short- and long-term consequences for health and well-being. Maternal dietary intake and status of iron and LCPUFAs warrants investigation since both nutrients play an important role in foetal growth and neurodevelopment, and their metabolism is interrelated (Rioux *et al.*, 2006). Nutrient deficiencies seldom occur in isolation and many women of childbearing age may suffer from both iron deficiency and inadequate n-3 PUFA status due to poor-quality diets (Briend *et al.*, 2011). Combined deficiencies may result in exacerbated adverse birth outcomes.

As mentioned in chapter 1, both iron and n-3 LCPUFAs are essential, are critical in early development and have several interactions. Iron is an important co-factor in several enzymes, including desaturases enzymes involved in LCPUFA synthesis (Chitturi *et al.*, 2015). Data from 1511 Canadian Inuit adults indicated that serum ferritin was positively correlated with erythrocyte membrane n-3 PUFA as well as with $\Delta 5$ desaturase activity, suggesting that the biosynthesis of LCPUFA will be compromised with iron depletion (Zhou *et al.*, 2011). Desaturases are considered metalloenzymes and therefore require adequate amounts of iron, zinc, copper and magnesium for optimal fatty acid metabolism (Arab, 2003). This relationship has been demonstrated in randomised controlled trials as well. One trial tested the effect of iron supplementation alone and in combination with DHA on inflammatory markers in non-pregnant women with IDA. After 12 weeks of supplementation C-reactive protein reduced significantly in the iron + DHA supplemented group. Combined supplementation is considered a means to reduce inflammation compared to supplementing with iron alone (Shidfar *et al.*, 2016). In an animal model, the results showed that a combined deficiency in iron and n-3 fatty acids resulted in neurotransmitter metabolism alterations to a greater extent than an iron or n-3 fatty acid deficiency alone. An iron deficient diet contributed to reductions of DHA in the total phospholipid fraction of erythrocytes (Baumgartner *et al.*, 2012). Similarly, an n-3 fatty acid deficient diet resulted in reduced plasma transferrin saturation in these rats. Altered fatty acid concentrations in membrane lipid bilayers may result in altered iron receptors and transporters involved (Baumgartner *et al.*, 2015).

These two nutrients have been shown to be indispensable in critical periods of development. Both iron and DHA are involved in normal membrane lipid biosynthesis, which includes the important myelination that happens in early pregnancy. In addition, inadequate maternal DHA and iron have shown to result in insufficient monoamine synthesis (the neurotransmitters dopamine, noradrenaline and serotonin) (Georgieff & Innis, 2005).

2.6 Summary

Many factors influence early development in the first thousand days. These may be genetic or modifiable risk factors such as socio-demographic factors, maternal pre-pregnancy health or health during pregnancy. It is evident that maternal nutrition plays a critical role. Pregnancy in itself is a period of significant anatomical and physiological changes. These changes also pose a challenge to assess nutritional status. Iron status and haemoglobin concentrations alter with normal pregnancy progression. However, deficiency has been shown to have adverse effects on birth outcomes. Similarly, lipid metabolism is altered during pregnancy resulting in increased total fatty acids but altered ratios of the different types of fatty acids. Higher concentrations of n-3 LCPUFAs have been associated with longer gestation and increased birth weight.

Many South African women may enter pregnancy with a combination of ID and low n-3 PUFA status due to poor diet quality. Other factors that may influence iron status specifically are the high prevalence of obesity and the accompanied low-grade inflammation affecting iron absorption. However, dietary intake data and nutrient status of pregnant women in South Africa are limited and therefore antenatal care, in terms of supplementation, is based on recommendations from global bodies. South Africa has a unique context considering HIV infection, obesity, the nutrition transition and women starting to attend ANC late in pregnancy. Context specific research would provide a platform for addressing the nutrition related challenges in pregnancy within an appropriate framework.

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CHAPTER 3

ARTICLE 1

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Nutrition during pregnancy and early development (NuPED) in urban South Africa: a study protocol for a prospective cohort

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Nutrition during pregnancy and early development (NuPED) in urban South Africa: a study protocol for a prospective cohort

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ABSTRACT

Background: Adequate nutrition during pregnancy is important to ensure optimal birth outcomes, maternal health and offspring development. However, little is known about the dietary intake and nutritional status of pregnant women residing in urban South Africa. Therefore, the Nutrition during Pregnancy and Early Development (NuPED) cohort study was initiated to assess early nutrition-related exposures predictive of early childhood development in urban South Africa.

Methods: The aims of this prospective cohort study are: 1) to assess dietary intake and nutritional status of urban pregnant women in Johannesburg, South Africa, and 2) to determine associations with birth outcomes, measures of maternal health, as well as measures of offspring health and development. Pregnant women (<18 weeks' gestation) (n=250) are being recruited from primary healthcare clinics in Johannesburg and are followed-up at a provincial hospital. Participants' dietary intake and nutrient status (focus on micronutrients and fatty acids) are assessed at <18, 22 and 36 weeks' gestation. Additional assessments during pregnancy include anthropometric and blood pressure measurements, obstetric ultrasound screens, and assessments of food security, maternal fatigue, prenatal depression, allergy, immune function, morbidity and gestational diabetes. At birth, maternal and neonatal health is assessed and an umbilical cord blood sample collected. Maternal and offspring health is followed-up at 6 weeks, as well as at 6, ≈7.5 and 12 months after birth. Follow-up assessments of mothers will include anthropometric measures, diet history, nutrient status, blood pressure, breast milk composition, and measures of postnatal depression and fatigue. Follow-up assessments of the offspring include feeding practices, nutrient status, measures of growth, psychomotor, socio-emotional and immune development, morbidity, allergy, as well as analysis of the gut microbiome and the epigenome.

Discussion: Ensuring adequate nutrition during pregnancy is one of the key actions endorsed by the South African Government to promote optimal early childhood development in an effort to eradicate poverty. The results from this study may serve as a basis for the development of

context-specific nutritional interventions which can improve birth outcomes and long-term quality of life of the mother and her offspring.

Keywords

Maternal health; maternal diet; nutrition; pregnancy; birth outcomes; early development; DOHaD

BACKGROUND

Recent estimates indicate that 250 million children in low- and middle income countries are at risk of not reaching their developmental potential [1]. This is worrisome as suboptimal childhood development is associated with poorer adult health, well-being and productivity – leading to an intergenerational cycle of poverty. As the trajectories of physical and mental health later in life are determined fundamentally during the first 1000 days of life, both the World Health Assembly Nutrition Targets and the Sustainable Developmental Goals call for action to, among others, improve maternal, infant and young child nutrition in an effort to ensure sustainable social and economic progress.

Maternal health and nutrition gained heightened attention three decades ago with the publication of the developmental origins of health and disease (DOHaD) hypothesis. Barker and Osmond [2] proposed that the cardiovascular disease they observed in an adult population from England and Wales was at least partly associated with poor early nutrition, and specifically undernutrition *in utero* [3]. Undernourishment *in utero* can stress the foetus in ways that permanently affect physiological growth and development, and can be described as a reprogramming of the foetus's developing phenotype [4]. Apart from the long-term health consequences, severe growth-restricted foetuses are at increased risk of stillbirth, and the live births have an increased risk of neonatal death, morbidity and permanent deficits in growth and neurocognitive development [5, 6].

Several maternal nutritional factors have been investigated in relation to adverse pregnancy outcomes, as well as offspring health and development [7–9]. The nutrients most studied during pregnancy include B-vitamins (particularly folic acid), vitamin D, iron, long-chain polyunsaturated fatty acids (particularly n-3 fatty acids) and iodine [8, 10]. However, adequate maternal intakes of zinc and vitamin A may also be important for optimal pregnancy outcomes, as well as for maternal and offspring health [11–13]. Furthermore, better overall diet quality has been associated with a lower risk for maternal perinatal depression and gestational weight gain, which in turn are risk factors for suboptimal offspring development [14–16].

The health of the adult South African population is a concern. South Africa has large economic disparities and 20% of the population are living in extreme poverty, indicating they cannot afford the minimum required food intake [17]. During a national survey in 2012, approximately 40% of the population were reported to have a monotonous diet based mainly on starches [18]. The country is undergoing a rapid nutrition transition characterised by changes in dietary patterns and nutrient intake alongside urbanisation [19, 20], which has resulted in a growing double-burden of under- and over-nutrition [20, 21]. Hence, not surprisingly, 31% and 13% of South African women of reproductive age are anaemic [22] and vitamin A deficient [18], respectively, while 68% of women are overweight or obese, and 46% hypertensive [22]. The effects are also seen in children, with 27% of under-fives being stunted [22]. Both stunting and poverty are known risk factors for poor child development [23]. Maternal short stature is, in turn, a risk factor for birth complications [24] – illustrating the intergenerational effect of poor nutrition.

Ensuring adequate nutrition during pregnancy is one of the key actions endorsed by the South African Government to promote optimal early childhood development in an effort to eradicate poverty [25]. The *Guidelines for maternity care in South Africa* [26] therefore recommend routine nutritional assessment – such as measuring mid-upper arm circumference and haemoglobin levels – and daily supplementation of 200 mg ferrous sulphate, 1000 mg calcium and 5 mg folic acid. However, studies show that the majority of South African women only seek or get access to

public antenatal care in their second trimester of pregnancy [27–30], which might be too late for the routine supplementation programme or other interventions to be effective.

Very little is known about the diet and nutritional status of pregnant women in South Africa, specifically residing in urban areas. Furthermore, understanding the associations of maternal diet and nutritional status during pregnancy with birth outcomes, as well as offspring health and development in the South African population will form the basis for the development of context-specific nutrition interventions that may improve birth outcomes and long-term quality of life of the mother and her offspring. Consequently, the *Nutrition during Pregnancy and Early Development (NuPED)* cohort study was initiated to investigate nutritional status during pregnancy and assess early nutrition-related exposures predictive of early childhood development in urban South Africa.

Aims of the study

The aims of the NuPED study are 1) to assess dietary intake and nutritional status of urban pregnant women in Johannesburg, South Africa, and 2) to determine associations with birth outcomes, measures of maternal health, as well as measures of offspring health and development. A simplistic conceptual framework showing the modifiable and non-modifiable exposure variables, as well as the outcome variables that will be determined to achieve the aims is shown in figure 1.

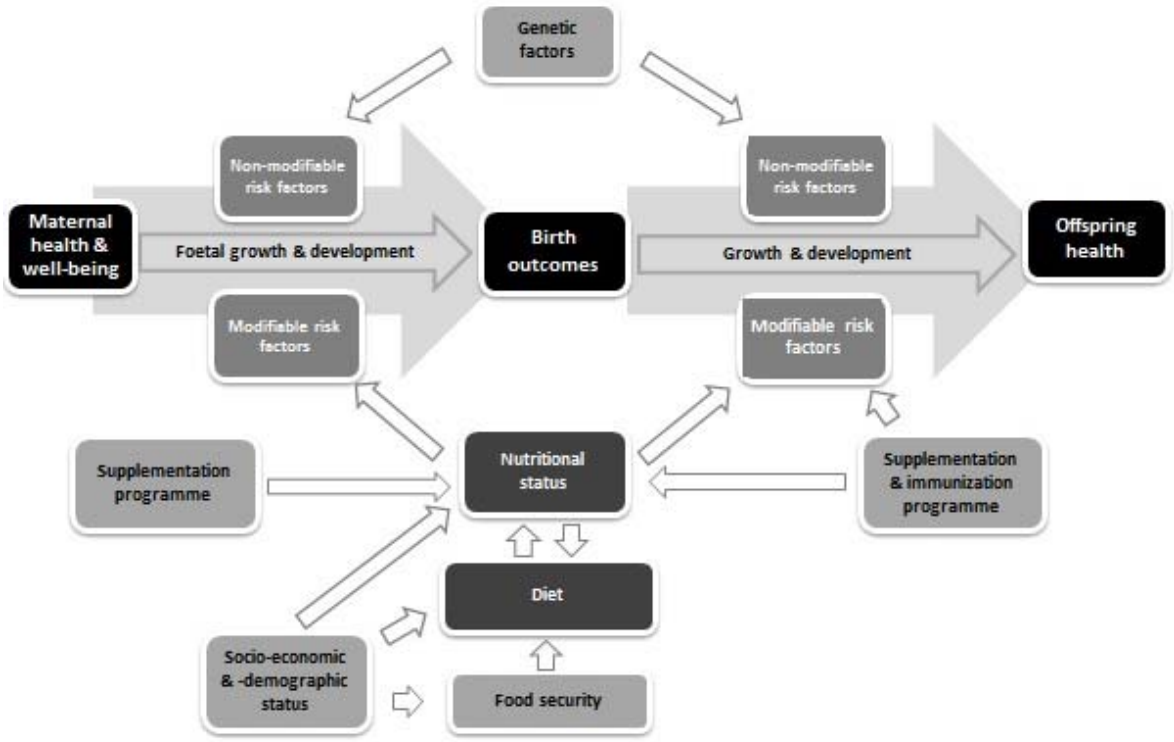


Figure 3.1: Simplistic conceptual framework of exposure and outcome variables in the NuPED study

The study investigates the indicated variables from early pregnancy to infants up to 12 months. The variables fall mainly in the modifiable risk factors category with the core interest being nutritional status. The outcome variables include birth outcomes such as birth weight and gestational age as well as postnatal growth and psychomotor development.

METHODS/DESIGN

Study design

This prospective cohort study follows 250 women throughout pregnancy to birth, and their infants up to 12 months of age. Briefly, data are collected early pregnancy, mid-pregnancy, late pregnancy and at birth. Postnatal assessments focus mainly on offspring health and development at 6 weeks, 6 months, 7.5 months (6 months + 6 weeks) and 12 months' postnatal age.

Recruitment of participants started on 7 March 2016 and completion of data collection is expected in June 2019.

Setting

The study is situated in Johannesburg, the largest city in South Africa. Recruitment of study participants takes place in two of the seven municipal regions of the city from which four primary health care clinics were identified. These clinics fall in the catchment area of Rahima Moosa Mother and Child Hospital (RMMCH). RMMCH is a provincial hospital focusing on maternal and paediatric healthcare, delivering more than 10,000 babies annually. Pregnancy data are collected at the antenatal care (ANC) clinic of RMMCH in addition to routine care. Birth data are collected in the relevant wards at RMMCH. Postnatal data are collected at the Empilweni Services and Research Unit (ESRU) at RMMCH. The execution of the study is coordinated by the Centre of Excellence for Nutrition of the North-West University.

Study population

The study population is urban pregnant women attending ANC at either one of four selected primary health care clinics or at the ANC clinic of the hospital. Women interested to partake in the study are screened according to inclusion and exclusion criteria, and referred to RMMCH ANC clinic for signing informed consent and data collection if eligible.

The inclusion criteria applied during recruitment screening are: 1) Confirmed pregnancy and planning to deliver her baby at RMMCH; 2) <18 weeks' gestational age; 3) Born in South Africa, Lesotho, Swaziland, Zimbabwe, Botswana or Namibia and has been living in South Africa for at least 12 months; 4) Able to communicate effectively in one of the following languages: English, Afrikaans, Sotho, Zulu or Xhosa.

The exclusion criteria are 1) <18 and >39 years; 2) Multiple pregnancy; 3) Using illicit drugs (self-confessed); 4) Smoking (current and/or in past year); 5) Known non-communicable diseases (NCDs) namely diabetes, renal disease, high cholesterol, and hypertension; 6) Known infectious disease namely tuberculosis and hepatitis; 7) Known serious illness namely cancer, lupus or psychosis.

Even though women with infectious disease are excluded, women who are HIV positive are still included. Due to the high prevalence of HIV in the country (36% of women aged 30-34 years [31]), their inclusion will make generalisation to the wider South African population a possibility.

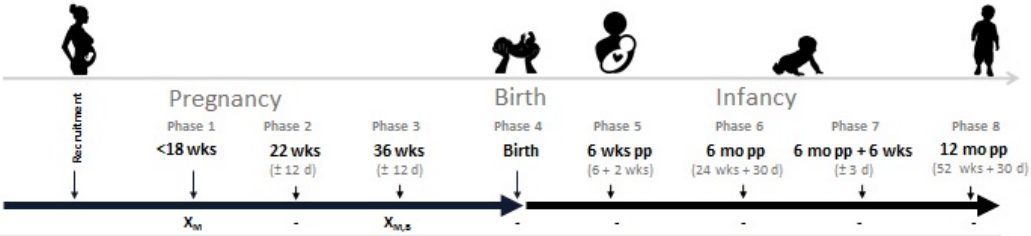
Recruitment and consent procedures

Consecutive sampling is applied, thus, all accessible pregnant women at the recruitment sites may form part of the sample, if they meet the inclusion and exclusion criteria, arrive at study site on their booked date and sign informed consent. All pregnant women in the waiting areas of the ANC clinics are informed about the study. Those interested, receive a study information leaflet and are screened for eligibility individually in a private space upon which a booking date is supplied if eligible. The informed consent form is given to the participant to read, consider and discuss with her partner and/or family. Upon arrival at RMMCH on the booked date, the trained fieldworkers explain the informed consent form in local languages and all are given the opportunity to ask questions. All are assured that participation is voluntary and that participation or non-participation in the study will not affect their clinical care. All research participants provide written informed consent before data collection. Written informed consent is again obtained before infant assessments at 6 weeks postnatally.

Data collection

Figure 2 summarises the measurements and time points of data collection throughout the project. There are eight data collection time points (here forth referred to as phases). All data are collected either by health professionals on site or by trained fieldworkers. Phase 1 data are collected at <18 weeks' gestation (as confirmed by obstetric ultrasound). These data will supply information on the nutritional status of women early in their pregnancy. It is important to note that in Johannesburg only 45% of women access ANC before 20 weeks gestation and only 23% in their first trimester (as reported from other urban areas) [30]. Thus, for practical purposes, the *early pregnancy window* was set at <18 weeks' gestation. Phase 2 data are collected at 22 weeks' gestation (window ±12 days) when anomaly ultrasounds are typically scheduled. Phase 3 data are collected at 36 weeks' gestation (window ±12 days). Study midwifes and/or fieldworkers collect birth data

(phase 4) within a window of 12 hours. Postnatal data are collected at 6 weeks (+14 days) (phase 5); 24 weeks (+ 30 days) (phase 6); 6 weeks post measles immunisation (± 3 days) (phase 7) *or* 30 weeks (+30 days) if measles immunization was not given between 24 and 28 weeks; and 52 weeks (+30 days) (phase 8) postnatal age. The purpose of the phase 7 data collection point specifically is to assess measles immunoglobulin G (IgG) as marker of response to immunisation at 6 months and immune function.



Assessment of:
M = Mother
B = Baby

	Phase 1 <18 wks	Phase 2 22 wks (± 12 d)	Phase 3 36 wks (± 12 d)	Phase 4 Birth	Phase 5 6 wks pp (6 + 2 wks)	Phase 6 6 mo pp (24 wks + 30 d)	Phase 7 6 mo pp + 6 wks (± 3 d)	Phase 8 12 mo pp (52 wks + 30 d)
Informed consent	X_M	-	$X_{M,s}$	-	-	-	-	-
Medical assessment/history	X_M	X_M	X_M	$X_{M,s}$	$X_{M,s}$	X_s	X_s	X_s
Blood pressure	X_M	X_M	X_M	-	X_M	X_M	-	$X_{M,s}$
Socio-economics & -demographics	X_M	-	-	-	-	-	-	-
Ultrasound screen	$X_{M,s}$	$X_{M,s}$	$X_{M,s}$	-	-	-	-	-
Anthropometry	X_M	X_M	X_M	$X_{M,s}$	$X_{M,s}$	$X_{M,s}$	$X_{M,s}$	$X_{M,s}$
Quantitative food frequency questionnaire (QFFQ)	X_M	-	X_M	-	-	-	-	-
Food frequency questionnaire (infant)	-	-	-	-	-	X_s	-	X_s
24h-recall	X_M	X_M	X_M	-	-	$X_{M,s}$	-	-
Feeding practices questionnaire	-	-	-	-	X_s	X_s	X_s	X_s
Food insufficiency and insecurity questionnaire	X_M	-	-	-	-	X_M	-	X_M
Multidimensional Assessment of Fatigue (MAF)	X_M	X_M	X_M	-	X_M	X_M	-	X_M
Edinburgh Postnatal Depression Scale (EPDS-10)	X_M	X_M	X_M	-	X_M	X_M	-	X_M
Psychomotor & socio-emotional development	-	-	-	-	-	X_s	-	X_s
ISAAC allergy questionnaire	X_M	X_M	X_M	-	-	-	X_M	-
CAIR allergy questionnaire (infant)	-	-	-	-	-	-	X_s	X_s
Morbidity (symptoms diary)	X_M	X_M	X_M	X_M	X_s	X_s	X_s	X_s
Oral glucose tolerance test	-	X_M	-	-	-	-	-	-
Delivery and newborn assessment	-	-	-	$X_{M,s}$	-	-	-	-
Cord blood sample	-	-	-	X	-	-	-	-
Blood sample	X_M	X_M	X_M	X_M	-	$X_{M,s}$	X_s	X_s
Urine sample	X_M	X_M	X_M	-	-	$X_{M,s}$	X_s	X_s
Rectal swab	-	-	-	$X_{M,s}$	-	$X_{M,s}$	-	X_s
Breast milk sample	-	-	-	-	X_M	X_M	-	-
Skin prick test	-	-	-	-	-	-	$X_{M,s}$	X_s

Figure 3.2: Data collection per phase during the NuPED study
Prenatal data collection time points are at <18 weeks' gestation (phase 1); ±22 weeks' gestation (phase 2); ±36 weeks' gestation (phase 3) and at birth (phase 4). Postnatal data collection time points are at infant postnatal age of 6 weeks (phase 5); 24 weeks (phase 6); 6 weeks post measles immunisation (± 3 days) (phase 7) or 30 weeks (+30 days) if measles immunization was not given between 24 and 28 weeks; and 52 weeks (+30 days) (phase 8). wks, weeks; d, days, pp, postpartum

Maternal socio-economics and -demographics

Socio-economic and -demographic data are collected at phase 1 by means of a structured interview. Data include date and country of birth, marital status, educational level, home language, employment status, household income, number of members in the household, and beneficiaries of social grants. Lastly, living standards data are obtained to allow classification according to the Living Standards Measure (LSM) developed by the South African Audience Reference Foundation (SAARF) [32]. This measure is widely used in South Africa to describe the socio-economic status of the population [33].

Maternal household food insufficiency and insecurity

The level of the participating women's household food insufficiency and insecurity is assessed at phase 1 in pregnancy and again at phases 6 and 8 postnatally. In a structured interview, women are asked questions on food insecurity and child hunger using the Community Childhood Hunger Identification Project (CCHIP) index [34] that was also used to determine the status of food security in previous national surveys in South Africa [35]. Furthermore, women were asked a validated, single-item question on food insufficiency: "*How many days in the past week have you gone hungry? By this I mean days when you felt you didn't have enough to eat*." that was previously used to determine food insufficiency in pregnant women in South Africa [36].

Maternal dietary intake

Maternal dietary intake data are obtained by means of two dietary assessment methods, namely the 24-hour recall (24-HR) and a quantified food frequency questionnaire (QFFQ). Both methods are interviewer administered by using standardised probing questions [37]. Standard measuring equipment, common size containers (e.g. cups, bowls and glasses) as well as two- and three-dimensional food models are being used to assist in quantifying portion sizes.

A single 24-HR, which obtains details about nutritional supplement use as well, is administered at phases 1, 2 and 3 in pregnancy, as well as at 6 months postnatally (phase 6). Each participant is requested to recall all foods and drinks consumed the previous day from when she woke up until the next day the same time. The recall is done chronologically unless the participant wishes

to recall randomly. The purpose of the single 24-HR is to describe the average intake of the group [38]. All supplement use, as well as food cravings and aversions, are additionally recorded daily by participants on a calendar.

The second dietary assessment method, the QFFQ, is completed at phases 1 and 3. It was validated for the population in the Transition and Health during Urbanisation of South Africans (THUSA) study [39] and its reproducibility was proven [40, 41]. It was also used previously to assess individual and total omega-3 and omega-6 fatty acid intake in a rural and urban South-African population [42]. This QFFQ includes a list of typically consumed foods and minor changes were made to the questionnaire according to vernacular used by the study population in that particular area. Participants are asked according to the ~140 food items listed in the QFFQ, the type/brand, cooking methods, frequency and the amount of all food and drink consumed in the past four weeks.

For the QFFQ data, portion sizes are converted to grams per week per food item, by two registered dietitians/nutritionists. For the 24-HR all portion sizes are converted to grams per day per food item. The resources to assist with this include the Condensed Food Composition Tables for South Africa [43] and the South African Medical Research Council (SAMRC) Food Quantities Manual [44].

Maternal anthropometric measurements

Maternal weight and mid-upperarm circumference (MUAC) are obtained at each phase (1 to 8), and height only at phase 1 and 5. All measurements are done twice and recorded to the nearest 0.05 kg for weight, 0.1 cm for MUAC and height. Standardised methods of the International Society for the Advancement of Kinanthropometry [45] are used with a calibrated digital scale for weight, a mobile stadiometer for height; and non-stretchable metal measuring tape for MUAC.

Maternal medical assessment and history

Medical history is obtained at each prenatal visit (phases 1 to 3) by means of participant responses and inspection of medical files. Information includes medication use (including vaccines), HIV status, obstetric history, hospital admission during pregnancy, use of alcohol, and

exposure to passive smoking. At the first postnatal visit (phase 6) a follow-up is made on the maternal medical history at birth. Blood pressure is measured at each prenatal visit (phases 1 to 3) as well as postnatally (phases 5, 6 and 8) according to international guidelines [46] using calibrated equipment. Appropriately sized cuffs are used for obese participants.

Standard procedures are used for a 2-hour 75g oral glucose tolerance test (OGTT) between 24 and 28 weeks gestation to determine development of gestational diabetes mellitus [47].

Maternal morbidity

Maternal morbidity symptoms are assessed from enrolment to birth using a daily calendar. Mothers are instructed on how to complete the calendar and to return completed calendars at each visit. The infectious morbidity symptoms assessed are fever, headache, diarrhoea, nasal discharge and coughing. Other possible pregnancy-related symptoms included are constipation, nausea, vomiting, extreme tiredness and heartburn. Any medication and supplementation use is also recorded daily.

Maternal allergy assessment

The International Study for Asthma and Allergies in Childhood (ISAAC) questionnaire [48] is used to assess allergy symptoms in maternal participants at phases 1 to 3 during pregnancy, and 7 postnatally. Additionally, skin prick tests to common allergens are used at phase 7 postnatally to assess sensitisation [49]. The questionnaire is designed to assess rhinitis, asthma and eczema in children and has been used successfully in an older black population in South Africa [50]. A positive score on any of these three symptoms indicates an allergic phenotype.

Maternal skin prick tests are performed by a medical doctor according to the procedure described in the Allergy Society of South Africa's position statement on skin prick testing [49]. In mothers, sensitisation to a house dust mite mixture including Dermatophagoides farinae, as well as German cockroach, mould mixture, cat and dog dander, maize pollen, Bermuda grass and Quercus robur (English oak), Eucalyptus, Cypressus arizonica (Arizona cypress), Platanus hybrida (London plane) and Acacia trees are measured. A diagnosis of 1) sensitised with clinical

symptoms, 2) sensitised and clinically tolerant, 3) sensitised and unknown clinical reactivity, 4) not sensitised with clinical symptoms, 5) unknown sensitization with clinical symptoms or 6) not sensitized with no clinical symptoms is made. The mother is given medical advice and referred if necessary.

Maternal depression and fatigue

Perinatal depression is assessed at phases 1 to 3 during pregnancy and at phases 5, 6 and 8 postnatally using the Edinburgh Postnatal Depression Scale (EPDS). The EPDS is a 10-item scale assessing depressive symptoms experienced in the past 7 days [51], which has been validated for assessing perinatal depression in African settings, including South Africa [52]. Maternal fatigue is assessed at the same time points using the Multidimensional Assessment of Fatigue (MAF) scale, which was shown to be a reliable and valid measure of fatigue in pregnant and postpartum women [53]. Both questionnaires are interviewer administered.

Foetal ultrasonography: Gestational age and foetal growth

Foetal ultrasonography examination is carried out by an obstetrician at the first data collection time point to confirm gestation. Estimation of foetal crown rump length and/or biparietal diameter or femur length between 6 – 18 weeks' gestation indicates an accuracy within 5 – 7 days [54]. Foetal crown-rump length is used to determine gestational age of participants in their first trimester [55]. For participants in their second trimester, a combination of multiple biometric parameters (biparietal diameter, head circumference, abdominal circumference, and femur length) are used to determine gestational age [55]. Ultrasound is also used to determine the number of foetuses and confirm foetal movement, as well as foetal growth at 22 and 36 weeks' gestation (phases 2 and 3).

Birth and neonatal assessments

Maternal data collected at birth (phase 4) are obtained from maternal medical files and include hospital admission and discharge dates and times, mode of delivery, induction/augmentation of delivery, type of anaesthetic or pain relief if any, rapid plasma reagin (RPR) status (indicative of

syphilis infection), HIV status, rhesus negative status and presence of maternal diabetes mellitus. If the delivery is induced or caesarean section conducted, the reason for this intervention is obtained. The study nurses obtain maternal weight before birth with a calibrated digital scale.

Neonatal data collected at birth from the medical file include date and time of birth, gender, Apgar score (at 1 and 5 minutes) [56], vital signs, medical interventions required, foetal distress and presence of meconium stained liquor. Four identically trained study nurses obtain newborn anthropometry (weight, midarm circumference (MAC), crown-heel length (CHL), head circumference (HC) and thoracic circumference (TC)) within 12 hours of birth [57]. If the measurements cannot be taken by the study nurses, hospital records are used to obtain anthropometrical data (using the same calibrated infant scale).

Newborn weight is measured with a calibrated digital infant scale to the nearest 10 g. In order to minimize intra-observer variability all circumferences and CHL are measured with an inelastic tape to the nearest 0.5cm (metal measuring tape not used to prevent possible lacerations). CHL is measured by placing the newborn in supine position on the tape measure on a flat surface with all limbs extended and measurement taken from vertex to heel of foot, with foot held in a perpendicular position to the leg.

Infant dietary intake and feeding practices

Data on infant feeding practices are collected at each postnatal phase (5 to 8). Mothers are asked how soon after birth the infant was breastfed, if the infant is currently being breastfed and if not, the duration of breastfeeding. All mothers are asked details about any other food or drink (including infant formula, medicine and supplements) given to the infant.

An unquantified food frequency questionnaire for the infant is administered at phases 6 and 8 for qualitative assessment (types and frequency) of milk and complementary feeding at 6 and 12 months postnatally. An adapted questionnaire previously used in the South African context is used [58]. Frequency of the type of food eaten during the past month can be reported by the

mother as *every day*; *most days* (not every day, but at least 4 times per week); *once a week* (less than 4 times per week, but at least once per week) or *never*.

A single 24-HR for the infant is administered at phase 6 for quantitative assessment (macro- and micronutrient intakes) of intakes at 6 months postnatally. Similar methods and aids are used as described for maternal dietary intake. However, smaller bowls and different sizes of small spoons are used for infant dietary intake to ease realistic reporting for the mother. Also, emphasis is placed on dished food not eaten and the amount left in the bowl to ensure actual intake is reported.

Infant anthropometric measurements

Infant growth is assessed at each postnatal visit. Before measurements are taken, the infant is assessed for presence of oedema. The infant is weighed on a calibrated scale with minimum of clothing, namely only a vest, and without a nappy; and recorded to the nearest 5 g.

Recumbent length of the infants is taken by means of an infantometer to the nearest 0.1 cm. All foot and headwear is removed before measurements are taken. The measurement is taken with the infant lying on his/her back on the infantometer, legs extended with the head and foot board making contact with the infant.

Infant medical assessments

A medical doctor performs a general and physical medical assessment of infants at each postnatal visit. The infant assessment includes HIV status history and a general, ear, nose and throat, respiratory, cardiovascular, abdominal and neurological examination as well as any current complaints. It also includes a medical plan for the infant.

Infant morbidity

Infant morbidity assessment is performed at each postnatal visit by a medical doctor with a structured questionnaire. A morbidity calendar, kept daily by the mother/caregiver, is used as reference. The calendar and symptoms are explained to the mother at birth and each postnatal visit up to phase 7, whereby each new section of the calendar is handed to the mother for return

at the next visit. Symptoms assessed are fever, diarrhoea, vomiting, nasal discharge, coughing, diaper and other rash. Any unscheduled visits to a medical facility and medicine given to the infant are also recorded. The medical doctor diagnoses and determines the duration of each morbidity event using the structured questionnaire with reference to the morbidity calendar.

Infant allergy assessment

A medical doctor assesses the allergy phenotype and sensitisation of infants with the Childhood Allergy and Immunology Research (CAIR) questionnaire and skin prick tests at phases 7 and 8 postnatally. The CAIR questionnaire was developed by the School of Paediatrics and Child Health of the University of Western Australia and is designed to assess asthma, rhinitis and eczema in infants. Infant skin prick tests are performed at phases 7 and 8 by a medical doctor according to the procedure described in the Allergy Society of South Africa's position statement on skin prick testing [49]. Skin prick tests to determine sensitization to common allergens are common medical practice in infants older than four months [59, 60]. In infants, sensitization to a house dust mite mixture including Dermatophagoides farinae, German cockroach, mould mixture, cat and dog dander, maize pollen, Bermuda grass, chicken egg, cow's milk, cod fish, peanuts, wheat and soybean flour and potato are measured. A diagnosis of 1) sensitised with clinical symptoms, 2) sensitised and clinically tolerant, 3) sensitised and unknown clinical reactivity, 4) not sensitised with clinical symptoms, 5) unknown sensitization with clinical symptoms or 6) not sensitized with no clinical symptoms is made. The infant's medical plan is managed accordingly.

Infant immune response

The infant's IgG response to measles immunisation is assessed at phase 7, which is 6 weeks after measles immunisation was administered at the study site. The measles immunisation in South Africa forms part of the National Expanded Programme for Immunisation and permission to administer it at the study site has been granted by the Department of Health of Gauteng Province and the City of Johannesburg. Response to an immunisation is regarded as a good marker to measure immune function *in vivo* [61] and the response will be in the log phase 6 weeks

after immunisation, which is the most sensitive stage to measure differences in response among infants.

Infant psychomotor and socio-emotional development

Psychomotor and socio-emotional development of the offspring is being assessed using the Protocol for Child Monitoring – Infant version (PCM-I), which combines both parental report and direct observation by trained assessors to provide a comprehensive evaluation of a child's motor skills, cognition, language, personal and socio-emotional development [62]. The PCM-I consists of items derived from: 1) the Kilifi Developmental Inventory (KDI) [63], previously used by the investigators to determine psychomotor development in an infant population in South Africa [64], 2) the Developmental Milestone Checklist (DMC-II) [65, 66], and 3) the Profile of Social-Emotional Development (PSED), which is based in part on the Brief Infant/Toddler Social Emotional Assessment [67].

Biological sample collection

Venous blood (42 ml) is drawn from the participating women into labelled EDTA-coated, serum and trace element free evacuated tubes at phases 1 - 4 during pregnancy and at phase 6 postnatally. At birth (phase 4), umbilical cord blood samples are taken immediately after the separation of the newborn from the umbilical cord and before placental delivery into labelled EDTA-coated, serum and trace element free evacuated tubes. Venous blood from the infant (3 ml) is drawn at phases 6, 7 and 8. Dry blood spots are collected on filter paper cards (Whatman, Inc) immediately after blood collection (maternal, cord and infant). The filter paper cards are allowed to dry at room temperature for 24 hours, placed in ziplock bags with desiccants, and stored at -20°C until analysis. In case venous blood cannot be obtained from the infants, capillary blood is being collected by foot venepuncture.

Venous blood is processed within 1 h after blood draw; plasma/serum separated and red blood cells washed twice with normal saline. Buffy coats are stored in 1:1 vol:vol RNA later (Ambion).

Midstream spot urine samples (5 ml) are collected from the participating women at phases 1, 2 and 3 during pregnancy and at phase 6 postnatally into a urine collection cup. From the infants, a 2-5 ml urine sample is collected at phases 6, 7 and 8 using adhesive paediatric urine collection bags. Urine samples are transferred into labelled microtubes and stored at -20°C within 4 hours.

Breast milk samples (fore-milk) are collected from lactating mothers at phases 5 and 6 as described previously [68].

Rectal swabs (FLOQSwab, COPAN) are collected from both the mother and the baby at phases 4 and 6 of data collection. The mucosal microbe sample is taken approximately ±3cm into the anal canal, beyond the anal verge. After collection the cotton bud end of the swab with the collected sample is immediately preserved in RNAlater (Ambion) and stored at -20 °C.

Biological samples are processed on site and stored at -20°C for a maximum of 14 days. Thereafter, frozen samples are transported to the North-West University for storage at -80° C until analysis. Storage temperature is monitored and logged for the entire duration of the study.

Biochemical analyses

Haemoglobin is determined on site in whole blood (20 µL) using HemoCue (Hb 201+, Ängelholm, Sweden). The iron status indices, ferritin and transferrin receptor, as well as the vitamin A status indicator retinol binding protein will be determined using the Q-Plex™ Human Micronutrient Array (7-plex, Quansys Bioscience, Logan, UT, USA) [69]. This multiplex immune-assay also includes the acute phase proteins C-reactive protein (CRP) and alpha1-acid glycoprotein (AGP), as well as the malaria marker HRP2 and thyroglobulin, which is a marker of iodine status. Urinary iodine concentrations are determined in spot urine samples using a modification of the Sandell-Kolthoff reaction with spectrophotometric detection [70]. Vitamin A and E status will be determined using high pressure liquid chromatography (HPLC) and ultraviolet light detection [71]. Vitamin D status will be determined by measuring total 25-Hydroxyvitamin D [25(OH)D] concentrations in serum using liquid chromatography tandem mass spectrometry (LCMSMS) [72]. Fatty acids in red blood cell total phospholipids are determined using gas chromatography tandem mass spectrometry

(GCMSMS) [73]. Zinc concentrations are determined in serum using atomic absorption spectrometry [74].

Thyroid hormones (thyroid stimulating hormone, thyroglobulin, total thyroxine) will be determined in whole blood spots by using electrochemiluminescence immunoassays. Lipid-derived immune modulators will be determined with LCMSMS [75]. Cytokines and hepcidin will be determined using ELISA.

Kynurenine pathway metabolites (mediates interactions between immunological and neurological function) will be determined using LCMSMS [76]. Brain-derived neurotrophic factor (BDNF) as a potential marker of neuronal growth and differentiation will be determined using ELISA [77].

Gut microbiome profiling will be done by isolating microbial DNA from the collected rectal swabs using Qiagen Stool minikit and analysing the 16S rRNA genes DNA sequences on the Ion Torrent 16S metagenomics solution offered by ThermoFisher Scientific.

Targeted epigenetic marks, specifically DNA methylation signatures, will also be assessed in the context of the primary and secondary outcomes of this study. Gene specific DNA methylation will be assessed using the Qiagen EpiTech system. Both, the EpiTect Methyl II Signature and EpiTect Methyl II Complete PCR Arrays (Qiagen) will be considered.

Targeted genotyping of genes of interest in the context of fatty acid, lipid and micronutrient metabolism will also be investigated following the Ion AmpliSeq Targeted Sequencing approach using the Ion *Chef*™ and Ion *S5*™ Systems (ThermoFisher).

Data management and analysis

Sample size calculation

The number of participants to sample has been calculated using the G*Power 3.1.9.2 statistical programme [78]. The statistical calculation involved is the linear multiple regression: fixed model, single regression coefficient. The calculation was based on a small effect size F^2 of 0.05; probability of error (alpha) of 5%; a power of 80% and ten predictors on the birth outcome “low

birth weight". The result was that 196 participants will be required. Taking into consideration that participants may opt out of the project (at 25% rate), it is calculated that a minimum of 245 participants should be recruited. We intended to recruit a minimum of 250 participants. However, should the researchers be able to obtain additional funding, additional participants may be included.

Data management

Data are managed by two dedicated data managers. Raw data are captured and saved in password protected Microsoft Access documents with passwords known only to the operator responsible for data imputing and the principal investigators. A second person checks 20% of all the captured data randomly and notes and corrects any errors. If there are more than 5% errors, respective data are re-captured. The final version of the database will be stored under protected zipped files. Data are collected on dual core electronic archives with automatic backup. Information of the single datasets are stored using anonymous IDs. The document linking anonymous IDs to participants will be collected and stored separately.

Dietary data are captured in Microsoft Excel (Microsoft Corporation, Washington, USA) and all electronic entries are double checked by a registered dietitian for the correct food code and a reasonable amount reported. Analyses will be done by the SAMRC by linking data to the most recent food composition database. Data will then be screened by means of range checks and for outliers in total energy, protein, fat, vitamin A and vitamin C intake.

Data analysis

Overall, data processing and statistical analysis are performed using the SAS statistical package (SAS, Cary, NC, USA). Analysis of baseline (phase 1) data will be conducted to describe the nutritional status and basic socio-economic characteristics of the pregnant women. Data will be tested for outliers and normality by means of Q-Q plots and histogram visual inspection. Test for normality will be performed by the Shapiro-Wilk test. Normally distributed data will be expressed

as means ± SD; non-normally distributed data will be expressed as medians (25th percentile, 75th percentile).

Data will be analysed cross-sectionally to determine associations between variables at each time point by using appropriate statistical methods (e.g. multiple linear regression analysis, ANCOVA, logistic regression analysis), adjusting for potential covariates.

Data will be analysed prospectively to determine associations between variables at different time points (longitudinally) by using appropriate statistical methods (e.g. linear mixed effects models), adjusting for potential time-dependent and static covariates.

Data will also be analysed retrospectively in matched-control sub-studies by determining associations between observed outcomes and variables collected at previous time points.

The level of significance will be set at $P < 0.05$.

DISCUSSION

The importance of perinatal nutrition and its role in offspring health, is recognised [79]. Nutrition during pregnancy is an important factor associated with both maternal and infant health outcomes [80]. To date, however, South African public health nutrition interventions for pregnant women are limited to folic-iron and calcium supplementation, while it is highly likely that the diet of pregnant women living in South Africa is lacking vital micronutrients and essential fatty acids beyond those supplied, or is even containing excessive amounts of specific micro- and macro-nutrients. In order to advocate evidence-based healthcare policy and practice, the identification of nutrient deficiencies and poor eating patterns of pregnant women in South Africa, which are associated with adverse neonatal outcomes and delayed early offspring development, is imperative.

Little is known about the dietary behaviour and nutritional status of pregnant women living in South Africa. To the best of our knowledge, this is the first South African study focusing on the assessment of both maternal dietary intake and nutritional status in women pre- and postnatally and to investigate associations with outcomes of maternal and infant health. This study is novel

due to the comprehensive set of nutrition related data and indicators of maternal and infant health being obtained in a South African setting. Therefore, this explorative project will contribute to identifying factors that may be targeted in future pre- and/or post-conception maternal interventions for optimal offspring development and possibly reduction in adult NCD risk.

This study also has its challenges. The inclusion and exclusion criteria for participants were designed as such to obtain a sample of generally healthy women, who are able to speak the local language and who could be followed-up from early pregnancy until the infants are 12 months old. South African statistics show that only 52% of women attend antenatal care before 20 weeks' gestation [30], thus access to women early in pregnancy is restricted and limits enrolment into the study. Furthermore, many healthcare facilities in Johannesburg serve a predominantly migrant population [81] posing a challenge for longitudinal data collection. Additionally, these women may choose to eat traditional foods [82] that do not form part of the South African Food Database for nutritional analysis; and may not be able to speak a local language [83] hampering detailed reporting during dietary assessments. Thus, migrating women or those unable to speak local languages could not be included in the study.

A limitation of the study is that women who fit the inclusion criteria are invited to join the study by visiting the data collection site at an agreed date. Having this option of attending may contribute to self-selection bias. Our recruitment data to date indicate that of those invited to take part in the study; only approximately 50% arrive at the data collection site on the booked date.

Even so, this study will provide a comprehensive and unique database from an urban South African setting which will allow for cross-sectional, prospective and retrospective analyses to describe the nutritional status and dietary intake of pregnant women, and to determine associations with health outcome measures. These results will supply context to intervention studies with the aim to improve maternal as well as offspring health in South Africa.

Abbreviations

24-HR – 24-hour recall; AGP - alpha1-acid glycoprotein; ANC – Antenatal care; BDNF - Brain-derived neurotrophic factor; CHL – Crown-heel length; CRP – C-reactive protein; DOHaD – Developmental Origins of Health and Disease; ELISA – enzyme-linked immunosorbent assay; ESRU – Empilweni Services and Research Unit; GC-MS/MS – Gas chromatography-mass spectrometry; HPLC – High-performance liquid chromatography; HC – Head circumference; HRP2 – histidine-rich protein 2; MAC – Midarm circumference; MUAC – Mid-upper arm circumference; NCD – Non-communicable disease; NuPED study – Nutrition during pregnancy and early development study; OGTT – Oral glucose tolerance test; QFFQ – Quantified food frequency questionnaire; RMMCH – Rahima Moosa Mother and Child Hospital; RPR – Rapid plasma reagin; SD – Standard deviation; TC – Thoracic circumference.

Ethics approval and consent to participate

The Human Research Ethics Committees of the North-West University, Potchefstroom (NWU-00186-15-A1 and NWU-00049-16-A1) and the University of Witwatersrand, Johannesburg (M150968 and M161045) provided ethical approval for the study. Permission to conduct the research in the relevant clinical setting was obtained from the Gauteng Health Department, City of Johannesburg District Research Committee and the Clinical Manager of Rahima Moosa Mother and Child Hospital.

All participants sign written informed consent before data collection.

Consent for publication

Not applicable.

Availability of data and material

Data sharing is not applicable to this article as no datasets were generated or analysed for the purposes of this protocol article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

EAS, JB, LM, LZ and CMS conceptualised and designed the study. EAS, JB, LM and LZ are implementing the research. LM and LZ are responsible for laboratory analyses. JB, LM and RC are responsible for data capturing and analyses. EAS drafted the paper with equal contributions from JB, LM, LZ and CMS. All authors read and approved the final manuscript.

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CHAPTER 4

ARTICLE 2

This manuscript was submitted to the journal PLOS ONE and is under review. Manuscript was accepted for publication during thesis examination. The accepted manuscript is attached as Annexure 10.

Journal Details	
Title:	PLOS ONE
Impact factor:	2.766
Publisher:	PLOS
Aim and scope:	Submitted under the call for papers: "Maternal and Child Health & Nutrition". PLOS ONE welcomes original research submissions from the natural sciences, medical research, engineering, as well as the related social sciences and humanities, including: • Primary research that contributors to the base of scientific knowledge, including interdisciplinary, replication studies, and negative or null results. • Systematic reviews whose methods ensure the comprehensive and unbiased sampling of existing literature. • Submissions describing methods, software, databases, or other tools that if they follow the appropriate reporting guidelines. • Qualitative research that adheres to appropriate study design and reporting guidelines.
Author guidelines:	https://journals.plos.org/plosone/s/submission-guidelines

Antenatal iron-deficiency in iron supplemented pregnant women is associated with premature birth and higher birth weight: the NuPED prospective study

Short title:

Antenatal iron-deficiency associations with premature birth and birth weight

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Abstract

Background: Recent studies are suggesting a U-shaped relationship between antenatal iron exposure and birth outcomes. Little is known about the iron status and associated birth outcomes of pregnant women in South Africa. Our aim was to assess iron status at early, mid- and late pregnancy, and to determine associations with gestational age and birth weight in women in Johannesburg, South Africa.

Methods: In this prospective study of 250 pregnant women, we measured haemoglobin, biomarkers of iron status and inflammation at <18, 22 and 36 weeks of gestation, plus birth weight and gestational age at delivery. Associations of anaemia and iron status with birth outcomes were determined using regression models adjusted for confounders.

Results: At enrolment, the prevalence of anaemia, iron depletion (ID) and iron deficiency erythropoiesis (IDE) was 28%, 15% and 15%, respectively, and increased significantly with pregnancy progression. Anaemia and ID at 22 weeks, as well as IDE at 36 weeks were associated with higher birth weight (β=168.1; 95% CI: 19.5, 316.7 and β=227.6; 95% CI: 58.4, 396.8 and β=168.7; 95% CI: 27.9, 309.5, respectively). Women in the lowest ferritin quartile at 22 weeks gave birth to babies weighing 312 g (95% CI: 94.8, 528.8) more than those in the highest quartile. In contrast, IDE at 22 weeks was associated with a higher risk for premature birth (OR: 4.62, 95% CI: 1.56, 13.68) and women in lower haemoglobin quartiles at <18 weeks had a shorter gestation by 7 days (β=-6.9, 95% CI: -13.3, -0.6) compared to those in the highest quartile.

Conclusion: Anaemia, ID and IDE prevalence increased during pregnancy despite routine iron supplementation. ID and anaemia at mid-pregnancy were associated with higher birth weight, while IDE was associated with premature birth. These results suggest that current antenatal screening and supplementation practices in South Africa need to be revisited.

Keywords

Pregnancy; iron deficiency; iron supplementation; gestation; birth weight

Introduction

Iron deficiency anaemia (IDA) is the result of prolonged iron depletion and affects a third of the world's population [1]. Pregnancy is a period of increased physiological iron requirements, and a deficiency may have variable effects on pregnancy, maternal and child health outcomes depending on the severity and time point of occurrence [2]. Preconception IDA has been associated with reduced infant growth and increased risk of adverse pregnancy outcomes [3], while IDA in the third trimester has been associated with poorer mental development of the child [4]. More recently, U-shaped associations of maternal haemoglobin and serum ferritin concentrations with risk for low birth weight and preterm birth have received heightened attention [5,6].

Data on iron intake and status of pregnant women in South Africa are sparse. National data on anaemia and iron status are available for women of reproductive age only. The South Africa Demographic and Health Survey conducted in 2016 estimated that 33% of women of reproductive age (15 – 49 years) are anaemic [7]. In the 2012 South African National Health and Nutrition Examination Survey (SANHANES) conducted among younger women (15 to 35 years) the prevalence of anaemia, iron depletion (ID) and IDA was 23%, 16% and 10%, respectively [8]. From these national data and a systematic review [9], approximately 23% to 33% of South African women are expected to enter pregnancy anaemic, and about half of these cases may be attributed to iron deficiency.

The South African Government is addressing iron deficiency in several ways. Firstly, the Government introduced mandatory fortification of maize meal and wheat flour with eight micronutrients, including iron (35 mg electrolytic iron per kg), in 2003 [10]. Secondly, and more specifically to improve status in pregnant women, the *Guidelines for maternity care in South Africa* [11] recommend routine nutritional assessment as well as daily supplementation with 200 mg ferrous sulphate (±65 mg of elemental iron), 1000 mg calcium and 5 mg folic acid. Regimes in South Africa may differ per province and pregnant women in Johannesburg (current study setting) receive 170 mg ferrous sulphate (±55 mg elemental iron) with calcium and folic acid. Supplementation is supplied to all pregnant women irrespective of individual iron or anaemia status. However, the effectiveness of routine antenatal iron supplementation in preventing anaemia and iron deficiency during pregnancy

has not been evaluated nationally. A cross-sectional study at a regional hospital in Durban, South Africa, reported an anaemia (haemoblogin <11 g/dL) prevalence of 43% in 2000 pregnant women between 34 and 36 weeks of gestation, despite receiving routine iron supplements [12].

Determining haemoglobin concentrations forms part of the routine nutritional assessment during antenatal care, while iron status is only further investigated if referred by a physician. Since haemoglobin is not a sensitive marker of iron status, the iron status of pregnant women in South Africa is not well described. Thus, the current study was motivated by the limited evidence on the effectiveness of routine iron supplementation in iron replete pregnant women [13] as well as the limited data available on pregnant women in South Africa. Therefore, the aim of our study was to assess iron status at early, mid- and late pregnancy, and to determine associations with both birth weight and gestational age in urban pregnant women in Johannesburg, South Africa. In an effort to explain the observed iron status, we additionally assessed iron intake during early pregnancy.

Materials and methods

Study design and participants

This study formed part of the *Nutrition during Pregnancy and Early Development* (NuPED) study, which is a prospective study conducted in South Africa's largest city, Johannesburg. The NuPED study protocol has been published previously [14]. Briefly, generally healthy pregnant women were recruited from primary healthcare clinics in Johannesburg between March 2016 and November 2017. Women were eligible for inclusion if they were aged 18 – 39 years, <18 weeks of gestation with singleton pregnancies, proficient in local languages, born in South Africa or neighbouring countries, and if they have been residing in Johannesburg for at least 12 months. Women were excluded if they reported using illicit drugs, were smoking, or had been diagnosed with a non-communicable disease (namely diabetes, renal disease, high cholesterol, and hypertension), an infectious disease (namely tuberculosis and hepatitis), or a serious illness (namely cancer, lupus or psychosis). Due to South Africa's high prevalence of HIV infection (36% of women aged 30-34 years [15]), women who were HIV positive were included in the study in order for it to be a better representation of the general population. The volunteering women who agreed to participate were followed-up at the antenatal

clinic of a tertiary hospital until June 2018. Data were collected at early pregnancy (<18 weeks of gestation), mid-pregnancy (±22 weeks), late pregnancy (±36 weeks) and at birth.

Outcome measurements

The primary outcome measures were birth weight and gestational age at birth. At birth, four trained study nurses obtained neonatal weight (to the nearest 10g) using calibrated digital infant scales within 12 hours of birth [16]. In case the study nurse could not obtain the birth weight herself, it was obtained from the medical record (measured using the same calibrated scales). Low birth weight (LBW) was defined as birth weight <2500 g [17]. Date and time of birth were recorded from maternal records. Women who delivered elsewhere were followed-up telephonically to obtain baby's date of birth and sex of the baby. Gestational age at birth was calculated in days using gestational age determined at the first visit by means of foetal ultrasonography examination. Preterm birth was defined as birth <37 + 0 weeks of gestation (259 days) [18].

Exposure measurements

Dietary and supplemented iron intake

Maternal dietary intake data were obtained at the first visit (<18 weeks of gestation) by means of an interviewer administered quantified food frequency questionnaire (QFFQ) using standardised probing questions [19]. The QFFQ was validated for a previous South African study [20], and its reproducibility was proven in similar study populations [21,22]. Women were asked according to the ~140 food items listed in the QFFQ, cooking methods, the type/brand, frequency and the amount of all food and beverages consumed in the past four weeks. To assist in portion size quantification, standard measuring equipment, two- and three-dimensional food models and common size containers (e.g. cups, bowls and glasses) were used. Three registered dietitians/nutritionists converted reported intakes to grams per week per food item using the Condensed Food Composition Tables for South Africa [23] and the South African Medical Research Council (SAMRC) Food Quantities Manual [24]. Analyses were done by the SAMRC by linking dietary intake data to the most recent food composition database to determine total daily dietary iron intake levels. The database

includes the iron content values of fortified foods as per the food fortification programme. The Estimated Average Requirement (EAR) cut-point method was used to determine the proportion of subjects with intake below the EAR, indicative of inadequate intake of iron in this population.

Supplement use was determined from participants' daily recorded supplement use (yes/no) on a supplied calendar from enrolment until birth. In addition, at each visit, the women were asked the type/brand, frequency and the amount of all dietary supplements used in the past week, taking into consideration supplementation supplied as part of routine care. From these data, average daily iron intake from supplements during pregnancy was calculated. Percentage compliance was calculated as total supplemented iron intake divided by total iron supplied X 100.

Haematological biomarkers

Maternal venous blood was drawn into labelled EDTA-coated and serum evacuated tubes at each visit during pregnancy. Haemoglobin concentrations were determined in whole blood (20µL) using calibrated HemoCue haemoglobin meters (Hb 201+, Ängelholm, Sweden). Haemoglobin values were adjusted for altitude as Johannesburg is located at 1753 meters above sea level [1]. Anaemia was defined as haemoglobin <11 g/dL at <18 weeks of gestation and haemoglobin <10.5 g/dL for mid- and late pregnancy based on cut-offs per trimester [25,26]. In addition, for the purpose of comparability, the prevalence of anaemia is reported according to the WHO [1] haemoglobin cut-off (<11 g/dL) throughout pregnancy. In cases where severe anaemia (haemoglobin <7 g/dL) was detected, the women were referred to the medical doctor on site and treated according to maternity care guidelines [11].

Serum was separated within 1h after blood draw and stored at -20 °C for a maximum of 14 days until transportation for storage at -80 °C until analysis. Ferritin and soluble transferrin receptor (sTfR) concentrations were determined using the Q-Plex™ Human Micronutrient Array (7-plex, Quansys Bioscience, Logan, UT, USA) [27]. This fully quantitative chemiluminescent multiplex assay also includes the acute phase proteins C-reactive protein (CRP) and α_1-acid glycoprotein (AGP). Ferritin concentrations were adjusted for inflammation using the correction factors recommended by Thurnham et al. [28]. Iron depletion (ID) was defined as adjusted ferritin <15 µg/L [29]. Iron deficient

erythropoiesis (IDE) was defined as sTfR >8.3 mg/L [30]. Iron deficiency anaemia (IDA) was defined as ferritin <15 µg/L plus haemoglobin <11 g/dL [1].

Covariates

Socio-economic and -demographic data, including maternal age and living standards measurements (reflective of socio-economic status) [31], were collected at the first visit early in pregnancy by means of an interviewer-administered questionnaire. Maternal anthropometric measurements (height and weight) were obtained using standardised methods from the International Society for the Advancement of Kinanthropometry [32] at each study visit. To determine body mass index (BMI) weight (kg) was divided by height (m) squared. An obstetrician conducted foetal ultrasonography examination to confirm gestational age and singleton pregnancy at the first visit [33,34]. A 2-hour 75 g oral glucose tolerance test was performed between 24 and 28 weeks of gestation using standard procedures [35]. Medical files were inspected to obtain data on maternal medical history, including parity, HIV status, mode of delivery, labour induction, as well as sex of the baby. During analyses, women were considered HIV positive irrespective of date of HIV contraction (prior to or during pregnancy).

Statistical methods

Sample size calculation was done using the G*Power 3.1.9.2 statistical programme [36]. The calculation was based on multiple linear regression analysis (fixed model, single regression coefficient); a small effect size F^2 of 0.05; probability of error (alpha) of 5%; a power of 80% and 10 predictors with birth weight as outcome. The result indicated a required sample size of 196 pregnant women. Considering an attrition rate of 25%, a minimum of 245 women were required. The sample size for this study was 250.

Data processing and statistical analysis of data were performed using SPSS version 25 (SPSS Inc, Chicago, IL, USA). Raw data were captured in Microsoft Access (Microsoft Corporation, Washington, USA) and 20% of all captured data were randomly checked for correctness. Dietary data were

captured in Microsoft Excel (Microsoft Corporation, Washington, USA) and all electronic entries were double checked for the correct food code and a realistic amount captured.

Data were tested for outliers and normality by means of Q-Q plots, histograms and Shapiro-Wilk test. Normally distributed data are expressed as means ± SD; non-normally distributed data are expressed as medians (25th percentile - 75th percentile), except Table 2 which displays medians with minimum – maximum ranges. Descriptive statistics were conducted to describe iron intake at early pregnancy. To examine the longitudinal trajectory of the iron status parameters with pregnancy progression, median concentrations were determined at each visit.

Univariable analyses per outcome were performed using Mann-Whitney U-test for continuous variables and Chi-square test for categorical variables. To test for significance of change in haematological biomarkers (haemoglobin, ferritin, sTfR, CRP and AGP) over time we used the 2-tailed paired *t* test. For the significance of change in proportions for the conditions (anaemia, ID, IDE, IDA and inflammation) over time we used the McNemar test. Next we used logistic regression analyses to investigate the relationship between the exposure (haemoglobin, ferritin, sTfR) and outcome variables (low birth weight and preterm birth) as binary outcomes with odds ratios (OR) and 95% confidence intervals (CI). Multiple linear regression analyses were conducted for continuous outcome variables (birth weight in grams and gestational age at birth in days). The β coefficient was reported with 95% CIs. In both regression analyses, 3 models were applied and different sets of covariates for the two outcome variables. For birth weight, model 1 adjusted for maternal age, gestational age at birth and sex of the baby. Model 2 included the covariates of model 1 plus parity and socio-economic status. Model 3 included the covariates of models 1 and 2 plus HIV status, maternal BMI at enrolment and glucose tolerance. For gestational age at birth, model 1 adjusted for maternal age, baby sex and delivery intervention (induction or caesarean section). Model 2 adjusted additionally for parity and socio-economic status. Model 3 adjusted in addition to models 1 and 2 for HIV status, maternal BMI at enrolment and glucose tolerance. Lastly, univariate comparisons were done between quartiles of each iron biomarker adjusted for the same covariates as with the regression analyses. *P* values of <0.05 were considered significant.

Ethical considerations

During recruitment, an informed consent form was supplied to potentially eligible women who were interested in being part of the study. Written informed consent was obtained at the first visit from all the women before data collection. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by both the Human Research Ethics Committees of the North-West University, Potchefstroom (NWU-00186-15-A1 and NWU-00049-16-A1) and the University of Witwatersrand, Johannesburg (M150968 and M161045). The Gauteng Health Department, City of Johannesburg District Research Committee and Clinical Manager of Rahima Moosa Mother and Child Hospital gave permission to conduct research at the indicated clinical setting.

Results

Participant characteristics and birth outcomes

A total of 595 potentially eligible women residing in Johannesburg, South Africa, were recruited and invited to take part in the study of which 313 volunteered (53%) and signed written informed consent. After screening, 63 women were excluded based on inclusion and exclusion criteria. In total, 250 women were enrolled and completed data collection at baseline (<18 weeks of gestation). Of these, 232 completed follow-ups to delivery: eleven women were lost to follow-up and seven had a miscarriage/intra-uterine foetal death. These 18 cases were included in cross-sectional analysis of data at enrolment, thus all 250 women were included when enrolment data were reported. One entry for birth data was excluded in regression analyses due to early emergency c-section (28 weeks of gestation). There was one maternal fatality shortly after delivery. Several women (n=29) delivered their babies elsewhere and therefore, birth weight data were missing (see Supplementary Table 1). At enrolment there were no significant differences in participant demographic characteristics between women with and without birth weight data (n=40). There was, however, a significant difference in the anaemia and iron status at enrolment of women with and without birth weight data.

Women without birth weight data were significantly more anaemic and had a lower iron status at enrolment than women with birth weight data.

Characteristics of pregnant women at enrolment (<18 weeks of gestation) in the total study group, as well as by birth outcome, are shown in Table 1. Most of the women were of black-African descent (88%) with a median age of 27 (24-32) years and gestation of 14 (12-16) weeks at enrolment. A quarter of the women (25%) were born in Zimbabwe, although the majority was born in South Africa (72%). Fifty-eight percent of women have completed secondary school and 23% post-school education. Many women (40%) were unmarried/single and 59% had an LSM score indicative of middle-class living standards. The median BMI (26.3 [23.0-30.6] kg/m^2) at enrolment was above a healthy range with 33% of women being overweight and 28% obese. More than a quarter of the women were HIV positive (26%). Thirty percent were nulliparous. At enrolment, the only significant difference between women who ultimately delivered premature and non-premature babies were their CRP status. When comparing women who delivered LBW vs non-LBW babies, those who gave birth to LBW babies had a significantly lower BMI at enrolment (24.8 [21.3-26.4] vs 27.2 [23.3-30.8] kg/m^2), more women had increased CRP (p=0.002) and they were more likely to be nulliparous (p=0.04) and unmarried/single at the time (p=0.04). The median birth weight was 3050 (2790-3380) grams and 15% (n=30) of the babies were born with LBW (<2500 g). The median gestational age at birth was 274 (266-282) days and 12% (n=27) of babies were born preterm (<259 days).

Table 1: Characteristics of pregnant women from the NuPED study at enrolment (<18 weeks of gestation) by birth outcome

Characteristics	**Total sample (n=250)*** *Median (IQR) or n (%)*	**LBW, 15% (n=30)**	**Non-LBW, 85% (n=173)**	***p*†**	**Preterm, 12% (n=27)**	**Term, 88% (n=204)**	***p*†**
Age (years)	27 (24-32)	28 (23-32)	28 (24-32)	0.87	28 (22-34)	27 (24-32)	0.62
Gestational age (weeks)	14 (12-16)	14 (12-16)	14 (12-16)	0.82	14 (10-16)	14 (12-16)	0.30
BMI (kg/m^2)	26.3 (23.0-30.6)	24.8 (21.3-26.4)	27.2 (23.3-30.8)	**0.01**	25.3 (21.7-28.4)	26.5 (23.1-30.6)	0.18
Underweight (<18.5 kg/m^2)	8 (3)	4 (13)	3 (2)		0	7 (4)	
Normal weight (18.5-24.9 kg/m^2)	89 (36)	12 (40)	61 (35)	**0.008**	13 (48)	70 (34)	0.37
Overweight (25-29.9 kg/m^2)	81 (33)	9 (30)	58 (34)		9 (33)	68 (33)	
Obese (≥30 kg/m^2)	71 (28)	5 (17)	51 (29)		5 (19)	59 (29)	
Ethnicity							
Black African	219 (88)	24 (80)	153 (88)		23 (88)	181 (88)	
Mixed ancestry	28 (11)	5 (17)	21 (12)	**0.04**	3 (12)	23 (11)	0.94
White	1 (<1)	-	-		-	-	
Indian	1 (<1)	1 (3)	0		0	1 (1)	
Country of birth							
South Africa	172 (72)	23 (82)	117 (70)		19 (76)	138 (70)	
Zimbabwe	60 (25)	4 (14)	47 (28)	0.20	6 (24)	53 (27)	0.82
Lesotho	4 (2)	0	2 (1)		0	4 (2)	
Swaziland	3 (1)	1 (4)	1 (1)		0	2 (1)	
Living Standards Measure (LSM)							
Low (LSM 1-4)	17 (7)	2 (7)	10 (6)		1 (4)	14 (7)	
Medium (LSM 5-7)	148 (59)	17 (57)	104 (60)	0.95	17(63)	123 (60)	0.82
High (LSM 8-10)	85 (34)	11 (36)	60 (34)		9 (33)	68 (33)	
Marital status							
Unmarried/single	100 (40)	19 (63)	66 (38)		14 (54)	79 (39)	
Married	68 (27)	5 (17)	46 (26)		5 (19)	59 (29)	
Divorced/Separated	2 (1)	1 (3)	1 (1)	**0.04**	0	2 (1)	0.51
Living together	57 (23)	3 (10)	44 (25)		4 (15)	48 (23)	
Traditional marriage	22 (9)	2 (7)	17 (10)		3 (12)	17 (8)	
Highest level of education							
Primary school or less	9 (4)	1 (3)	4 (2)		0	9 (4)	
Grade 8 – 10	37 (15)	5 (17)	23 (13)	0.89	6 (23)	27 (13)	0.35
Grade 11 – 12	145 (58)	16 (53)	105 (61)		13 (50)	123 (60)	
Post-school education	58 (23)	8 (27)	42 (24)		7 (27)	46 (23)	
Parity							
Nulliparous	74 (30)	15 (50)	47 (27)		10 (37)	57 (28)	
Primiparous	88 (35)	5 (17)	65 (37)	**0.04**	7 (26)	75 (36)	0.68
Multiparous	88 (35)	17 (33)	62 (36)		10 (37)	73 (36)	
HIV status							
Positive	64 (26)	6 (20)	43 (25)	0.58	7 (26)	53 (26)	0.99
Negative	186 (74)	24 (80)	131 (75)		20 (74)	152 (74)	
Inflammatory status							
Elevated CRP (>5 mg/L)	149 (60)	10 (33)	110 (63)	**0.002**	11 (41)	129 (63)	**0.03**
Elevated AGP (>1 g/L)	28 (11)	3 (10)	22 (13)	0.68	3 (11)	24 (12)	0.93

LBW: Low birth weight; IQR: interquartile range; CRP: C-reactive protein; AGP: α_1-acid glycoprotein; LSM: Living Standards Measure

Data are presented as n (%) for categorical variables and median (IQR) for continuous variables.

† Mann-Whitney-U test for continuous variables, and Chi-square test for categorical variables.

* n-values are equal to 250 for LSM, highest level of education, parity and HIV status; 239 for Country of birth; and 249 for all other variables.

Maternal dietary and supplemented iron intake

The results on dietary iron intake at early pregnancy and supplemented iron intake throughout pregnancy are displayed in Table 2. Median maternal dietary iron intake as reported at <18 weeks of gestation was 19.1 (4.6-46.1) mg per day from foods, which included fortified foods. There was no significant difference in dietary iron intake between the anaemic and non-anaemic women (p=0.45) nor between the ID and non-ID women (p=0.24). Most women (62%) consumed less iron than the Estimated Average Requirement (EAR) (22 mg/day) for women during pregnancy, and two women (1%) had a dietary iron intake above the upper limit (UL) (45 mg) from foods [37]. The estimated median percentage compliance to iron supplementation during the course of pregnancy was 100% (0-227), and the median supplemented iron intake of 55 (0-125) mg/day is reflective of routine iron supplementation. There was no significant difference in mean supplemented iron intake or supplementation compliance between the anaemic and non-anaemic (p=0.96 and p=0.95); and the ID and non-ID (p=0.64 and p=0.67) women at 36 weeks of gestation.

Table 2: Dietary iron intake at early pregnancy (<18 weeks of gestation) and supplemented iron intake throughout pregnancy

	All women		Anaemic		Non-anaemic			ID		Non-ID		
	n (%)	Median (Min-Max)	n (%)	Median (Min-Max)	n (%)	Median (Min-Max)	$p^{\dagger *}$	n (%)	Median (Min-Max)	n (%)	Median (Min-Max)	$p^{\dagger *}$
Dietary iron intake at early pregnancy, mg/day	250	19.1 (4.6-46.1)	70	19.5 (6.9-43.2)	173	18.7 (4.6-46.1)	0.45	37	19.5 (8.6-39.8)	213	18.9 (4.6-46.1)	0.24
< EAR (<22 mg/d)	155 (62)		41 (59)		111 (64)			23 (62)		132 (62)		
Between EAR and UL	93 (37)		29 (41)		60 (35)			14 (38)		79 (37)		
>UL (>45 mg/d)	2 (1)		0		2 (1)			0		2 (1)		
Supplemented iron at 36 weeks of gestation:												
Mean supplemented iron intake, mg/day	227	55 (0-125)	63	55 (0-110)	158	55 (0-125)	0.96	34	55 (0-73)	193	55 (0-125)	0.64
% Compliance	228	100 (0-227)	63	100 (0-200)	159	100 (0-227)	0.95	34	100 (0-133)	194	100 (0-227)	0.67

EAR: Estimated Average Requirements; UL: Tolerable Upper Intake Level
Anaemic: Haemoglobin <11 g/dL; ID: Ferritin <15 µg/L
Data are presented as median (minimum-maximum) for continuous variables and n (%) for categorical variables.
* Nonparametric tests for independent samples were used for comparing continuous variables.

Haematological outcomes with pregnancy progression

Table 3 shows the haematological biomarkers relevant to iron and inflammatory status with pregnancy progression as measured at the three time points. Fig 1 illustrates the anaemia, ID, IDE and IDA trajectory with pregnancy progression. It should be noted that even though 232 women completed follow-up visits up to birth, only 199 blood samples were available for the 36 weeks visit. This is due to 27 women giving birth prematurely (before this visit) and six samples not available for analyses. At enrolment, there were only 3 cases with severe anaemia (haemoglobin <7 g/dL). The median haemoglobin concentration at early pregnancy was 11.7 (10.8-12.7) g/dL and declined to 11.2 (10.1-12.1) g/dL at the second visit (p<0.001) and plateaued to the end of pregnancy (11.2 [10.1-12.1] g/dL) (p=0.88). Anaemia prevalence (using the WHO cut-off: haemoglobin <11 g/dL throughout pregnancy) increased from 28% to 44% (p<0.001) and 45% (p=0.99) at the three time points, respectively. However, when using the more conservative haemoglobin cut-off of <10.5 g/dL for the second and third time points, the anaemia prevalence of 28% at early pregnancy remained unchanged at the second (32%; p=0.70) and third (34%; p=0.69) visit. Median adjusted ferritin concentrations declined gradually and significantly from 47.6 (21.3-98.7) µg/L to 31.7 (17.8-58.6) µg/L and 20.8 (13.9-40.1) µg/L at the three time points, respectively. ID prevalence increased from 15% to 19% (p=0.06) and 33% (p=0.001). The prevalence of IDA (using WHO haemoglobin cut-off) increased from 9% to 14% (p=0.08) and 23% (p=0.008), while the prevalence of IDA using the lower haemoglobin cut-off changed from 9% to 11% (p=0.68) and 19% (p=0.01). Median sTfR concentrations increased significantly from 4.8 (3.8-6.6) mg/L at <18 weeks to 6.8 (5.4-8.3) and 8.1 (6.5-10.5) mg/L with pregnancy progression. Consequently, IDE prevalence increased significantly from 15% to 25% and 47% during pregnancy. Both median CRP and AGP concentrations declined significantly with pregnancy progression. At enrolment, 60% of participants had elevated CRP (>5 mg/L) and 11% had elevated AGP (>1 g/L) (Table 1).

Table 3: Haematological biomarkers of iron status at three time points in pregnant women residing in Johannesburg, South Africa

Biomarker	<18 weeks Median (IQR)	22 weeks Median (IQR)	36 weeks Median (IQR)	*p*† (<18 & 22 weeks)	*p*† (22 & 36 weeks)
n	250	232	199		
Haemoglobin, g/dL	11.7 (10.8-12.7)	11.2 (10.1-12.1)	11.2 (10.1-12.1)	**<0.001**	0.88
Serum ferritin, µg/L	47.6 (21.3-98.7)	31.7 (17.8-58.6)	20.8 (13.9-40.1)	**<0.001**	**<0.001**
Serum sTfR, mg/L	4.8 (3.8-6.6)	6.8 (5.4-8.3)	8.1 (6.5-10.5)	**<0.001**	**<0.001**
Serum CRP, mg/L	6.5 (3.1-14.4)	6.6 (3.1-12.8)	4.8 (2.8-9.8)	0.06	**<0.001**
Serum AGP, g/L	0.75 (0.63-0.86)	0.61 (0.53-0.74)	0.55 (0.47-0.65)	**<0.001**	**<0.001**

sTfR: soluble transferrin receptor; CRP: C-reactive protein; AGP: α_1-acid glycoprotein.
Data are presented as medians (interquartile range [IQR]) for continuous variables
† Wilcoxon nonparametric tests were used for comparing continuous variables between <18 and 22 weeks and between 22 and 36 weeks of gestation.

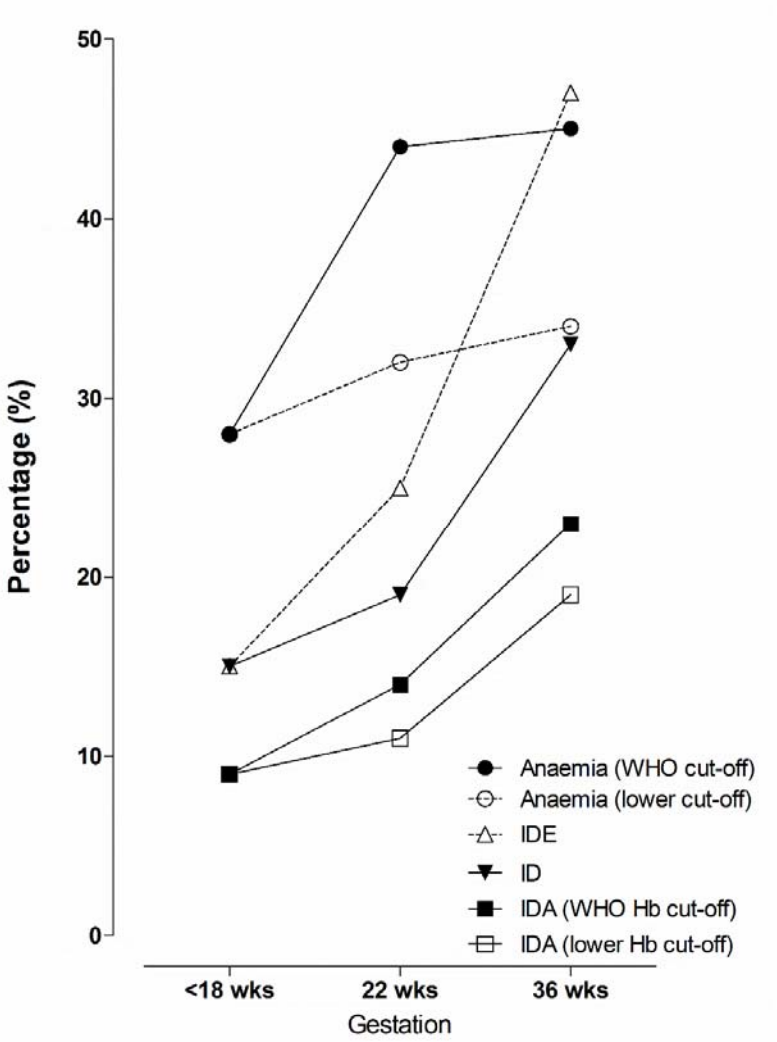

Figure 4.1: Iron and anaemia status trajectory over the course of pregnancy in women residing in Johannesburg, South Africa

ID – iron depletion; IDE – iron deficiency erythropoiesis; IDA – iron deficiency anaemia; Hb – haemoglobin; wks - weeks. Anaemia (WHO cut-off): haemoglobin <11 g/dL); Anaemia (lower cut-off): haemoglobin <10.5 g/dL at 22 and 36 weeks of gestation; IDE: sTfR >8.3 mg/L ID: Ferritin <15 µg/L; IDA (WHO Hb cut-off): ferritin <15 µg/L plus haemoglobin <11 g/dL; IDA (lower Hb cut-off): ferritin <15 µg/L plus haemoglobin <10.5 g/dL at 22 and 36 weeks of gestation.

Associations of iron and anaemia status with birth outcomes

Results from the logistic regression analyses of the associations of maternal iron status with LBW as well as preterm birth as binary outcomes are shown in Table 4. The only significant association found was that women who were IDE at 22 weeks of gestation had 4.6 times the risk of giving birth prematurely (OR: 4.62, 95% CI: 1.56, 13.68) as indicated in the fully adjusted model.

Table 4: Associations between maternal iron status and birth outcomes (LBW and premature birth) (binary logistic regression, odds ratios and 95% confidence intervals)

		Associations with LBW*								
		Model 1 (n=186)			**Model 2** (n=186)			**Model 3** (n=156)		
	Predictor	**OR**	**95% CI**	***p***	**OR**	**95% CI**	***p***	**OR**	**95% CI**	***p***
<18 weeks	**Anaemia**, Hb <11 g/dL	0.40	0.10, 1.61	0.20	0.38	0.09, 1.64	0.20	0.33	0.06, 1.79	0.20
	ID, Fer <15 µg/L	0.27	0.04, 2.01	0.20	0.25	0.03, 1.93	1.83	0.99	0.13, 7.16	0.98
	IDE, sTfR >8.3 mg/L	0.18	0.02, 1.64	0.13	0.15	0.02, 1.36	0.09	0.56	0.05, 6.23	0.64
22 weeks	**Anaemia**, Hb <11 g/dL	0.50	0.15, 1.65	0.26	0.47	0.14, 1.65	0.24	0.59	0.13, 2.67	0.49
	Anaemia, Hb <10.5 g/dL	0.39	0.10, 1.62	0.20	0.35	0.08, 1.49	0.15	0.59	0.12, 2.95	0.52
	ID, Fer <15 µg/L	0.32	0.05, 1.89	0.21	0.31	0.05, 1.93	0.21	0.35	0.4, 3.29	0.36
	IDE, sTfR >8.3 mg/L	0.64	0.15, 2.66	0.53	0.57	0.13, 2.49	0.45	0.89	0.22, 5.65	0.89
36 weeks	**Anaemia**, Hb <11 g/dL	1.04	0.31, 3.49	0.95	1.27	0.36, 4.5	0.71	2.47	0.44, 13.83	0.30
	Anaemia, Hb <10.5 g/dL	1.37	0.39, 4.78	0.62	1.82	0.49, 6.83	0.37	1.93	0.36, 10.40	0.45
	ID, Fer <15 µg/L	1.52	0.44, 5.24	0.51	1.52	0.43, 5.37	0.51	0.98	0.13, 7.16	0.98
	IDE, sTfR >8.3 mg/L	0.93	0.28, 3.06	0.90	0.97	0.28, 3.32	0.96	0.90	0.16, 5.05	0.91
		Associations with premature birth#								
		Model 1 (n=187)			**Model 2** (n=187)			**Model 3** (n=154)		
<18 weeks	**Anaemia**, Hb <11 g/dL	1.22	0.46, 3.26	0.69	1.21	0.45, 3.23	0.71	1.59	0.50, 5.04	0.43
	ID, Fer <15 µg/L	1.81	0.60, 5.54	0.30	1.75	0.56, 5.46	0.33	2.44	0.67, 8.90	0.18
	IDE, sTfR >8.3 mg/L	1.10	0.70, 1.73	0.67	0.75	0.20, 2.79	0.67	0.98	2.3, 4.12	0.99
22 weeks	**Anaemia**, Hb <11 g/dL	0.51	0.19, 1.34	0.17	0.48	0.18, 1.30	0.15	0.60	0.20, 1.84	0.37
	Anaemia, Hb <10.5 g/dL	0.94	0.35, 2.52	0.89	0.92	0.34, 2.51	0.87	1.07	0.34, 3.30	0.91
	ID, Fer <15 µg/L	0.89	0.28, 2.89	0.85	0.88	0.27, 2.86	0.83	1.34	0.37, 4.82	0.66
	IDE, sTfR >8.3 mg/L	3.26	1.29, 8.26	**0.01**	3.25	1.28, 8.25	**0.01**	4.62	1.56, 13.68	**0.006**
36 weeks	**Anaemia**, Hb <11 g/dL	0.66	0.16, 2.81	0.58	0.62	0.14, 2.74	0.52	0.55	0.09, 3.44	0.52
	Anaemia, Hb <10.5 g/dL	1.06	0.25, 4.55	0.94	1.03	0.23, 4.62	0.97	1.06	0.16, 6.92	0.94
	ID, Fer <15 µg/L	1.04	0.24, 4.43	0.96	1.01	0.23, 4.39	0.97	0.35	0.04, 3.21	0.35
	IDE, sTfR >8.3 mg/L	1.61	0.41, 6.32	0.50	1.58	0.38, 6.58	0.53	2.05	0.38, 11.09	0.40

Hb: haemoglobin; Fer: serum ferritin; LBW: low birth weight; ID: iron depletion; IDE: iron deficiency erythropoiesis; sTfR: soluble transferrin receptor; OR: odds ratio; CI: confidence interval

* Model 1 for low birth weight adjusted for: maternal age, gestational age at birth and sex of the baby; model 2 adjusted in addition to model 1: parity and living standards measure (socio-economic status); model 3 adjusted in addition to models 1 and 2: HIV status, maternal BMI at enrolment and glucose tolerance.

Model 1 for premature birth adjusted for: maternal age, baby sex and delivery intervention (induction or caesarean section); models 2 and 3 adjusted for the same additional factors as for low birth weight.

p values of <0.05 were considered significant.

Table 5 shows the results from the multiple linear regression analyses on the associations of iron and anaemia status with birth weight (in grams) and gestational age at birth (in days) as outcomes. In the fully adjusted model, anaemia (haemoglobin <10.5 g/dL) at 22 weeks of gestation was associated with a 168 g higher birth weight in neonates (β=168.1; 95% CI: 19.5, 316.7). However, this association was not significant when anaemia was defined according to the WHO haemoglobin cut-off (<11 g/dL). Similarly, ID at 22 weeks was associated with a 228g higher birth weight (β=227.6; 95% CI: 58.4, 396.8). IDE at 36 weeks was associated with a 169 g higher birth weight (β= 168.7; 95% CI: 27.9, 309.5).

Anaemia defined using the WHO cut-off (haemoglobin <11 g/dL) at 22 weeks of gestation was associated with an increase in gestational age by 5 days (β=5.05; 95% CI: 0.44, 9.66). This association did not hold when the lower haemoglobin cut-off was used (haemoglobin <10.5 g/dL).

Table 5: Associations between maternal iron status and birth outcomes (birth weight and gestational age at birth) (multivariable linear regression, β-values and 95% confidence intervals)

		Birth weight (n=203)*								
		Model 1			**Model 2**			**Model 3**		
	Predictor	**β**	**95% CI**	***P***	**β**	**95% CI**	***P***	**β**	**95% CI**	***P***
<18 weeks	**Anaemia**, Hb <11 g/dL	18.2	-126.1, 162.6	0.80	24.0	-120.2, 168.2	0.74	63.5	-87.4, 214.4	0.41
	ID, Fer <15 µg/L	-10.3	-194.3, 173.8	0.91	22.2	-163.2, 207.5	0.81	42.0	-152.1, 236.0	0.67
	IDE, sTfR >8.3 mg/L	150.3	-29.3, 326.9	0.10	183.9	3.8, 363.9	0.05	128.7	-60.5, 317.9	0.18
22 weeks	**Anaemia**, Hb <11 g/dL	100.0	-31.0, 231.1	0.13	140.3	7.0, 273.7	**0.04**	127.8	-10.9, 266.4	0.07
	Anaemia, Hb <10.5 g/dL	166.4	27.4, 305.5	**0.02**	202.2	62.5, 341.9	**<0.01**	168.1	19.5, 316.7	**0.03**
	ID, Fer <15 µg/L	210.2	47.0, 373.3	**0.01**	223.6	61.1, 386.2	**<0.01**	227.6	58.4, 396.8	**<0.01**
	IDE, sTfR >8.3 mg/L	40.1	-109.4, 189.7	0.59	52.3	-97.0. 201.6	0.49	58.1	-97.0, 213.3	0.46
36 weeks	**Anaemia**, Hb <11 g/dL	-45.5	-183.0, 92.1	0.52	-24.9	-166.4, 116.7	0.73	25.6	-120.9, 172.0	0.73
	Anaemia, Hb <10.5 g/dL	13.3	-131.8, 158.3	0.86	33.8	-113.6, 181.1	0.65	105.9	-42.8, 254.6	0.16
	ID, Fer <15 µg/L	75.1	-68.6, 218.8	0.30	86.9	-56.5, 230.2	0.23	123.2	-19.8, 266.2	0.09
	IDE, sTfR >8.3 mg/L	136.0	1.6, 270.4	**<0.05**	182.1	42.8, 321.5	**0.01**	168.7	27.9, 309.5	**0.02**
		Gestational age at birth (n=233)#								
		Model 1			**Model 2**			**Model 3**		
<18 weeks	**Anaemia**, Hb <11 g/dL	1.34	-3.20, 5.87	0.56	1.34	-3.24, 5.91	0.57	1.39	-3.65, 6.43	0.59
	ID, Fer <15 µg/L	-3.27	-9.03, 2.50	0.27	-3.68	-9.54, 2.18	0.22	-3.40	-9.84, 3.05	0.30
	IDE, sTfR >8.3 mg/L	0.66	-504, 6.35	0.82	0.35	-5.43, 6.14	0.90	1.56	-4.80, 7.91	0.63
22 weeks	**Anaemia**, Hb <11 g/dL	5.05	0.96, 9.14	**0.02**	5.01	0.76, 9.25	**0.02**	5.05	0.44, 9.66	**0.03**
	Anaemia, Hb <10.5 g/dL	2.23	-2.25, 6.70	0.33	1.98	-2.60, 6.55	0.40	3.07	-1.97, 8.11	0.23
	ID, Fer <15 µg/L	0.53	-4.70, 5.76	0.84	0.01	-0.03, 0.04	0.79	1.14	-4.64, 6.91	0.70
	IDE, sTfR >8.3 mg/L	-2.45	-7.16, 2.26	0.31	-2.56	-7.31, 2.19	0.29	-2.90	-8.07, 2.28	0.27
36 weeks	**Anaemia**, Hb <11 g/dL	-0.49	-4.88, 3.89	0.82	-0.70	-5.26, 3.85	0.76	2.50	-5.33, 4.50	0.87
	Anaemia, Hb <10.5 g/dL	0.07	-4.55, 4.68	0.98	-0.08	-4.82, 4.66	0.97	-0.80	-5.83, 4.22	0.75
	ID, Fer < 15 µg/L	2.56	-2.03, 7.16	0.27	2.50	-2.15, 7.14	0.29	2.26	-2.61, 7.12	0.36
	IDE, sTfR >8.3 mg/L	-1.01	-5.30, 3.30	0.64	-1.36	-5.90, 3.17	0.55	-0.33	-5.11, 4.46	0.89

Hb: haemoglobin; Fer: serum ferritin; ID: iron depletion; IDE: iron deficiency erythropoiesis; sTfR: soluble transferrin receptor; CRP: C-reactive protein; AGP: α_1-acid glycoprotein; CI: confidence interval

* Model 1 for birth weight adjusted for: maternal age, gestational age at birth and sex of the baby; model 2 adjusted in addition to model 1: parity and living standards measure (socio-economic status); model 3 adjusted in addition to models 1 and 2: HIV status, maternal BMI at enrolment and glucose tolerance.

Model 1 for gestational age at birth adjusted for: maternal age, baby sex and delivery intervention (induction or caesarean section); models 2 and 3 adjusted for the same additional factors as for birth weight.

p values of <0.05 were considered significant.

To further investigate the association of low and high concentrations of iron biomarkers with birth outcomes, haematological biomarker concentrations were divided in quartiles, and univariate comparisons of birth weight and gestational age conducted in adjusted models as shown in Table 6. There was a significant difference between the second and highest quartiles of haemoglobin at <18 weeks of gestation with gestational age at birth. A lower haemoglobin at early pregnancy was associated with shorter gestation by 7 days (β=-6.9, 95% CI: -13.3, -0.6) when compared to the highest quartile (haemoglobin >12.7 g/dL). In addition, we found significant differences in birth weight between quartiles of serum ferritin concentrations at 22 weeks and 36 weeks of gestation. Women in the lowest ferritin quartile (<17.8 µg/L) at 22 weeks gave birth to babies weighing 311.8 g (95% CI: 94.8, 528.8) more than those in the highest quartile (>58.6 µg/L). At 36 weeks of gestation, those in the lowest ferritin quartile (ferritin <13.85 µg/L) gave birth to babies weighing 410.3 g (95% CI: 184.3, 636.3) more than those in the highest quartile (ferritin >40.08 µg/L) (p<0.001). Women in the third ferritin quartile (ferritin 20.80-40.08 µg/L) at 36 weeks of gestation also had significantly heavier babies (276.2 g, 95% CI: 72.2, 480.2) than women in the highest quartile (>40.08 µg/L).

Table 6: Associations between quartiles of haemoglobin, ferritin and sTfR at three time points with birth weight and gestational age at birth (β-values and 95% confidence intervals)

Iron index	Median birth weight* (g)	n	β (95% CI)	*p*	Median gestational age# (days)	n	β (95% CI)	*p*
	<18 weeks				**<18 weeks**			
Haemoglobin (g/dL)				0.28				0.15
Quartile 1: <10.8	3058	35	-39.7 (-258.5, 179.1)	0.72	271	36	-3.7 (-10.3, 2.9)	0.27
Quartile 2: 10.8-11.7	3122	42	23.6 (-187.2, 234.4)	0.83	268	41	-6.9 (-13.3, -0.6)	**0.03**
Quartile 3: 11.8-12.7	2923	36	-174.4 (-392.0, 43.1)	0.12	273	37	-1.3 (-7.9, 5.3)	0.70
Quartile 4: >12.7	3098	40	1		275	39	1	
Ferritin (µg/L)				0.57				0.07
Quartile 1: <21.30	3151	37	123.2 (-93.4, 339.87)	0.26	267	38	-6.0 (-12.2, 0.25)	0.06
Quartile 2: 21.30-47.56	3019	43	-8.14 (-212.4, 196.1)	0.94	276	43	2.5 (-3.5, 8.5)	0.41
Quartile 3: 47.57-98.68	3079	34	52.1 (-163.5, 267.7)	0.63	273	33	-0.5 (-6.9, 5.9)	0.88
Quartile 4: >98.68	3027	42	1		273	42	1	
sTfR (mg/L)				0.64				0.99
Quartile 1: >6.63	3161	41	130.7 (-81.4, 342.8)	0.23	273	42	0.1 (-6.3, 6.4)	0.99
Quartile 2: 4.82-6.63	3094	39	29.6 (-189.4, 248.6)	0.79	272	38	0.6 (-6.0, 73)	0.85
Quartile 3: 3.76-4.81	3035	36	41.5 (-177.1, 260.4)	0.71	270	36	-0.3 (-6.9, 6.3)	0.92
Quartile 4: <3.76	2967	40	1		274	40	1	
	22 weeks				**22 weeks**			
Haemoglobin (g/dL)				0.52				0.72
Quartile 1: <10.8	3163	38	137.9 (-79.7, 355.42)	0.21	274	40	2.4 (-4.2, 9.0)	0.47
Quartile 2: 10.8-11.2	3068	41	12.6 (-171.9, 257.1)	0.70	272	41	0.4 (-6.1, 6.8)	0.91
Quartile 3:11.3-12.1	3007	36	-18.17 (-229.4, 193.1)	0.87	270	35	-1.5 (-7.9, 4.9)	0.64
Quartile 4: >12.1	3025	41	1		272	40	1	
Ferritin (µg/L)				**0.045**				0.60
Quartile 1: <17.78	3248	33	311.8 (94.8, 528.8)	**0.005**	270	35	-4.2 (-10.8, 2.5)	0.22
Quartile 2: 17.78-31.66	3041	41	104.8 (-104.0, 313.6)	0.32	271	41	-3.6 (-10.1, 2.8)	0.27
Quartile 3: 31.67-58.63	3067	40	131.2 (-73.8, 336.1)	0.21	272	39	-2.9 (-9.2, 3.5)	0.37
Quartile 4: >58.63	2936	40	1		275	39	1	
sTfR (mg/L)				0.35				0.37
Quartile 1: >8.33	2962	36	-109.2 (-330.1, 111.8)	0.33	268	37	-4.7 (-11.3, 1.9)	0.10
Quartile 2: 6.85-8.33	3044	39	-180.6 (-394.5, 33.33)	0.10	273	39	0.6 (-5.9, 7.1)	0.86
Quartile 3: 5.36-6.84	3025	42	-156.2 (-366.8, 54.34)	0.15	273	41	-1.0 (-7.01, 0.2)	0.76
Quartile 4: <5.36	3231	37			275	37	1	
	36 weeks				**36 weeks**			
Haemoglobin (g/dL)				0.99				0.75
Quartile 1: <10.03	3169	31	-0.6 (-242.4, 241.2)	1.00	275	32	-2.1 (-7.2, 3.0)	0.41
Quartile 2: 10.03-11.2	3140	36	-29.0 (-255.1, 197.1)	0.80	275	34	-2.1 (-7.0, 2.8)	0.40
Quartile 3:11.3-12.1	3143	35	-26.1 (-246.4, 194.3)	0.82	274	35	-2.3 (-7.0, 2.4)	0.34
Quartile 4: >12.1	3169	39	1		277	39	1	
Ferritin (µg/L)				**0.004**				0.57
Quartile 1: <13.85	3346	30	410.3 (184.3, 636.3)	**<0.001**	277	31	1.4 (-3.7, 6.5)	0.58
Quartile 2: 13.85-20.79	3129	33	193.7 (-20.7, 408.0)	0.08	273	32	-2.1 (-7.0, 2.7)	0.39
Quartile 3: 20.80-40.08	3212	46	276.2 (72.2, 480.2)	**0.008**	275	46	-0.6 (-5.2, 3.9)	0.79
Quartile 4: >40.08	2936	35	1		275	34	1	
sTfR (mg/L)				0.17				0.84

Quartile 1: >10.53	3300	30	156.1 (-71.8, 384.1)	0.18	275	30	0.8 (-4.2, 5.8)	0.75
Quartile 2: 8.06-10.53	3096	36	-60.1 (-272.3, 152.1)	0.58	274	35	-1.4 (-6.0, 3.2)	0.56
Quartile 3: 6.45-8.05	3102	41	-78.0 (-283.7, 127.6)	0.45	275	41	-0.3 (-4.8, 4.1)	0.88
Quartile 4: <6.45	3149	37	1		276	37	1	

*Birth weight adjusted for maternal age, gestational age at birth, sex of the baby, parity, living standards measure (socio-economic status); HIV status, maternal BMI at enrolment and glucose tolerance.
#Gestational age adjusted for maternal age, delivery intervention, sex of the baby, parity, living standards measure (socio-economic status); HIV status, maternal BMI at enrolment and glucose tolerance

Discussion

In this prospective study of pregnant women residing in Johannesburg, South Africa, the prevalence of anaemia, ID and IDE increased despite iron supplementation forming part of routine antenatal care. We found that ID and anaemia at mid-pregnancy, as well as IDE at late-pregnancy were associated with higher birth weight. In contrast, women with IDE at mid-pregnancy had a 4.6 times higher risk of giving birth prematurely and women with a lower haemoglobin at early pregnancy gave birth significantly earlier than those in the highest haemoglobin quartile.

In this sample of generally healthy, non-smoking, singleton pregnancies from an urban area of South Africa, we observed a similar iron deficiency prevalence during early pregnancy as in women of reproductive age participating in previous national surveys [7,8,38]. About a quarter (28%) of the women were anaemic at early pregnancy, while 15%, 15% and 9% of women were ID, IDE and IDA, respectively. The WHO recommends routine daily iron supplementation (30-60 mg elemental iron) plus folic acid for all pregnant women to cover increased iron requirements. Furthermore, in settings where at least 40% of pregnant women have haemoglobin concentrations <11 g/dL , a daily dose of 60 mg elemental iron should be preferred over a lower dose [1,39,40]. Even though the prevalence of anaemia is less than 40% in South African women of reproductive age, the recommendation is to supplement all pregnant women with 60 mg elemental iron daily (in conjunction with folic acid and calcium) [11]. In our setting, all pregnant women receive 55 mg elemental iron (170 mg dried ferrous sulphate) daily with folic acid and calcium. Compliance to supplements was high in our study (median of 100%), even so, we found significant declines in iron status with pregnancy progression. Two cohort studies in West African countries with routine iron supplementation in place observed a similar decline in iron status during pregnancy [41,42]. The sharp decline in haemoglobin concentrations that we observed in our sample of women at mid-pregnancy can be explained by maternal red blood cell mass and plasma volume expansion leading to haemodilution [43].

It is also known that serum ferritin concentrations gradually decline with pregnancy progression. While haemodilution may explain this phenomenon, it has been suggested that declines in serum ferritin concentrations may reflect iron mobilisation from stores to cover increased requirements for red blood cell production, as well as placental transfer to the foetus [44]. In our sample of pregnant

women, 47% had elevated sTfR concentrations by late pregnancy. It is unclear why these women receiving iron supplements experienced such a marked increase in sTfR concentrations. Increased sTfR expression is reflective of erythropoietic activity, typically expected with red blood cell mass expansion in pregnancy [45]. However, increased sTfR is also strongly associated with functional tissue iron deficiency, indicating that iron cannot be mobilised for erythropoiesis despite adequate iron stores [46]. Elevated concentrations of the hormone hepcidin, which is the main regulator of systemic iron homeostasis [44], may explain this observation. With sufficient systemic iron (thus in iron-replete cases), hepcidin concentrations increase, which in turn reduces the release of iron from enterocytes, macrophages and hepatocytes. Conversely, production of hepcidin is suppressed during iron deficiency to allow release of iron from stores and to increase dietary iron absorption. Recent studies showed that hepcidin is actively reduced during the second and third trimesters of pregnancy to support increased iron requirements [42]. However, hepatocyte hepcidin production increases with inflammation irrespective of iron status [44]. More than half of the women enrolled in our study (n=149; 60%) entered the study with elevated CRP (>5 mg/L) concentrations, indicating a high prevalence of acute and sub-clinical inflammation. African ethnicity has been associated with higher circulating CRP concentrations [47]. In addition, this sample of pregnant women had a high prevalence of overweight and obesity (33% and 28%, respectively), as well as HIV infection (26%), which may have contributed to a more inflammatory state. These factors may have led to an increase in hepcidin concentrations, and consequently to a reduction in iron absorption and release from hepatic stores. In addition, high intakes of calcium (1 g calcium supplementation/day to all pregnant women in South Africa) have been shown to inhibit iron absorption [48]. The *Guidelines for maternity care South Africa* [11] indicates that calcium “is best taken 4 hours before or after iron supplements”. However, it is not known how well this recommendation is implemented. This context may explain the increasing prevalence of ID, IDE and IDA with pregnancy progression in an iron supplemented population.

In our sample, the mean daily iron intake (19 mg, 4.6-46.1 mg) at early pregnancy was approximately 3 mg less than the EAR for pregnant women (22 mg/day), but higher than the EAR for non-pregnant women (8.1 mg/day) [37]. This supports the current recommendation that iron should be

supplemented during pregnancy, but arguably at a lower dose in this setting. Women's iron requirements differ depending on stage of pregnancy. Prior to pregnancy, women's iron requirements are higher than for men due to menstruation. In the first trimester of pregnancy iron requirements are less than prior to pregnancy due to cessation of menses [44], while they increase drastically from the second trimester due to blood volume expansion and increased erythropoietic activity. Therefore, if reported dietary intakes during early pregnancy are reflective of dietary intakes prior to pregnancy, it is likely that most women achieved the recommended iron intake prior to pregnancy. This may explain the relatively low prevalence of ID at enrolment and highlights the need for exploring other determinants of anaemia [49] in women, such as other micronutrient deficiencies and/or inflammation.

In our sample, anaemia and ID at mid-pregnancy were associated with a 168 g and 228 g higher birth weight, respectively. Consistently, IDE at late pregnancy was associated with a 169 g higher birth weight. When comparing quartiles of ferritin concentration at mid-pregnancy, women in the lowest quartile (ferritin <17.78 µg/L) gave birth to significantly heavier babies (312 g) than women in the highest quartile (ferritin >58.63 µg/L). To our knowledge, we are the second study in an African setting (with routine iron supplementation [41]) to find this association. In the cohort of pregnant women in Papua New Guinea (n=279), malaria infection was common and the prevalence of anaemia (haemoglobin <11 g/dL) at enrolment (±25 weeks of gestation) very high (95%). Lower ferritin concentrations at enrolment were associated with higher mean birth weights, and iron deficient women gave birth to 230 g heavier newborns when compared to iron-replete women. The authors indicated that only 7% and 12% of the association was mediated through placental and peripheral malaria infection, respectively, demonstrating an association between iron deficiency and higher birth weight through Malaria-independent mechanisms. Similar associations have been found elsewhere. A Chinese cohort study (n=511) in non-anaemic pregnant women receiving iron supplements as part of routine antenatal care also found a significantly higher birth weight in the lowest compared to the highest ferritin quartile [50]. Furthermore, an Indian cohort (n=1196) (non-anaemic with supplementation) showed similar results with the highest tertile of supplemental iron intake associated with low birth weight [51].

The observed inverse associations between maternal iron status and birth weight in settings of routine iron supplementation can be interpreted from two viewpoints. Firstly, the association between ID, anaemia and IDE with higher birth weight could be an indication that antenatal iron supplementation is protective in iron depleted women, resulting in improved foetal growth. Systematic reviews on the efficacy of antenatal iron supplementation versus placebo have shown significant reductions in anaemia and iron deficiency at term. Evidence for a beneficial effect on birth outcomes is, however, less clear [52–54]. The most recent Cochrane review indicates borderline significance of iron supplementation to reduce risk for low birth weight (RR 0.84; 95% CI: 0.69, 1.03; 11 studies) [53]. In contrast, there is emerging evidence of a U-shape association of iron status and haemoglobin with birth weight [5,6]. Thus, the second viewpoint is from the right side of this U-shape association. Routine iron supplementation in a mixed population of deficient and replete women may contribute to mixed results. In our sample, iron supplementation may have had a negative impact on foetal growth in iron-replete women. Hwang and colleagues [55] found that excessive maternal iron intake at mid-pregnancy was associated with reduced foetal growth in a South Korean cohort (n=337). The foetuses of women in the third tertile of total iron intake had smaller outcomes in biparietal diameter, abdominal circumference and femur length at mid-pregnancy. Even though this South Korean cohort demonstrated lower than recommended iron intake from foods, supplementation contributed to intakes above the Tolerable Upper Intake Level (45 mg). Other studies indicate that iron supplementation in iron-replete women is associated with adverse maternal and foetal outcomes [51,56], although results are inconsistent [57]. When considering the median ferritin and haemoglobin concentrations as well as the prevalence of ID and anaemia at early pregnancy in our sample, it is apparent that most women were iron-replete while receiving iron supplementation. We speculate that provision of supplemental iron in these iron-replete women may have had negative consequences to the mother, which may have compromised foetal growth. There is evidence that unabsorbed supplemental iron reaching the colon can negatively alter gut microbiome composition and increase gut inflammation [58]. This supports the notion that strategies to prevent iron deficiency during pregnancy should consider the inflammation burden in the target

population, and take relevant actions to reduce inflammation in an effort to improve iron absorption and utilisation.

When we assessed the predictors for gestational age, associations were different than for birth weight. Firstly, lower haemoglobin in early pregnancy was associated with shorter gestation which supports previous research [59]. It is suspected that these women entered pregnancy with anaemia since the quartiles associated with the shorter gestation reflects pre-pregnancy anaemia, i.e. haemoglobin <12 g/dL, and haemodilution only peaks late in second trimester or early third trimester [43]. Secondly, as expected, poor iron status increased the risk for premature birth. IDE at mid-pregnancy quadrupled the risk for premature birth. It would be expected that in a supplemented sample the risk would be attenuated in the ID group [53], however, as explained above, elevated sTfR concentrations could be a consequence of inflammation causing iron not to be mobilised.

The key strength of this study was that data were collected prospectively with multiple variables and data collection points over pregnancy. In addition, the iron biomarkers assessed allowed for a complete description of iron status. Previous studies may not have considered concomitant inflammation when interpreting iron status data, however, our analyses included CRP and AGP, which were used to adjust ferritin concentrations accordingly [28]. The analyses were strengthened due to assessment and inclusion of several confounders.

The study limitations should be considered when interpreting the results. Due to the observational study design, conclusions on causality of observed relationships are not possible. However, our findings may generate hypotheses for further investigation, given the consistency of results. An additional limitation is self-selection bias at recruitment since women were recruited at primary healthcare clinics and then volunteered and agreed to participate at a different setting. Lastly, the sample was of relatively small size and not representative of the general population. Women were selected to be non-smoking, generally healthy and presented at primary healthcare clinics somewhat earlier than the typical 20 weeks of gestation [60].

In conclusion, we found an increase in ID, IDE and IDA with pregnancy progression despite routine iron supplementation in an urban South African setting. We observed an inverse association between maternal iron status and birth weight, while IDE at mid-pregnancy increased the risk for

premature birth. These results add to the raising concern on the consequences of iron supplementation in iron-replete pregnant women. Nonetheless, there is no question that ID and anaemia should be prevented in pregnancy. However, the challenge remains on how to do so safely in a public health setting. Considering that South Africa has a well-implemented food fortification programme, high prevalence of inflammation, possible influence of antenatal calcium supplements on iron absorption, as well as the known risks associated with both low and high iron exposure, we recommend that the current antenatal supplementation regime in South Africa be revisited.

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Supplementary table 1: Characteristics and iron status of pregnant women from the NuPED study at enrolment (<18 weeks of gestation) by birth weight data availability¥

Characteristics at enrolment	**Birth weight data obtained 84% (n=203)**	**Birth weight data missing 16% (n=40)**	***p*†**	**Total sample (n=250)*** *Median (IQR) or n (%)*
Age (years)	28 (24-32)	27 (24-30)	0.45	27 (24-32)
Gestational age (weeks)	14 (12-16)	14 (13-16)	0.20	14 (12-16)
BMI (kg/m^2) (n=242)	26.3 (23.1-30.6)	26.5 (22.2-33.7)	0.98	26.3 (23.0-30.6)
Underweight (<18.5 kg/m^2)	7 (4)	0	0.62	8 (3)
Normal weight (18.5-24.9 kg/m^2)	72 (36)	15 (38)		89 (36)
Overweight (25-29.9 kg/m^2)	67 (33)	12 (30)		81 (33)
Obese (≥30 kg/m^2)	56 (28)	13 (33)		71 (28)
Ethnicity (n=242)				
Black African	176 (87)	36 (92)	0.07	219 (88)
Mixed ancestry	27 (13)	2 (5)		28 (11)
White	1 (1)	0		1 (<1)
Indian	0	1 (3)		1 (<1)
Country of birth (n=232)				
South Africa	140 (72)	27 (71)	0.15	172 (72)
Zimbabwe	51 (86)	8 (14)		60 (25)
Lesotho	2 (1)	2 (5)		4 (2)
Swaziland	1 (1)	1 (3)		3 (1)
Living Standards Measure (LSM) (n=243)				
Low (LSM 1-4)	12 (6)	4 (10)	0.63	17 (7)
Medium (LSM 5-7)	120 (59)	23 (58)		148 (59)
High (LSM 8-10)	71 (35)	13 (33)		85 (34)
Marital status (n=242)				
Unmarried/single	84 (41)	12 (31)	0.29	100 (40)
Married	51 (25)	16 (41)		68 (27)
Divorced/Separated	2 (1)	0		2 (1)
Living together	47 (23)	9 (23)		57 (23)
Traditional marriage[#]	19 (8)	2 (5)		22 (9)
Highest level of education (n=242)				
Primary school or less	5 (3)	4 (10)	0.17	9 (4)
Grade 8 – 10	27 (13)	8 (21)		37 (15)
Grade 11 – 12	121 (60)	20 (51)		145 (58)
Post-school education	50 (25)	8 (18)		58 (23)
Parity (n=243)				
Nulliparous	61 (30)	10 (25)	0.88	74 (30)
Primiparous	70 (35)	15 (38)		88 (35)
Multiparous	72 (35)	15 (38)		88 (35)
HIV status (n=243)				
Positive	49 (24)	13 (33)	0.27	64 (26)
Negative	154 (76)	27 (67)		186 (74)
Inflammatory status (n=243)				
Normal CRP	83 (41)	14 (35)	0.49	
Elevated CRP (>5 mg/L)	120 (59)	26 (65)		149 (60)
Normal AGP	178 (88)	38 (95)	0.18	
Elevated AGP (>1 g/L)	25 (12)	2 (5)		28 (11)
Anaemia (n=236)				
Normal haemoglobin	146 (75)	22 (55)	**0.01**	173 (71)
Anaemic (Hb <11g/dL)	50 (25)	18 (45)		70 (29)
Iron stores (n=243)				
Normal serum ferritin	179 (88)	28 (70)	**<0.01**	213 (85)
Iron depleted (Fer <15 µg/L)	24 (12)	12 (30)		37 (15)
Iron deficiency erythropoiesis (n=243)				
Normal sTfR	179 (85)	33 (83)	0.66	212 (85)
Increased sTfR (sTfR >8.3 mg/L)	30 (15)	7 (17)		38 (15)

IQR: interquartile range; CRP: C-reactive protein; AGP: α_1-acid glycoprotein; LSM: Living Standards Measure; Hb: Haemoglobin; Fer: ferritin; sTfR: soluble transferrin receptor.

Data are presented as n (%) for categorical variables and median (IQR) for continuous variables.

¥ Women who had a miscarriage or intrauterine foetal death (IUFD) also had missing birth weight data (n=7), but were not considered in this table.

† Mann-Whitney-U test for continuous variables, and Chi-square test for categorical variables.

* n-values are equal to 250 for LSM, highest level of education, parity, HIV status, iron stores and iron deficiency erythropoiesis; 243 for anaemia; 239 for Country of birth; and 249 for all other variables.

#: Traditional marriage, recognised under South African customary law, is entered between parties based on tradition which does not require the approval of an officiator for validation. It is also different from civil marriage in that a polygamous marriage is permissible [61].

CHAPTER 5

ARTICLE 3

This manuscript is prepared for submission to the journal Prostaglandins, Leukotrienes & Essential Fatty Acids.

Journal Details	
Title:	Prostaglandins, Leukotrienes & Essential Fatty Acids
Impact factor:	2.437
Publisher:	Elsevier
Aim and scope:	Prostaglandins, Leukotrienes & Essential Fatty Acids aims to cover all aspects of the roles of lipids in cellular, organ and whole organism function, and places a particular emphasis on human studies. Papers concerning all medical specialties are published. Much of the material is particularly relevant to the development of novel treatments for disease.
Author guidelines:	https://www.elsevier.com/journals/prostaglandins-leukotrienes-and-essential-fatty-acids/0952-3278/guide-for-authors

Associations of maternal PUFA intake and status in early pregnancy with birth weight and gestational age in Johannesburg, South Africa: the NuPED prospective study

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Abbreviations: NuPED study, Nutrition during Pregnancy and Early Development study; PUFA(s), polyunsaturated fatty acids; ALA, alpha-linoleic acid (18:3n-3); LA, linoleic acid (18:2n-6); EPA, eicosapentaenoic acid (20:5n-3); AA, arachidonic acid (20:4n-6); DHA, docosahexaenoic acid (22:6n-3); BMI, body mass index; SD, standard deviation; IQR, interquartile range

ABSTRACT

Background: Since polyunsaturated fatty acids (PUFA) are critical for early development, the aim of this study was to describe PUFA intake and status in early pregnancy and to determine associations of these with birth weight and gestational age in women residing in Johannesburg, South Africa.

Methods: In a prospective cohort, we interviewed 250 women at <18 weeks of gestation using quantified food frequency questionnaires to determine dietary fatty acid intake. Relative percentage fatty acid composition was determined in red blood cell (RBC) total phospholipids by means of gas chromatography at <18, 22 and 36 weeks of gestation. Logistic regression analyses were conducted with dietary and RBC fatty acids as predictor and low birth weight (LBW) and preterm birth as binary outcome variables.

Results: Dietary intake of n-6 fatty acids, specifically linoleic acid (LA), was high, while n-3 fatty acid intakes were low with an LA:ALA ratio of 39:1 (28:1-51:1). RBC fatty acid composition changed significantly during pregnancy, however, n-3 docosahexaenoic acid (DHA) did not decrease in late pregnancy as expected. In fully adjusted models, higher dietary n-6 PUFA and higher RBC AA:EPA ratios were associated with an increased risk for premature birth (OR: 5.03, 95% CI: 1.16, 21.82, p=0.031 and OR: 4.51, 95% CI: 1.29, 15.79). By contrast, higher dietary n-6 PUFA and LA were associated with a lower risk for LBW (OR: 0.05, 95% CI: 0.00, 0.97, p=0.047 and OR: 0.02, 95% CI: 0.00, 0.60, p=0.025).

Conclusion: High maternal n-6 PUFA intake in early pregnancy and a high RBC AA:EPA ratio was associated with an increased risk for premature delivery in women residing in urban South Africa. However, these high n-6 PUFA intakes were protective from LBW.

Keywords

Pregnancy; birth weight; gestational age; polyunsaturated fatty acids; birth outcomes; maternal fatty acid status; maternal nutrition

1. INTRODUCTION

Long-chain polyunsaturated fatty acids (LCPUFA) play an important role in early life. Poor status during pregnancy has been associated with a number of pregnancy complications, including intra-uterine growth restriction, preterm birth and low birth weight [1–3]. Prematurity is considered the most important cause of death in neonates [4]. In South Africa, complications of prematurity contribute to 48% of neonatal deaths [5]. Severe growth-restricted foetuses are at increased risk of stillbirth and the live births have an increased risk of neonatal death, short-term morbidity and permanent deficits in growth and neurocognitive development [6]. Clinical trials have indicated a lower risk for preterm birth with omega-3 (n-3) LCPUFA supplementation and that supplemented women gave birth to modestly heavier babies compared to women who received placebo [7–9]. In the third trimester, foetal requirements for the n-6 LCPUFA arachidonic acid (AA) and the n-3 LCPUFA docosahexaenoic acid (DHA) are specifically high because of rapid synthesis of brain tissue at the start of the growth spurt [10,11]. Since the foetus has limited ability to synthesise DHA, it is dependent on maternal supply [12–14]. Thus, there is a general change in maternal fatty acid status with normal pregnancy progression [14,15]. Total absolute amounts of all fatty acids in maternal plasma phospholipids increase throughout gestation [16–20] with greater fat mobilisation in the second half of pregnancy for the purposes of placental transfer [15]. In addition, the relative composition of various fatty acids in plasma and red blood cell (RBC) phospholipids changes with pregnancy progression. The proportion of saturated and monounsaturated fatty acids (MUFA) increases while total polyunsaturated fatty acids (PUFAs) generally decrease [1,16,17,20,21].

Little is known about fatty acid intake and status in low- and middle-income countries [22]. There are no national data available on the type of fats consumed by South African adults. Regional cross-sectional studies showed that women from rural areas typically consume less total fat (19% of total energy) compared to those in urban areas (34% of total energy) [23] and that mean n-3 PUFA intakes are low [23–25]. Total PUFA intake has been reported to be less than 10% of total energy in adult women from urban and rural areas, and dietary linoleic acid (LA) to alpha-linoleic acid (ALA) ratios were between 34:1 and 42:1, indicative of poor n-3 fatty acid intake. Among these women, mean

absolute DHA consumption was reported to be much lower (≤ 67 mg/day) than the recommended intakes of 200 mg per day [23,26].

The assumption is therefore that women in South Africa are consuming less than the recommended dietary n-3 fatty acids. Total PUFA intake may be adequate, however, this is attributed to high n-6 fatty acid intake, mainly LA. It is therefore likely that disproportionate LCPUFA intake and status of pregnant women in South Africa may contribute to shorter gestation and lower birth weights. Therefore, the aim of the study was firstly to describe the dietary LCPUFA intake at early pregnancy, as well as the LCPUFA status at early, mid- and late pregnancy in women residing in Johannesburg, South Africa. Additionally, the aim was to determine associations of LCPUFA intake and status at early pregnancy with birth weight and gestational age.

2. MATERIALS AND METHODS

2.1. Study design and participants

The *Nutrition during Pregnancy and Early Development* (NuPED) study is a prospective study conducted in South Africa's largest city, Johannesburg, between March 2016 and May 2019. The study protocol has been published previously [27]. Briefly, 595 generally healthy pregnant women were invited from primary healthcare clinics to take part in the study between March 2016 and November 2017. Of these, 313 (53%) volunteered and signed written informed consent. After screening, 63 women were excluded according to study criteria. Women were eligible for inclusion if they were aged 18-39 years, <18 weeks of gestation with singleton pregnancies, proficient in local languages, born in South Africa or neighbouring countries, and if they have been residing in Johannesburg for at least 12 months. Women were excluded if they were smoking, reported using illicit drugs, or were diagnosed with a non-communicable disease (namely diabetes, renal disease, high cholesterol, and hypertension), an infectious disease (namely tuberculosis and hepatitis), or a serious illness (namely cancer, lupus or psychosis). Due to South Africa's high prevalence of HIV infection [28], women who were HIV positive were included in the study. Data were collected at early pregnancy (<18 weeks of gestation), mid-pregnancy (±22 weeks), late pregnancy (±36 weeks) and at birth at a tertiary hospital in Johannesburg. Birth data collection was completed in June 2018.

2.2. Birth outcome measures

Birth weight and gestational age at birth were the primary outcome measures. At birth, trained study nurses obtained birth weight (to the nearest 10 g) using calibrated digital infant scales [29]. In case the study nurse could not obtain the birth weight herself, it was obtained from the medical record (measured using the same calibrated scales). Low birth weight (LBW) was defined as birth weight <2500 g [30]. Gestational age at birth was calculated in days using gestational age at first visit as confirmed with foetal ultrasonography. Preterm birth was defined as birth at <37 + 0 weeks of gestation (<259 days) [4].

2.3. Maternal PUFAs

2.3.1. Dietary and supplemented fatty acid intake

The maternal dietary intake methodology has been reported previously [27]. Briefly, maternal dietary intake data were obtained at the first visit (<18 weeks of gestation) by means of a validated quantified food frequency questionnaire (QFFQ) [31–33]. Listed QFFQ items assessed how often, on average, during the past four weeks a person consumed foods and beverages. Interviewers prompted for cooking methods, the type/brand, frequency and the amount of the reported items. Registered dietitians/nutritionists converted reported intakes to grams per week per food item using the Condensed Food Composition Tables for South Africa [34] and the South African Medical Research Council (SAMRC) Food Quantities Manual [35]. The SAMRC linked dietary intake data to the most recent South African food composition database to determine total fatty acid intake levels. Supplement use was determined at each visit. The women were asked the type/brand, frequency and the amount of all dietary supplements used in the past week. Women who indicated use of n-3 supplements at any point in pregnancy, were considered supplement users. Primary analyses of dietary fatty acid intake levels did not include intake from supplements.

2.3.2. Maternal red blood cell total phospholipid fatty acid profiles

Maternal venous blood was drawn into labelled EDTA-coated tubes at each study visit during pregnancy. Samples were processed 1 hour after blood draw. RBCs were washed twice with 0.15

mol/L NaCl and centrifuged at 500 X g for 10 min to remove the buffy coat. Samples were stored at -20 °C for a maximum of 14 days, thereafter they were stored at -80 °C until analyses. RBC total phospholipid fatty acids were extracted as previously described [36]. In short, phospholipids were extracted from RBC with chloroform: methanol (2:1 vol: vol; containing 0.01% butylated hydroxytoluene) using a modified method of Folch [37]. The total phospholipid fraction was separated from the neutral lipids using thin-layer chromatography silica gel 60 plates (10x 20 cm; Merck) and eluted with diethyl ether: petroleum ether: acetic acid (30:90:1, vol: vol: vol). The lipid band that contained phospholipids were scraped from the thin-layer chromatography plate and trans-methylated with methanol: sulphuric acid (95:5 vol:vol) at 70ºC for 120 min to yield fatty acid methyl esters (FAMEs). The resulting FAMEs were extracted with 1 ml distilled water and 2 ml hexane. The organic layer was evaporated at 45°C and re-dissolved in hexane. FAMEs were analysed with an Agilent Technologies 7890A gas chromatograph system equipped with an Agilent Technologies 7000B triple quad mass selective detector (Agilent Technologies). The gas chromatography separation of FAMEs was carried out on a HP88 capillary column (100 m x 0.25 mm x 0.20 µm; Agilent) by using helium as the carrier gas at a flow rate of 2.2 mL/min. The gas chromatography injector was maintained at a temperature of 270 °C, and the mass spectrometry source at 250 °C. The injection volume of the sample solution was 1 µL by using a split ratio of 1:80. The oven temperature was programmed to rise from 50 °C to 170 °C at 30 °C /min, then from 170 ºC to 215 ºC at 2 ºC/min, and lastly at 4 °C/min to 230 °C. After that the temperature was held isothermally at 230 ºC for 7 min. The total analysis time was 38.25 min. Mass spectrometry was carried out in positive impact multiple reaction monitoring mode, with at least two transitions per compound. Quantification of FAMEs was performed with Masshunter (B.06.00). FAME peaks were identified and calibrated against a standard reference mixture of 33 FAMEs (Nu-Check-Prep) and two single FAME standards (Larodan Fine Chemicals AB). Relative percentages of fatty acids (% w/w) were calculated by taking the concentration of a given fatty acid derivative as a percentage of the total concentration of all fatty acids identified in the sample.

2.3.3. Covariates

Socio-economic and -demographic data were collected at the first visit early in pregnancy (<18 weeks of gestation) by means of an interviewer-administered questionnaire. Maternal height and weight were obtained using standardised methods from the International Society for the Advancement of Kinanthropometry [38] at each study visit. To determine body mass index (BMI) weight (kg) was divided by height (m) squared. Gestational age at first visit was confirmed with foetal ultrasonography examination [39,40]. A 2-hour 75 g oral glucose tolerance test was performed between 24 and 28 weeks of gestation using standard procedures [41]. Maternal medical history, including parity, HIV status, mode of delivery, labour induction, as well as sex of the baby were obtained from medical files.

2.4. Statistical methods

Sample size calculations have been reported previously [27]. The sample size for this study was 250. All statistical analyses were done in SPSS version 25 (SPSS Inc, Chicago, IL, USA) with a level of statistical significance set at 0.05. Data were tested for outliers and normality by means of Q-Q plots, histograms and Shapiro-Wilk test. Data were checked for outliers and implausible data points were deleted. Normally distributed data are expressed as means ± SD; non-normally distributed data are expressed as medians (interquartile range). Descriptive statistics were conducted to describe the characteristics of the women and new-borns as well as maternal nutrient intake at early pregnancy. The Mann-Whitney-U test was conducted to compare the medians of intake and status per outcome group, i.e. LBW and premature birth, respectively. To explore the changes in fatty acid composition with pregnancy progression, median percentage contributions were determined at each visit, and to test significance of change in status over time we used the Wilcoxon signed rank test. Spearman's correlation was used to assess associations between dietary intake and RBC fatty acid composition.

Dietary intakes of PUFA as well as RBC PUFA were categorised in two groups: below and above the median, with below the median group as reference. We used logistic regression analyses to investigate the relationship of the categories of maternal PUFA intake and status with outcome

variables (LBW and preterm birth) as binary outcomes with odds ratios (OR) and 95% confidence intervals (CI). In the regression analyses we applied two models for each outcome. Each outcome variable had different sets of covariates. For birth weight as outcome variable, model 1 adjusted for maternal age, gestational age at birth and sex of the baby. Model 2 included the covariates of model 1 plus parity, socio-economic status, HIV status, maternal BMI at enrolment and glucose tolerance. For gestational age at birth, model 1 adjusted for maternal age, baby sex and delivery intervention (induction or caesarean section). Model 2 adjusted additionally for parity, socio-economic status, HIV status, maternal BMI at enrolment and glucose tolerance. Regression analyses with dietary PUFA levels also included total energy intake as a covariate. Due to the correlations between RBC fatty acids expressed as percentage of total fatty acids [42], models assessing single fatty acids included the other single fatty acids as covariates.

2.5. Ethical considerations

This study was conducted according to the principles of the Declaration of Helsinki. All procedures involving human subjects were approved by both the Human Research Ethics Committees of the North-West University, Potchefstroom (NWU-00186-15-A1) and the University of Witwatersrand, Johannesburg (M150968). The Gauteng Health Department, City of Johannesburg District Research Committee and Clinical Manager of Rahima Moosa Mother and Child Hospital gave permission to conduct research at the indicated clinical setting. All women provided written informed consent at the first visit before data collection.

3. RESULTS

3.1. Participant characteristics and birth outcomes

In total 250 women were enrolled and completed data collection at baseline (<18 weeks of gestation). Of these, 233 attended follow-up visits: ten women were lost-to-follow-up and seven (3%) had a miscarriage/intra-uterine foetal death. Data of these 17 women were included in cross-sectional analysis of data at enrolment. There was one maternal fatality shortly after delivery. Several women

(n=28) delivered their babies at a different setting and therefore, some birth data were missing, however in some cases date of birth and sex of the baby could be determined telephonically.

Maternal and new-born characteristics are shown in Table 1. At enrolment, the median maternal age was 27 (24-32) years and the median length of gestation was 14 (12-16) weeks. The median BMI was 26.3 [23.0-30.6] kg/m^2 and a third of women (33%) were overweight and 28% obese. Most of the women were from black-African descent (88%), born in South Africa (72%) and had middle-class living standards (LSM 5-7) (59%). The majority (58%) completed secondary school and an additional 23% completed post-school education. Many women (40%) were unmarried/single and almost a third were nulliparous (30%). More than a quarter of the women were HIV positive (26%). Only 4% (n=11) of the women reported using n-3 fatty acid supplementation during pregnancy. From this pregnancy cohort, 55% of the babies were male and 60% births were by vaginal delivery. The median birthweight was 3050 (2790-3380) grams with 14% born LBW. The median gestational age at birth was 274 (266-282) days with 11% being born prematurely.

Table 1: Characteristics of pregnant women at enrolment and of new-borns from the NuPED study

Maternal characteristics	n	Median (IQR) or n (%)
Age (years)	250	27 (24-32)
Gestational age (weeks)	250	14 (12-16)
BMI (kg/m^2)	249	26.3 (23.0-30.6)
Underweight (<18.5 kg/m^2)		8 (3)
Normal weight (18.5-24.9 kg/m^2)		89 (36)
Overweight (25-29.9 kg/m^2)		81 (33)
Obese (≥30 kg/m^2)		71 (28)
Ethnicity	249	
Black African		219 (88)
Mixed ancestry		28 (11)
White		1 (<1)
Indian		1 (<1)
Country of birth	239	
South Africa		172 (72)
Zimbabwe		60 (25)
Lesotho		4 (2)
Swaziland		3 (1)
Living Standards Measure (LSM)	250	
Low (LSM 1-4)		17 (7)
Medium (LSM 5-7)		148 (59)
High (LSM 8-10)		85 (34)
Marital status	249	
Unmarried/single		100 (40)
Married		68 (27)
Divorced/Separated		2 (1)
Living together		57 (23)
Traditional marriage		22 (9)
Highest level of education	249	
Primary school or less		9 (4)
Grade 8-10		37 (15)
Grade 11-12		145 (58)
Post-school education		58 (23)
Parity	250	
Nulliparous		74 (30)
Primiparous		88 (35)
Multiparous		88 (35)
HIV positive	250	64 (26)
Inflammatory status		
Elevated CRP (>5 mg/L)	250	149 (60)
Elevated AGP (>1 g/L)	250	28 (11)
n-3 supplement users	250	11 (4)
New-born characteristics		
Birthweight, g	203	3050 (2790-3380)
LBW (<2500 g)	203	29 (14)
Gestational age at birth, days	233	274 (266-282)
Premature birth (<259 days)	233	26 (11)
Sex (% males)	212	116 (55)
Delivery mode	202	
Vaginal		121 (60)
Caesarean section		81 (40)

BMI: body mass index; LBW: low birth weight; IQR: interquartile range; CRP: C-reactive protein; AGP: α_1-acid glycoprotein
Data are presented as n (%) for categorical variables and median (IQR) for continuous variables.

3.2. Maternal dietary fatty acid intake at early pregnancy

The median (IQR) reported daily intakes of energy, macronutrients and fatty acids at enrolment for the total group and by birth outcome are presented in Table 2. The reported total median energy intake was 11907 (8789-15028) kJ per day. The median macronutrient contribution to total energy (%E) was 56.6% (50.5–61.4) from carbohydrates, 13.0% (11.8–14.7) from protein and 31.1% (27.2–35.2) from fat. The reported median LA intake was 21.5 (14.8-31.4) g per day and ALA was 584.5 (368.8-814.8) mg per day. The median LA:ALA ratio was 39:1 (28:1-51:1). The median EPA and DHA reported intake were 61.8 (33.4-120.9) mg EPA/day and 128.9 (71.4-246.4) mg DHA/day.

The women who delivered LBW babies did not report significantly different intakes for energy, total carbohydrates, total protein or total fat compared to those who delivered normal birthweight babies. However, women who delivered LBW babies reported significantly higher intakes of 20:2n-6 and 20:3n-6 fatty acids (49.1 vs 25.9 mg/day and 16.1 vs 6.9 mg/day, respectively) at early pregnancy. Women who delivered prematurely also reported higher intake of these fatty acids (54.7 vs 25.9 mg/day of 20:2n-6 and 15.4 vs 7.1 mg/day of 20:3n-6). In addition, the premature group reported higher intakes of protein (119.0 vs 89.4 g/day), cholesterol (582.7 mg vs 363.5 mg), AA (180.3 mg vs 142.4 mg/day), EPA (85.7 vs 59.7 mg/day) and DPA (38.5 vs 23.6 mg/day) compared to the group who delivered at term.

Table 2: Reported daily energy, macronutrient and fatty acid intake of pregnant women at <18 weeks gestation in South Africa per birth outcome

Nutrient	Total group (n=248) Median (IQR)	LBW (n=29) Median (IQR)	Normal birth weight (n=173) Median (IQR)	$p^{\dagger}$	Premature birth (n=26) Median (IQR)	Term (n=205) Median (IQR)	$p^{\dagger}$
Energy, kJ	11907 (8789 - 15028)	13666 (10771 - 16489)	11950 (8646 - 14883)	0.42	14408 (10135 - 16881)	11950 (8690 - 14883)	0.07
Total CHO, g	384.7 (297.5 - 491.7)	432.4 (331.1 - 608.3)	386.6 (292.0 - 486.1)	0.69	471.1 (296.8 - 623.1)	386.6 (297.1 - 486.1)	0.07
Total protein, g	90.6 (67.9 - 121.4)	116.1 (73.5 - 141.7)	90.6 (65.5 - 121.1)	0.42	119.0 (86.3 - 13.9)	89.4 (65.9 - 120.1)	**<0.01**
Total fat, g	98.1 (65.8 - 130.7)	111.2 (71.8 - 136.7)	97.7 (63.3 - 130.7)	0.23	114.9 (86.1 - 139.9)	97.7 (63.4 - 130.4)	0.17
SFA, g	29.4 (19.0 - 39.7)	30.9 (20.8 - 44.8)	29.6 (19.1 - 39.5)	0.69	32.1 (24.4 - 42.8)	29.3 (18.4 - 39.7)	0.28
MUFA, g	31.5 (19.2 - 43.9)	32.6 (20.0 - 47.5)	31.5 (18.1 - 42.5)	0.69	33.2 (28.2 - 45.6)	31.5 (18.4 - 44.1)	0.28
PUFA, g	23.9 (16.5 - 33.6)	24.9 (17.0 - 34.9)	23.0 (16.5 - 32.9)	0.69	27.8 (19.3 - 35.6)	22.7 (16.3 - 33.1)	0.16
Trans fatty acids, g	1.3 (0.6 - 2.4)	1.5 (0.6 - 3.7)	1.3 (0.6 - 2.4)	0.99	1.7 (0.8 - 3.8)	1.3 (0.6 - 2.3)	0.11
Cholesterol, mg	367.7 (234.7 - 589.2)	429.1 (288.8 - 673.7)	367.9 (231.4 - 585.5)	0.42	582.7 (317.5 - 727.7)	363.5 (224.1 - 567.2)	**0.01**
CHO, %E	56.6 (50.5 - 61.4)	57.5 (51.5 - 60.1)	56.4 (50.2 - 61.7)	0.69	56.8 (50.5 - 60.3)	56.9 (50.5 - 61.5)	0.92
Protein, %E	13.0 (11.8 - 14.7)	13.1 (12.1 - 15.7)	13.0 (11.7 - 14.7)	0.69	13.5 (12.7 - 15.9)	13.0 (11.6 - 14.6)	**0.04**
Fat, %E	31.1 (27.2 - 35.2)	30.0 (27.0 - 34.0)	31.3 (27.4 - 35.5)	0.69	30.2 (26.5 - 34.3)	31.0 (27.3 - 35.3)	0.87
SFA, %E	9.24 (7.5 - 10.8)	9.0 (7.8 - 10.3)	9.3 (7.7 - 11.0)	0.23	9.0 (7.3 - 10.7)	9.3 (7.7 - 10.8)	0.74
MUFA, %E	4.4 (3.5 - 5.3)	4.3 (3.6 - 5.0)	4.4 (3.5 - 5.3)	0.69	4.2 (3.6 - 5.3)	4.4 (3.5 - 5.3)	0.89
PUFA, %E	3.5 (2.7 - 4.4)	3.2 (2.8 - 4.1)	3.4 (2.7 - 4.4)	0.99	3.5 (2.8 - 4.2)	3.4 (2.7 - 4.3)	0.84
Trans fat, %E	0.2 (0.1 - 0.3)	0.2 (0.1 - 0.4)	0.2 (0.1 - 0.3)	0.42	0.3 (0.1 - 0.4)	0.2 (0.1 - 0.3)	0.11
n-6, %E	3.2 (2.5 - 4.1)	3.0 (2.6 - 3.9)	3.2 (2.5 - 4.0)	0.69	3.3 (2.6 - 3.9)	3.2 (2.5 - 4.1)	0.89
n-3, %E	0.1 (0.1 - 0.2)	0.1 (0.1 - 0.2)	0.1 (0.1 - 0.2)	0.99	0.1 (0.1 - 0.2)	0.1 (0.1 - 0.2)	0.55
Total n-6, g	21.7 (14.9 - 31.3)	23.4 (14.8 - 32.0)	21.6 (14.8 - 30.9)	0.69	26.1 (17.5 - 32.8)	20.8 (14.8 - 31.1)	0.17
18:2n-6 (LA), g	21.5 (14.8 - 31.1)	23.2 (14.6 - 31.7)	21.3 (14.7 - 30.7)	0.69	25.9 (17.4 - 32.5)	20.7 (14.7 - 30.9)	0.18
20:2n-6, mg	28.5 (10.6 - 55.9)	49.1 (20.0 - 65.5)	25.9 (9.8 - 52.4)	**0.05**	54.7 (33.8 - 73.2)	25.9 (9.9 - 51.8)	**<0.01**
20:3n-6 (DGLA), mg	8.7 (3.7 - 16.6)	16.1 (6.8 - 19.8)	6.9 (3.6 - 15.1)	**0.02**	15.4 (8.7 - 19.6)	7.1 (3.6 - 16.3)	**<0.01**
20:4n-6 (AA), mg	142.7 (78.4 - 203.2)	159.0 (85.5 - 249.6)	142.8 (75.9 - 202.6)	0.42	180.3 (122.5 - 236.6)	142.4 (72.7 - 200.6)	**0.02**
22:2n-6, mg	18.0 (8.9 - 29.0)	19.0 (10.9 - 26.2)	17.2 (8.9 - 28.5)	0.69	19.8 (8.6 - 32.0)	17.0 (8.9 - 28.4)	0.40
Total n-3, g	0.8 (0.6 - 1.2)	0.9 (0.6 - 1.4)	0.9 (0.6 - 1.2)	0.99	0.9 (0.7 - 1.4)	0.9 (0.5 - 1.3)	0.16
18:3n-3 (ALA), mg	584.5 (368.8 - 814.8)	648.8 (373.5 - 820.1)	604.0 (361.5 - 829.5)	0.23	641.3 (458.1 - 814.2)	596.9 (367.6 - 827.8)	0.37
18:4n-3, mg	3.7 (0.3 - 17.0)	4.7 (1.2 - 16.8)	3.5 (0.3 - 18.9)	0.57	6.1 (0.4 - 21.9)	3.5 (0.3 - 18.4)	0.50
20:5n-3 (EPA), mg	61.8 (33.4 - 120.9)	74.0 (37.4 - 151.4)	62.3 (32.7 - 120.4)	0.69	85.7 (56.3 - 152.2)	59.7 (31.2 - 122.7)	**0.02**
22:5n-3 (DPA), mg	25.1 (13.2 - 43.7)	33.4 (16.6 - 50.0)	24.2 (12.3 - 44.0)	0.23	38.5 (21.6 - 49.1)	23.6 (12.1 - 44.0)	**0.01**
22:6n-3 (DHA), mg	128.9 (71.4 - 246.4)	135.6 (81.4 - 283.3)	129.1 (69.3 - 247.3)	0.69	152.2 (122.3 - 280.8)	128.1 (69.1 - 247.7)	0.10
n-6:n-3	25 (18 - 35)	25 (16 - 33)	24 (18 - 34)	0.99	24 (16 - 31)	25 (18 - 34)	0.51
LA:ALA	39 (28 - 51)	41 (29 - 48)	36 (27 - 53)	0.23	41 (31 - 47)	37 (28 - 50)	0.67

CHO, carbohydrates; %E, percentage energy; LA, linoleic acid; ALA, alpha-linolenic acid; DGLA, dihomo-ɣ-linolenic acid; AA; arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapenataenoic acid; DHA, docosahexaenoic acid. † Mann-Whitney-U test were contact to compare the medians per outcome group.

3.3. Maternal red blood cell total phospholipid fatty acid composition at early pregnancy

The median RBC total phospholipid fatty acids (% of total fatty acids) and ratios between fatty acids of the women at enrolment (<18 weeks of gestation) by birth outcome are presented in Table 3. Median RBC saturated fatty acids (SFA) and PUFA were 44.4 (42.8-46.2)% and 40.6 (38.6-42.3)%, respectively. There were no significant differences in maternal RBC fatty acid composition at enrolment between the women who ultimately delivered LBW babies and normal birthweight babies. Similarly, there were no significant differences in RBC fatty acids between those who delivered prematurely compared to those who delivered their babies at term.

Spearman's correlations were conducted between dietary and RBC fatty acids for LA, DGLA, AA, ALA, EPA and DHA as assessed at enrolment. Significant correlations were found between intake and status for EPA and DHA (r_S=0.25, p<0.001 and r_S=0.23, p<0.001, respectively). Dietary LA intake had a weak negative correlation with RBC LA (r_S=-0.12, p=0.027). However, there were no associations between the intake and status of ALA, DGLA and AA, respectively (r_S=0.02, p=0.387; r_S=-0.01, p=0.862 and r_S=0.08, p=0.099). Even though the correlation between dietary LA and the status of its longer-chain derivative AA was not significant, there was a trend towards significance (r_S=0.10, p=0.060). There were no significant correlations between dietary ALA and RBC EPA (r_S=-0.02, p=0.399), and RBC DHA (r_S=-0.07, p=0.153).

Table 3: Red blood cell total phospholipid fatty acid composition of pregnant women at <18 weeks gestation in South Africa by birth outcome

Fatty acids % w/w	Total group (n=243) Median (IQR)	LBW (n=28) Median (IQR)	Normal birth weight (n=170) Median (IQR)	p†	Premature birth (n=26) Median (IQR)	Term (n=201) Median (IQR)	p†
SFA	44.4 (42.8 - 46.2)	44.4 (43.4 - 45.9)	44.6 (42.7 - 46.5)	0.54	44.8 (43.3 - 46.9)	44.4 (42.7 - 46.1)	0.47
MUFA	14.6 (13.7 - 15.7)	14.5 (14.0 - 15.8)	14.6 (13.6 - 15.7)	0.84	14.6 (14.3 - 15.8)	14.6 (13.7 - 15.7)	0.33
PUFA	40.6 (38.6 - 42.3)	40.7 (38.3 - 41.7)	40.5 (38.5 - 42.4)	0.84	40.5 (36.8 - 41.7)	40.6 (38.7 - 42.3)	0.52
n-6 PUFA	33.0 (31.1 - 34.4)	33.1 (31.3 - 34.0)	32.9 (31.0 - 34.4)	0.84	32.9 (30.4 - 34.1)	33.0 (31.1 - 34.4)	0.66
n-6 LCPUFA	21.6 (20.0 - 22.9)	21.2 (20.5 - 22.9)	21.6 (19.9 - 22.7)	0.84	22.0 (20.4 - 23.0)	21.6 (20.0 - 22.8)	0.52
18:2n-6 (LA)	11.3 (10.3 - 12.5)	11.1 (10.0 - 12.2)	11.3 (10.2 - 12.7)	0.84	10.9 (9.8 - 12.1)	11.4 (10.4 - 12.6)	0.11
18:3n-6 (GLA)	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	0.54	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	0.37
20:3n-6 (DGLA)	1.2 (1.0 - 1.4)	1.2 (1.0 - 1.5)	1.2 (1.0 - 1.4)	0.54	1.2 (1.1 - 1.5)	1.2 (1.0 - 1.4)	0.11
20:4n-6 (AA)	14.3 (13.4 - 15.3)	14.1 (13.0 - 15.1)	14.3 (13.3 - 15.4)	0.84	14.4 (13.0 - 15.4)	14.3 (13.4 - 15.3)	0.80
n-3 PUFA	7.5 (6.5 - 8.4)	7.2 (6.4 - 7.9)	7.4 (6.5 - 8.4)	0.84	7.2 (6.1 - 7.8)	7.4 (6.5 - 8.4)	0.26
n-3 LCPUFA	7.4 (6.4 - 8.3)	7.1 (6.3 - 7.8)	7.4 (6.5 - 8.3)	0.84	7.1 (6.1 - 7.7)	7.4 (6.5 - 8.4)	0.28
18:3n-3 (ALA)	0.1 (0.1 - 0.1)	0.1 (0.1 - 0.1)	0.1 (0.1 - 0.1)	0.31	0.1 (0.1 - 0.1)	0.1 (0.1 - 0.1)	0.89
20:5n-3 (EPA)	0.3 (0.2 - 0.4)	0.3 (0.2 - 0.3)	0.3 (0.2 - 0.4)	0.84	0.2 (0.2 - 0.3)	0.3 (0.2 - 0.4)	0.80
22:5n-3 (DPA)	1.8 (1.6 - 2.1)	1.8 (1.6 - 2.0)	1.9 (1.6 - 2.1)	0.31	1.8 (1.6 - 2.1)	1.8 (1.6 - 2.1)	0.44
22:6n-3 (DHA)	5.1 (4.5 - 6.0)	5.0 (4.7 - 5.8)	5.1 (4.5 - 5.9)	0.84	4.9 (4.5 - 5.3)	5.1 (4.5 - 6.0)	0.29
n6:n3	4.5 (3.8 - 5.0)	4.6 (4.1 - 4.9)	4.5 (3.8 - 5.0)	0.54	4.6 (4.2 - 5.0)	4.5 (3.7 - 5.1)	0.34
LC n6:n3	2.9 (2.6 - 3.4)	3.0 (2.6 - 3.3)	2.9 (2.6 - 3.4)	0.31	3.1 (2.8 - 3.4)	2.9 (2.5 - 3.4)	0.16

LA, linoleic acid; ALA, alpha-linolenic acid; DGLA, dihomo-ɣ-linolenic acid; AA; arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid
† Mann-Whitney-U test were contact to compare the medians per outcome group.

3.4. Maternal red blood cell total phospholipid fatty acid composition during pregnancy

The RBC total phospholipid fatty acid composition at each visit during pregnancy as well as the significance of changes in RBC fatty acids from one visit to the next are presented in Table 4. All fatty acids significantly changed during pregnancy except for 18:3n-6, 20:3n-6 and 18:3n-3. With the significant increase in saturated fatty acids (SFAs) and MUFAs between <18 and 22 weeks of gestation, total PUFA significantly decreased [40.6 (38.6-42.3)% to 38.3 (37.1-39.6)%, p<0.001]. This decrease was therefore reflected in the decrease in both total n-6 PUFA and total n-3 PUFA. The individual fatty acids contributing to the significant decrease in total PUFA between <18 and 22 weeks of gestation were AA (14.3 [13.4-15.3]% to 12.5 [11.7-13.2]), EPA [0.25 (0.19-0.36)% to 0.21 (0.15-0.30)%], DPA [1.82 (1.56-2.12)% to 1.66 (1.43-1.94)%] and DHA (5.10 [4.48-5.97]% to 5.08 [4.50-5.75]%].

Between 22 and 36 weeks of gestation SFA significantly decreased, however MUFA and PUFA increased. Total n-6 PUFA significantly increased between 22 and 36 weeks of gestation [31.1 (29.5-32.5)% to 31.7 (29.7-33.7)%], although the change were small. During this time total n-3 PUFA did not change (p=0.939). Only median LA significantly increased from 22 to 36 weeks of gestation [11.4 (10.6-12.3)% to 12.1 (10.7-13.0)%], and was therefore the main contributor to the increase in total PUFA and total n-6 PUFA.

Even though total n-3 PUFA only decreased between <18 and 22 weeks of gestation, EPA and DPA decreased significantly at both time points. Median DHA did not change between 22 and 36 weeks of gestation (p=0.181). The n-6:n-3 PUFA ratio did not change with pregnancy progression (p=0.981 and p=0.336). However, when considering individual fatty acid ratios, changes were noted. Within the n-6 fatty acid pathway, the LA:AA ratio increased significantly during pregnancy (p<0.001 in both instances). Within the n-3 fatty acid pathway, EPA:ALA ratio decreased significantly only in early pregnancy [3.4 (2.4-5.1)% to 2.7 (2.0-3.8)%]. DHA:ALA ratio only decreased from <18 to 22 weeks of gestation [73.1 (49.8-94.8)% to 66.4 (50.8-84.1)%] and then showed a significant increase from 22 to 36 weeks of gestation [66.4 (50.8-84.1)% to 67.5 (52.8-90.6)%). Similarly, DHA:EPA ratio decreased from <18 to 22 weeks [24.7 (17.9-33.9)% to 23.6 (18.5-30.7)%) and then increased from 22 to 36 weeks of gestation [23.6 (18.5-30.7)%, to 28.3 (20.7-35.6)%).

Table 4: Red blood cell total phospholipid fatty acid composition at three time points of pregnant women in South Africa

Fatty acids % w/w	<18 weeks (n= 243)		22 weeks (n=229)		36 weeks (n=190)		<18 & 22 wks	22 & 36 wks
	Median	(IQR)	Median	(IQR)	Median	(IQR)	*p*†	*p*†
SFA	44.4	(42.8 - 46.2)	45.9	(45.0 - 47.0)	44.5	(42.7 - 46.9)	**<0.001**	**<0.001**
MUFA	14.6	(13.7 - 15.7)	15.5	(14.7 - 16.6)	16.1	(14.8 - 17.2)	**<0.001**	**0.002**
PUFA	40.6	(38.6 - 42.3)	38.3	(37.1 - 39.6)	39.0	(36.5 - 41.0)	**<0.001**	**0.012**
n-6 PUFA	33.0	(31.1 - 34.4)	31.1	(29.5 - 32.5)	31.7	(29.7 - 33.7)	**<0.001**	**0.005**
n-6 LCPUFA	21.6	(20.0 - 22.9)	19.6	(18.3 - 20.7)	19.7	(17.6 - 21.4)	**<0.001**	0.449
18:2n-6 (LA)	11.3	(10.3 - 12.5)	11.4	(10.6 - 12.3)	12.1	(10.7 - 13.0)	0.190	**<0.001**
18:3n-6 (GLA)	0.0	(0.0 - 0.0)	0.0	(0.0 - 0.0)	0.0	(0.0 - 0.0)	0.360	0.110
20:3n-6 (DGLA)	1.2	(1.0 - 1.4)	1.2	(1.0 - 1.5)	1.2	(1.0 - 1.5)	0.335	0.658
20:4n-6 (AA)	14.3	(13.4 - 15.3)	12.5	(11.7 - 13.2)	12.4	(11.2 - 13.2)	**<0.001**	0.145
n-3 PUFA	7.5	(6.5 - 8.4)	7.1	(6.2 - 8.0)	6.9	(6.2 - 8.0)	**<0.001**	0.939
n-3 LCPUFA	7.4	(6.4 - 8.3)	7.0	(6.2 - 7.9)	6.8	(6.1 - 7.9)	**<0.001**	0.899
18:3n-3 (ALA)	0.1	(0.1 - 0.1)	0.1	(0.1 - 0.1)	0.1	(0.1 - 0.1)	0.483	0.123
20:5n-3 (EPA)	0.25	(0.19 - 0.36)	0.21	(0.15 - 0.30)	0.18	(0.14 - 0.26)	**<0.001**	**0.001**
22:5n-3 (DPA)	1.82	(1.56 - 2.12)	1.66	(1.43 - 1.94)	1.56	(1.34 - 1.84)	**<0.001**	**0.006**
22:6n-3 (DHA)	5.10	(4.48 - 5.97)	5.08	(4.50 - 5.75)	5.12	(4.49 - 5.93)	**0.014**	0.181
n-6:n-3 PUFA	4.5	(3.8 - 5.0)	4.4	(3.9 - 5.0)	4.5	(4.0 - 5.2)	0.981	0.336
n-6:n-3 LCPUFA	2.95	(2.57 - 3.36)	2.79	(2.45 - 3.22)	2.86	(2.45 - 3.24)	**<0.001**	0.664
LA:ALA	152.6	(113.8 - 196.3)	148.2	(118.4 - 186.3)	157.8	127.2 - 200.3)	0.373	**0.006**
LA:AA	0.79	(0.70 - 0.89)	0.92	(0.83 - 1.02)	0.98	(0.86 - 1.11)	**<0.001**	**<0.001**
AA:EPA	56.1	(40.0 - 76.6)	59.8	(41.5 - 81.4)	69.0	(48.3 - 90.8)	**0.001**	**0.016**
AA:DHA	2.8	(2.4 - 3.2)	2.5	(2.2 - 2.8)	2.4	(2.1 - 2.7)	**<0.001**	**<0.001**
EPA:ALA	3.4	(2.4 - 5.1)	2.7	(2.0 - 3.8)	2.4	(1.9 - 3.3)	**<0.001**	0.103
DHA:ALA	73.1	(49.8 - 94.8)	66.4	(50.8 - 84.1)	67.5	(52.8 - 90.6)	**0.011**	**0.038**
DHA:EPA	24.7	(17.9 - 33.9)	23.6	(18.5 - 30.7)	28.3	(20.7 - 35.6)	**0.001**	**<0.001**

SFA, saturated fatty acids; PUFA, polyunsaturated fatty acid; LCPUFA, long chain PUFA; LA, linoleic acid; GLA, γ-linolenic acid; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; ALA, α-linoleic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; wks, weeks
Total PUFA percentage includes the sum of all PUFAs detected in the sample, i.e. LA, GLA, ALA, 20:2n6; 20:3n9, 20:3n6, AA, EPA, 22:4n6, DPA (n-6), DPA (n-3), DHA
Total n-6 PUFA percentage includes the sum of all n-6 PUFA, i.e. LA, GLA, 20:2n-6, 20:3n-6, AA, 22:4n-6, DPA (n-6), while the Total n-6 LCPUFA did not include LA and GLA
Total n-3 PUFA percentage includes the sum of all n-3 PUFA, i.e. ALA, EPA, DPA (n-3), DHA, while the Total n-3 LCPUFA did not include ALA
† Wilcoxon nonparametric tests were used.

3.5. Associations between maternal dietary fatty acid intake at early pregnancy and birth outcomes

Table 5 presents the results from the logistic regression analyses of reported n-6 and n-3 dietary fatty acid intake (above the median, with below the median as reference) at enrolment (<18 weeks of gestation) and LBW as outcome. In the fully adjusted model, women who reported consuming n-6 PUFA and LA in amounts above the median had a significantly lower risk for LBW (OR 0.05, 95% CI: 0.00, 0.97 and OR: 0.02, 95% CI: 0.00, 0.60, respectively). No other significant associations were found between reported fatty acid intake at enrolment and LBW.

Table 6 presents the logistic regression analyses results of reported dietary n-6 and n-3 fatty acid intakes (above the median, with below the median as reference) at enrolment (<18 weeks of gestation) and premature birth as outcome. In fully adjusted models total n-6 PUFA, but not total n-3 PUFA, were associated with preterm birth. Women consuming above the median n-6 PUFA had an increased risk for preterm birth (OR: 5.03, 95% CI: 1.16, 21.82). No other associations between dietary fatty acid intake at enrolment and preterm birth were found.

Table 5: Associations of above-the-median dietary fatty acid intake at enrolment (<18 weeks of gestation) with low birth weight (binary logistic regression, odds ratios and 95% confidence intervals)

	Model 1 (n=192)			**Model 2** (n=155)		
Dietary fatty acid*	**OR**	**95% CI**	***p***	**OR**	**95% CI**	***p***
Total PUFA	0.52	(0.16, 1.67)	0.273	0.09	(0.01, 1.38)	0.083
n-6 PUFA	0.42	(0.13, 1.4)	0.158	0.05	(0.00, 0.97)	**0.047**
18:2n-6 (LA)	0.12	(0.03, 0.96)	**0.045**	0.02	(0.00, 0.60)	**0.025**
20:2n-6	2.14	(0.50, 9.12)	0.303	2.27	(0.24, 21.43)	0.475
20:3n-6 (DGLA)	3.37	(0.86, 13.24)	0.082	3.51	(0.39, 31.29)	0.262
20:4n-6 (AA)	0.49	(0.11, 2.20)	0.354	0.84	(0.12, 6.11)	0.862
n-3 PUFA	1.28	(0.41, 3.97)	0.666	1.38	(0.21, 9.27)	0.740
18:3n-3 (ALA)	3.04	(0.64, 14.41)	0.161	1.31	(0.08, 20.79)	0.850
20:5n-3 (EPA)	1.82	(0.20, 16.5)	0.594	0.89	(0.04, 22.27)	0.942
22:6n-3 (DHA)	0.43	(0.05, 4.11)	0.463	0.28	(0.01, 7.94)	0.456
n-6:n-3 PUFA	0.97	(0.32, 2.95)	0.963	0.77	(0.18, 3.25)	0.767
LA:ALA	1.45	(0.48, 4.40)	0.510	0.66	(0.15, 2.97)	0.590

Total n-6 PUFA and n-3 PUFA are the totals of all relevant fatty acids in the food composition data base.

PUFA, polyunsaturated fatty acid; LA, linoleic acid; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; ALA, α-linoleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LCPUFA, long-chain polyunsaturated fatty acid
Model 1 adjusted for maternal age, gestational age at birth and sex of the baby. Model 2 included the covariates of model 1 plus parity, socio-economic status, HIV status, maternal BMI at enrolment, glucose tolerance and total energy intake.
***** Dietary intakes of PUFA were categorised in two groups: below and above the median, with below the median group as reference.

Table 6: Associations of above-the-median dietary fatty acid intake at enrolment (<18 weeks of gestation) with premature birth (binary logistic regression, odds ratios and 95% confidence intervals)

	Model 1 (n=198)			**Model 2** (n=158)		
Dietary fatty acid*	**OR**	**95% CI**	***p***	**OR**	**95% CI**	***p***
Total PUFA	2.74	(1.07, 7.06)	**0.036**	3.23	(0.81, 12.95)	0.098
n-6 PUFA	3.38	(1.27, 9.04)	**0.015**	5.03	(1.16, 21.82)	**0.031**
18:2n-6 (LA)	2.33	(0.69, 7.90)	0.174	3.15	(0.57, 17.39)	0.187
20:2n-6	2.43	(0.74, 7.90)	0.142	6.16	(0.94, 40.27)	0.058
20:3n-6 (DGLA)	3.19	(0.92, 11.10)	0.069	4.55	(0.67, 30.67)	0.120
20:4n-6 (AA)	0.88	(0.25, 3.08)	0.845	0.76	(0.15, 3.92)	0.741
n-3 PUFA	1.86	(0.75, 4.64)	0.183	1.34	(0.33, 5.44)	0.686
18:3n-3 (ALA)	0.77	(0.234, 2.51)	0.662	0.58	(0.10, 3.27)	0.540
20:5n-3 (EPA)	2.02	(0.48, 8.60)	0.341	1.24	(0.18, 8.42)	0.824
22:6n-3 (DHA)	0.73	(0.17, 3.20)	0.677	0.52	(0.06, 4.18)	0.536
n-6:n-3 PUFA	0.97	(0.40, 2.34)	0.943	1.25	(0.42, 3.71)	0.686
LA:ALA	1.32	(0.55, 3.19)	0.535	1.36	(0.45, 4.08)	0.588

Total n-6 PUFA and n-3 PUFA are the totals of all relevant fatty acids in the food composition data base.

PUFA, polyunsaturated fatty acid; LA, linoleic acid; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; ALA, α-linoleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LCPUFA, long-chain polyunsaturated fatty acid
Model 1 adjusted for maternal age, sex of the baby and delivery intervention (induction or caesarean section). Model 2 included the covariates of model 1 plus parity, socio-economic status, HIV status, maternal BMI at enrolment, glucose tolerance and total energy intake.
* Dietary intakes of PUFA were categorised in two groups: below and above the median, with below the median group as reference.

3.6. Associations between red blood cell phospholipid fatty acid composition and birth outcomes

Table 7 presents the logistic regression results of RBC fatty acid composition (above the median, with below the median as reference) at enrolment (<18 weeks of gestation) and LBW as outcome. No significant associations were found between fatty acid composition at enrolment and LBW.

Table 8 presents the logistic regression analyses of RBC fatty acids (above the median, with below the median as reference) at enrolment (<18 weeks of gestation) and premature birth as outcome. There was a significant association between the ratio of AA:EPA and premature birth. Women with a higher AA:EPA ratio had an increased risk for premature birth (OR: 4.51, 95% CI: 1.29, 15.79, *p*=0.018). There were no other significant associations.

Table 7: Associations between maternal red blood cell fatty acid composition at enrolment (<18 weeks of gestation) and low birth weight (binary logistic regression, odds ratios and 95% confidence intervals)

	Model 1 (n=188)			**Model 2** (n=152)		
RBC Fatty acid*	**OR**	**95% CI**	***p***	**OR**	**95% CI**	***p***
n-6 PUFA	0.75	0.27, 2.31	0.622	1.89	(0.42, 8.48)	0.406
18:2n-6 (LA)	0.73	0.22, 2.41	0.610	0.65	(0.13, 3.12)	0.587
20:3n-6 (DGLA)	0.84	0.25, 2.88	0.782	1.04	(0.23, 4.65)	0.960
20:4n-6 (AA)	0.35	0.10, 1.23	0.100	0.48	(0.09, 2.52)	0.388
n-3 PUFA	0.91	0.28, 2.90	0.868	1.89	(0.37, 9.82)	0.447
18:3n-3 (ALA)	1.34	0.42, 4.32	0.623	0.86	(0.16, 4.51)	0.856
20:5n-3 (EPA)	0.79	0.22, 2.83	0.712	1.80	(0.33, 9.78)	0.494
22:6n-3 (DHA)	1.74	0.44, 6.82	0.430	2.43	(0.39, 15.15)	0.341
n-6:n-3 LCPUFA[&]	1.28	0.41, 4.06	0.671	1.65	(0.35, 7.80)	0.529
AA:EPA	1.87	0.58, 6.06	0.299	1.06	(0.25, 4.45)	0.932
AA:DHA	0.41	0.12, 1.37	0.146	0.48	(0.10, 2.25)	0.349

PUFA, polyunsaturated fatty acid; LA, linoleic acid; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; ALA, α-linoleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LCPUFA, long-chain polyunsaturated fatty acid; RBC, red blood cell

Model 1 adjusted for maternal age, gestational age at birth and sex of the baby. Model 2 included the covariates of model 1 plus parity, socio-economic status, HIV status, maternal BMI at enrolment and glucose tolerance.

* RBC PUFA were categorised in two groups: below and above the median, with below the median group as reference.

[&]n-6:n-3 LCPUFA ratio included fatty acids of 20 carbon chains and longer.

Table 8: Associations between maternal red blood cell fatty acid composition at enrolment (<18 weeks of gestation) and premature birth (binary logistic regression, odds ratios and 95% confidence intervals)

	Model 1 (n=194)			**Model 2** (n=155)		
RBC Fatty acid*	**OR**	**95% CI**	***p***	**OR**	**95% CI**	***p***
n-6 PUFA	0.69	(0.28, 1.70)	0.422	0.39	(0.12, 1.20)	0.099
18:2n-6 (LA)	0.58	(0.23, 1.52)	0.270	0.43	(0.12, 1.53)	0.191
20:3n-6 (DGLA)	2.55	(0.95, 6.89)	0.065	3.03	(0.91, 10.08)	0.071
20:4n-6 (AA)	1.72	(0.68, 4.37)	0.251	2.14	(0.68, 6.71)	0.192
n-3 PUFA	0.99	(0.41, 2.41)	0.983	1.12	(0.38, 3.27)	0.836
18:3n-3 (ALA)	1.98	(0.76, 5.12)	0.160	1.47	(0.45, 4.79)	0.519
20:5n-3 (EPA)	0.73	(0.26, 2.10)	0.563	0.37	(0.10, 1.41)	0.143
22:6n-3 (DHA)	0.81	(0.27, 2.39)	0.696	1.52	(0.38, 6.00)	0.551
n-6:n-3 LCPUFA[&]	1.96	(0.77, 4.95)	0.156	2.16	(0.67, 7.05)	0.200
AA:EPA	2.71	(1.04, 7.06)	**0.041**	4.51	(1.29, 15.79)	**0.018**
AA:DHA	1.69	(0.68, 4.21)	0.257	1.51	(0.50, 4.57)	0.469

PUFA, polyunsaturated fatty acid; LA, linoleic acid; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; ALA, α-linoleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LCPUFA, long-chain polyunsaturated fatty acid
Model 1 adjusted for maternal age, sex of the baby and delivery intervention (induction or caesarean section). Model 2 included the covariates of model 1 plus parity, socio-economic status, HIV status, maternal BMI at enrolment and glucose tolerance.
* RBC PUFA were categorised in two groups: below and above the median, with below the median group as reference.
[&]n-6:n-3 LCPUFA ratio included fatty acids of 20 carbon chains and longer.

4. DISCUSSION AND CONCLUSIONS

In this prospective study of generally healthy pregnant women in urban South Africa, we found higher n-6 PUFA dietary intakes early in pregnancy to be associated with increased risk for preterm birth. A higher RBC phospholipid AA:EPA ratio was also associated with an increased risk for preterm birth. Many studies have been conducted on maternal n-3 PUFA intake and status in association with gestational age at birth [7,8] with much less focus on n-6 PUFAs. In 37 women who delivered preterm compared to 34 controls in the USA, maternal RBC AA was higher among the preterm group [43]. The n-6 fatty acid pathway produces prostaglandins, which are known to be involved in the onset of parturition [44,45] which may explain this relationship. The women who delivered premature babies in our study, also reported significantly higher dietary intakes of protein, cholesterol and the n-6 fatty acids LA, 20:2n-6, DGLA and n-3 EPA and DPA. This may be reflective of diets high in meat and other animal-derived products including fish. Maternal dietary patterns high in meat, fried

chicken and fish and processed meats have been associated with preterm birth when compared to the well-known Dietary Approaches to Stop Hypertension (DASH) dietary pattern in a large cohort (n=3143) [46].

It is well known that the desaturase and elongase enzymes compete for the bioconversion of LA and ALA to their respective longer chain fatty acids in humans, although to a limited extend. The LCPUFA status is therefore partially dependent on the LA:ALA ratio consumed [45]. The women in this study reported consuming a high LA:ALA ratio at 39:1 (28:1-51:1). Equally high ratios have been reported by non-pregnant women in South Africa [23]. Other factors may influence the bioconversion of fatty acids, including the availability of trace elements, such as zinc and iron, acting as cofactors. Genetic polymorphisms in fatty acid desaturase (*FADS*) genes have also been demonstrated to affect this conversion since these genes control enzyme activity [47]. Further investigations are necessary to assess the role that these factors play in the RBC fatty acid composition of pregnant women in South Africa.

In contrast to our findings of high n-6 PUFA intake with preterm birth, we found that high dietary intakes of the n-6 fatty acid LA at early pregnancy was associated with a reduced risk for LBW. Literature on the relationship between maternal LA intake and offspring birth weight is limited and conflicting. There are studies showing no association between n-6 PUFA dietary intake and birth weight [48,49]. A supplementation trial of either n-3 or n-6 fatty acids (as cod liver or corn oil) in 341 women during pregnancy resulted in no differences in birth weight between the two groups [50]. However, in a Korean cohort of pregnant women (n=1407) high maternal n-6 fatty acid intake was negatively associated with birth weight [51]. When considering status, not intake, several studies have shown a negative association between n-6 PUFA status and birth weight [1,52,53]. To better understand this relationship, additional new-born anthropometrical measures and data on micronutrient status may be required. Birth weight alone is not providing insight on body composition of the offspring. We found no association between maternal n-3 fatty acid status and LBW. Although some studies have shown a positive association with birth weight [54], others have not [51]. There is, however, evidence that AA in early life has a growth-promoting effect [55]. Since high LA intakes may translate to AA, this may have possibly promoted foetal growth.

A recent systematic review of maternal PUFA status during pregnancy highlighted the relatively limited number of studies that have explored fatty acid profiles at different stages of pregnancy [56]. Our data therefore makes a valuable contribution to understand the changes in RBC fatty acids during pregnancy. Even though RBC ALA did not change with pregnancy progression, EPA and DPA significantly decreased. This could be interpreted that EPA and DPA were either converted to lipid mediators, or increasingly transferred to the foetus with pregnancy progression or used for conversion to DHA. However surprisingly, DHA only significantly decreased from early to mid-pregnancy, but not between mid- and late pregnancy [5.10% (4.48-5.97) to 5.08% (4.50-5.75) at <18wks and 22wks respectively]. The systematic review referred to earlier, confirmed that RBC DHA decreases with pregnancy progression [56]. This change suggests preferential transfer of DHA to the foetus towards the end of pregnancy. Since RBC DHA composition did not decrease late in pregnancy in our sample, it may raise the question on the effective transfer of DHA to the foetus in this context. Also, genetic data may provide insight if there is an upregulation of desaturases enzymes to produce more DHA in late pregnancy. The women in our study reported low intakes of EPA and DHA – less than the recommended 300 mg/day EPA+DHA during pregnancy (of which DHA should be 200 mg/day) [26,57]. This may have contributed to the different RBC composition compared to data reported in the literature.

This is the first study in South Africa describing the fatty acid dietary intakes at early pregnancy as well as describing fatty acid status with pregnancy progression. A comprehensive number of factors were assessed in this study, allowing to control for confounding factors. Even so, the study's findings should be interpreted with caution due to some limitations. Owing to the observational study design, conclusions on causation cannot be drawn. Dietary intake data collection methods also have limitations due to recall bias, incomplete recall etc. There were missing data which lowered the power for regression analyses. Our study sample was of relatively small size and not representative of the general population since a generally healthy population was recruited. There is the risk of self-selection biased, since the women were invited to the study with a booking date and could then volunteer to attend on the specific day.

In conclusion, we found that pregnant women in South Africa are consuming high amounts of LA which contributed to the high total n-6 PUFA intakes that were associated with an increased risk of preterm birth. These high LA intakes may have contributed to the high RBC AA:EPA ratio in early pregnancy which was also associated with an increased risk for preterm birth. On the other hand, high intakes of LA were associated with lower risk of LBW. It is unclear why this association was found. There is limited literature on maternal dietary LA and foetal growth. The RBC fatty acid composition of the women changed significantly with pregnancy progression. However, the expected reduction in the DHA proportion, reflective of DHA supply to the foetus, were not observed between mid- and late pregnancy in our sample. In the context of obesity, HIV infection and nutrition transition, dietary interventions to reduce excessive n-6 PUFA intake relative to n-3 PUFA intake and improve the fatty acid status in pregnant women, may have positive implications on birth outcomes and early development.

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6. DECLARATION OF INTEREST

None

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CHAPTER 6
SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Background

Maternal health during pregnancy influences the health of the offspring throughout the life cycle. The developmental origins of health and disease (DOHAD) concept has emerged over the past few decades. Research has demonstrated a link between perinatal and early childhood factors and the risk of non-communicable diseases (NCDs). It is therefore important to determine maternal predictors of birth outcomes and infant health in order to address population health challenges. South Africa has a rapidly increasing trend of NCDs and obesity in the adult population (Nojilana *et al.*, 2016), which may have been predisposed perinatally. For example, lower birth weight has been linked with increased risk for hypertension, glucose intolerance and hyperlipidaemia in later life (Kunz & King, 2007). In South Africa, approximately 15% of babies are born LBW (Pattinson & Rhoda, 2014) and approximately 12% are born preterm (Blencowe *et al.*, 2012). Addressing nutritional predictors of LBW and prematurity may influence long term health of South Africans.

Limited information exists on the nutritional status of pregnant women in South Africa. A systematic review on published literature between 2005-2015 described the micronutrient intake and status (iron, vitamin A, Iodine, folate and zinc) in women of reproductive age and pregnant women from four African countries, including South Africa (Harika *et al.*, 2017). A mere nine studies could be reviewed for South Africa over this period of time, of which only one reported on pregnant women (n=46) (Mostert *et al.*, 2005). This illustrates the need for further research on this topic.

Estimates indicate that 30% of pregnant women in South Africa are anaemic (Stevens *et al.*, 2013). Haemoglobin concentrations are assessed at least twice in pregnant women during antenatal visits in all public healthcare facilities (National Department of Health, 2015). However, the haemoglobin results are currently only used for clinical interpretation and are not electronically available for population-based studies. Even if the haemoglobin data were available, it would still not be an accurate portrayal of the iron status of pregnant women. Haemoglobin is not a sensitive marker for iron status and biochemical assessment of ferritin, sTfR and possibly other markers should be inspected to determine iron deficiency prevalence. Even regional studies on iron status in pregnancy are sparse. Most studies assess haemoglobin, not ferritin or other markers for iron, in pregnancy (Hoque *et al.*, 2007; National Department of Health (NDoH) *et al.*, 2019; Tunkyi & Moodley, 2015). It can be deducted that there is insufficient evidence to classify South Africa as a setting where anaemia is a severe public health problem amongst pregnant women. Even so, all pregnant women attending antenatal care at a public healthcare setting receive supplementation of ~60 mg elemental iron. The purpose of the supplementation is to prevent anaemia in pregnancy. Half of all anaemia cases are estimated to be due to iron deficiency (World Health Organization, 2017) and therefore other interventions are necessary to address non-iron related anaemia. It is not evident whether the antenatal iron supplementation programme in South Africa is effective in preventing anaemia. It is also not clear how universal iron supplementation affects iron replete pregnant women. Concerns

have been raised that iron supplementation in iron replete women may have detrimental effects (Brannon & Taylor, 2017; Daru *et al.*, 2017).

There are many complex physiological changes during pregnancy, including blood volume expansion (explaining in part the reduction in haemoglobin levels with pregnancy progression) as well as altered lipid metabolism (Larqué *et al.*, 2012). Lipids, specifically LCPUFAs, have been shown to be critical in foetal development. There are limited data on the fatty acid status of pregnant women in South Africa. However, regional dietary intake studies in women of child-bearing age have shown that diets are low in n-3 PUFA and high in n-6 PUFA (Ford *et al.*, 2016). Specifically n-3 PUFA have been shown to be important during the life course and are linked with health benefits, particularly mental and visual development in early life (Calder, 2016). Clinical trials with n-3 fatty acid supplementation during pregnancy have shown increased gestational length compared to controls, although the effect on birth weight seems smaller (Middleton *et al.*, 2018). The consequence of an unfavourable ratio of n-6 to n-3 fatty acid intake and status during pregnancy is unclear.

With this in mind, the central aim of this study was to assess dietary intake and status of iron and LCPUFA during pregnancy; and to determine associations of maternal iron and LCPUFA status throughout pregnancy with birth outcomes, in particular gestational age and birth weight, in pregnant women giving birth at Rahima Moosa Mother and Child Hospital (RMMCH) in Johannesburg. The following sections summarise the results found according to the objectives set for this study.

6.2 Summary of main findings

Objective 1: *to conceptualise, implement and execute the larger NuPED prospective cohort study in which the PhD project is nested.*

Chapter 3 is the written product of the objective achieved. Due to the identified gap in literature on the nutritional status and dietary intake during pregnancy in South Africa, a prospective study design was required to answer the objectives set and to add to existing literature. Deliberations regarding the study concept started in 2014 and were formally structured in 2015 when concrete discussions with clinical management were held at the proposed research site. Ethical clearance and permissions to conduct the study were obtained in late 2015. Participant recruitment started in early March 2016 at four of the sixteen referral primary healthcare clinics for RMMCH, which are all situated in either Region B or C in the City of Johannesburg (see Figure 6.1 below for the map).

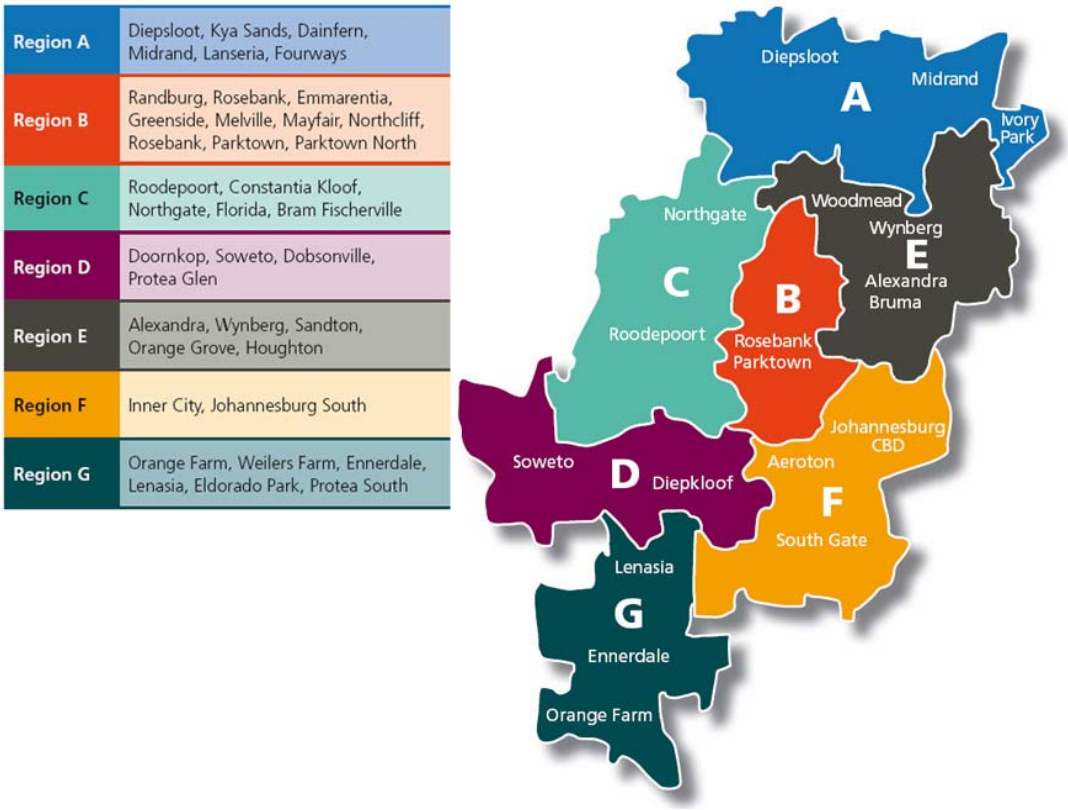

Figure 6.1: The seven regions of the City of Johannesburg
(obtained from: http://www.htxt.co.za/wp-content/uploads/2014/06/jra-map.jpg)

Participant recruitment took place on the days scheduled for first antenatal bookings at the primary healthcare facilities. Recruitment continued until November 2017 and 1340 women were screened for invitation, 595 invited to the study at RMMCH and 313 women volunteered and arrived at RMMCH. Numerous women could not be invited to the study since the aim was to follow women from early pregnancy (<18 weeks of gestation) and many women presented late at the first booking. Additionally, many healthcare facilities in Johannesburg serve a predominantly migrant population which is not ideal for participant retention in a prospective study design. The recruitment period was therefore prolonged.

There were eight data collection time points for the larger study, while data from four time points were utilised for the purposes of the current PhD study. Confirmation of gestational age at first study visit allowed for calculation of the date for the follow-up visits which were scheduled around ±22 weeks and ±36 weeks of gestation. Trained fieldworkers phoned participants before follow-up visits as reminders. Midwives were appointed and trained to follow-up on women who were in labour and those who underwent caesarean section or induction. Birth data collection were completed in June 2018. Figure 2 in Chapter 3 summarises the data collected at each study visit. Data collection in a

public clinical setting, without a permanent research set-up, required navigation of challenges. Good relationships with clinical staff and fieldworkers assisted in this process.

The execution of this project resulted in a rich and unique database on pregnant women (and children) from an urban South African setting which will provide the basis and context for possible intervention studies.

Objective 2: *to describe the dietary iron intake at early pregnancy, as well as the iron status at early, mid- and late pregnancy and to determine associations of iron status with both birth weight and gestational age.*

The results of objective 2 are presented in Chapter 4. We showed that in early pregnancy, iron intake from foods alone was lower than the EAR during pregnancy (19 vs 22 mg/day). This intake is, however, meeting the recommendations for non-pregnant women (18 mg/day) and therefore many women may have reached their iron requirements before and during early pregnancy. Iron requirements in early pregnancy is low due to cessation of menses and requirements increase markedly from the second trimester due to higher erythropoietic activity (Bothwell, 2000). Universal iron supplementation of ~55 mg of elemental iron per day (supplied as 170 mg dried ferrous sulphate) was provided to women at the research setting as part of routine care. Our sample reported high compliance with the supplementation.

We determined that 28% and 15% of the women were anaemic and iron deficient, respectively, at study entry. We demonstrated that iron status declined with pregnancy progression despite routine iron supplementation. Anaemia, ID and IDE prevalence increased during pregnancy. We postulate that the high inflammatory status among these women (60%) could have contributed to increased hepcidin concentrations, resulting in poor iron absorption.

Lastly, we demonstrated that iron deficiency at mid-pregnancy and IDE in late pregnancy were associated with delivering higher birth weight babies. In contrast, IDE women at 22 weeks had an increased risk for preterm birth. Lower haemoglobin at early pregnancy was associated with shorter gestation. This implies that women with higher iron status gave birth to smaller babies over a longer gestational period. Although it is recognised that iron deficiency should be prevented and treated, these results suggest further investigation on the implications of iron supplementation of iron replete pregnant women and revisiting of current supplementation regimes.

Objective 3: *to describe the dietary LCPUFA intake at early pregnancy, as well as the LCPUFA status at early, mid- and late pregnancy and to determine associations of LCPUFA status at early pregnancy with birth weight and gestational age.*

In Chapter 5, we demonstrated that women consumed high amounts of n-6 LA (higher than recommended intakes), although the percentage contribution towards total energy were low due to relatively high total energy intakes. On the other hand, n-3 fatty acid intakes were reported to be very low, resulting in a high LA:ALA intake ratio of 39:1. Women who ultimately delivered preterm babies, reported higher intake of protein, cholesterol, some n-6 PUFAs as well as some n-3 PUFAs when compared to those delivering at term. Even so, there were no significant differences in RBC fatty acid composition at early pregnancy in women who ultimately delivered LBW babies compared to those who delivered normal weight babies. Similarly, no differences were found in RBC fatty acid composition in those who delivered prematurely compared to those who delivered at term.

RBC fatty acid composition changed significantly during pregnancy. It was expected to see a significant reduction in RBC DHA by late pregnancy, however, in our sample RBC DHA did not change between 22 and 36 weeks of gestation.

Regression analyses revealed that higher dietary intakes of n-6 PUFA early in pregnancy were associated with an increased risk of delivering prematurely. Likewise, a higher RBC AA:EPA ratio at early pregnancy was also associated with increased risk for preterm birth. In contrast, we found that women who consumed higher n-6 LA had a lower risk of delivering LBW babies.

A diagrammatic illustration of the results from this PhD study is supplied in Figure 6.2 below.

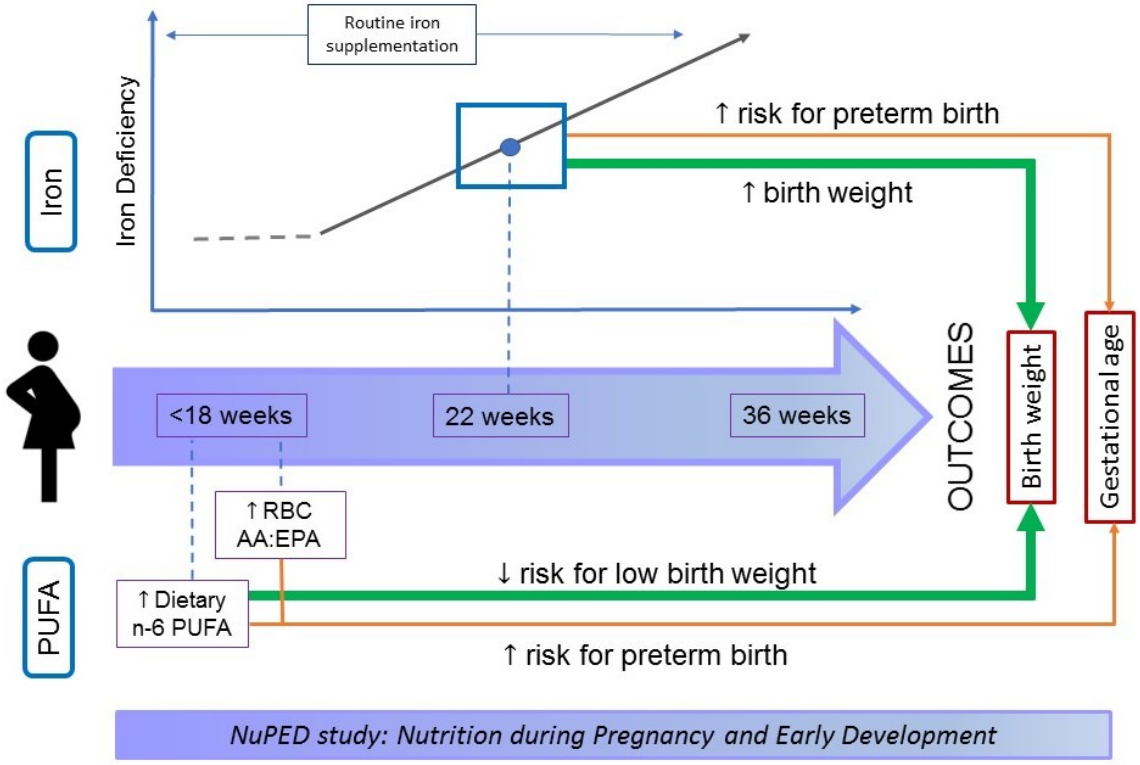

Figure 6.2: Diagrammatic illustration of the PhD study results

6.3 Strengths, limitations, chance and confounding

The key strength of this study was that data were collected prospectively with multiple variables and data collection points over pregnancy. Where we described iron status, the iron biomarkers assessed allowed for a complete description of iron status and were adjusted for inflammation and altitude where applicable. Furthermore, we investigated dietary intake as well as nutrient status which allowed for interpretation of observations. The analyses were strengthened due to assessment and inclusion of several confounders. The application of models with different sets of covariates assisted in reducing the possibility of results being obtained by chance. Although several confounding factors were accounted for, it should be appreciated that all possible confounding factors could not have been assessed and included.

General lifestyle factors such as physical activity, sleeping patterns, water consumption, caffeine consumption and emotional well-being may all play a role in maternal and foetal health (Pei *et al.*, 2015). The matter of homogeneity was therefore considered when determining the population for this study. We therefore applied inclusion and exclusion criteria to draw a sample of generally healthy pregnant women from this urban area of Johannesburg. We excluded women who smoke, however, we did not assess the use of snuff (smokeless tobacco inhaled through the nasal cavity) or e-cigarettes. The use of snuff during pregnancy in South Africa has not shown to be associated with low birth weight, while cigarette smoking has been associated. Even so, the snuff users, had a shorter gestation (Steyn *et al.*, 2006). Excluding smokers from our study may have introduced bias in the results, since non-smokers typically may have healthier lifestyles. However, only approximately 6% of pregnant women in peripheral areas of Johannesburg smoke (Steyn *et al.*, 2006), therefore, exclusion should not have affected generalisability to a large extend.

The study limitations should be considered when interpreting the results. Due to the observational study design, conclusions on causality of observed relationships are not possible. An additional limitation is possible self-selection bias at recruitment since women were recruited at primary healthcare clinics and then volunteered and agreed to participate at a different setting. Lastly, the sample was of relatively small size and not representative of the general population. Considering the relatively low prevalence of LBW and preterm birth, larger cohorts will allow for better power in statistical analyses. Women included in the study presented at primary healthcare clinics somewhat earlier than the typical 20 weeks of gestation (Smith, 2016) and may have different lifestyles to those who present later at first booking.

6.4 Recommendations for future work

- Further research is needed to assess the effectiveness and possible risks of iron supplementation in iron deficient and iron replete pregnant women, respectively.
- Further research is needed to investigate strategies to improve dietary fatty acid consumption before and during pregnancy. This may include development of dietary guidelines for pregnancy in South Africa. Additionally, n-3 fatty acids should be considered in the supplementation regime.
- Having some background on the iron and fatty acid status and intake of pregnant women in this population, further research is necessary to determine if interactions between these nutrients have an influence on maternal and birth outcomes.
- Governmental health authorities should invest in the above-mentioned research at national level to allow for maternal care guidelines and supplementation regimes to be revisited within the unique South African context (high prevalence of overweight, HIV, TB and NCDs in nutrition transition).
- Improved nutritional health monitoring before and during pregnancy should be considered in public healthcare to address nutritional deficiencies or imbalances and causes of inflammation.

6.5 Conclusion

Our results are unique and shed light on the iron and fatty acid status of pregnant women in South Africa at several time points within the context of routine iron supplementation. Anaemia and iron deficiency increased with pregnancy progression despite routine iron supplementation. Noteworthy, iron deficiency at mid-pregnancy was associated with higher birth weight and increased risk of preterm birth. Similar results were found for women who consumed higher amounts of n-6 PUFA at early pregnancy, namely increased risk for preterm birth and decreased risk for low birth weight. It is prevalent that the women consumed higher than the recommended n-6 LA and lower than the recommended n-3 ALA and DHA.

In the context of an urban environment, the generally healthy pregnant women presented with a high prevalence of overweight and obesity and inflammation. Even though the sample was considered to be low-risk pregnancies, 14% of the babies were born low birth weight and 11% prematurely. It is important to identify and address nutritional factors that may lower these statistics to improve the health of future generations in South Africa.

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ANNEXURES

Annexure 1: Ethical approval – NWU HREC

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

Private Bag X6001, Potchefstroom
South Africa 2520

Tel: 018 299-1111/2222
Web: http://www.nwu.ac.za

Ethics Office
Tel: 018-299 2092
Fax: 018-299 2088
Email: Minrie.Greeff@nwu.ac.za

25 January 2016

Prof CM Smuts
Nutrition

Dear Prof Smuts

HREC APPROVAL OF YOUR APPLICATION

Ethics number: NWU-00186-15-A1 Nutrition during Pregnancy and Early Development: the NuPED study

Kindly use the ethics reference number provided above in all correspondence or documents submitted to the Health Research Ethics Committee (HREC) secretariat.

Project title: Intake and status of fatty acids and iron during pregnancy in association with birth outcomes among urban South African women

Project leader/supervisor: Prof CM Smuts

Student: EA Symington

Application type: Sub-study

Risk level descriptor: Medium

You are kindly informed that at the meeting held on 19/11/2015 of the HREC, Faculty of Health Sciences, the aforementioned was approved.

The period of approval for this project is from 25/01/2016 to 20/11/2016.

After ethical review:

Translation of the informed consent document to the languages applicable to the study participants should be submitted to the HREC (if applicable).

The HREC requires immediate reporting of any aspects that warrants a change of ethical approval. Any amendments, extensions or other modifications to the protocol or other associated documentation must be submitted to the HREC prior to implementing these changes. Any adverse/unexpected/unforeseen events or incidents must be reported on either an adverse event report form or incident report form.

A progress report should be submitted within one year of approval of this study and before the year has expired, to ensure timely renewal of the study. A final report must be provided at completion of the study or the HREC must be notified if the study is temporarily suspended

or terminated. The progress report template is obtainable from Carolien van Zyl at Carolien.VanZyl@nwu.ac.za. Annually a number of projects may be randomly selected for an external audit.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process.

Please note that for any research at governmental or private institutions, permission must still be obtained from relevant authorities and provided to the HREC. Ethics approval is required BEFORE approval can be obtained from these authorities.

The HREC complies with the South African National Health Act 61 (2003), the regulations on Research with Human Participants of 2014 of the Department of Health and Principles, the Declaration of Helsinki, 2013, the Belmont Report and the Ethics in Health Research: Principles, Structures and Processes (SANS document).

We wish you the best as you conduct your research. If you have any questions or need further assistance, please contact the Ethics Office at Carolien.VanZyl@nwu.ac.za or 018 299 1206.

Yours sincerely

Prof Minrie Greeff
HREC Chairperson

Current details: (13210572) C:\Users\13210572\Documents\HREC\HREC - Applications\2015 Applications\Applications 10 - 19 November 2015\NWU-00186-15-A1 (CM Smuts-EA Symington)\NWU-00186-15-A1 (CM Smuts-EA Symington) - AL\NWU-00186-15-A1 (CM Smuts-EA Symington) - AL.docm
26 January 2016

File reference: 9.1.5.3

Annexure 2: Ethical approval – University of Witwatersrand HREC (Medical)

R14/49 Prof Marius Smuts et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M150968

NAME: (Principal Investigator)	Prof Marius Smuts et al
DEPARTMENT:	Centre of Excellence for Nutrition University of the Witwatersrand and North West University Region B and C, City of Johannesburg Florida Clinic, Bosmont Clinic, Sophiatown and Rahima Moosa Mother and Child Hospital
PROJECT TITLE:	Nutrition during Pregnancy and Early Development: The NuPED Study
DATE CONSIDERED:	02/10/2015
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	
APPROVED BY:	Professor P Cleaton-Jones,Chairperson, HREC (Medical)
DATE OF APPROVAL:	08/02/2016

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004, 10th floor, Senate House/2nd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature Date 18/02/2016

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Annexure 3: Permission Letter from Gauteng Department of Health

GAUTENG PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

OUTCOME OF PROVINCIAL PROTOCOL REVIEW COMMITTEE (PPRC)

Researcher's Name (Principal investigator)	Prof. Marius Smuts
Organization / Institution	Centre of Excellence for Nutrition: North West University
Research Title	Nutrition During Pregnancy and Early Development (NuPED) study
Contact number	Address: N/A Contact no: 018 299 2086 Cell: Email: Marius.Smuts@nwu.ac.za
Protocol number	GP2015RP 38 473
Date submitted	26/10/2015
Date reviewed	26/11/2015
Outcome	Approved

It is a pleasure to inform you that the Gauteng Health Department has approved your research on "Nutrition During Pregnancy and Early Development (NuPED) study"

Study sites: JHB Metro, Rahima Moosa Hospital, Florida, Bosmont, Sophiatown and Zandspruit Clinics.

The Provincial Protocol Review Committee kindly requests that you to submit a report after completion of your study and present your findings to the Gauteng Health Department.

Recommended/~~Not Recommended~~

Dr. B. Ikalafeng
(on behalf of the PPRC)

Date: 27/11/2015

Approved/Not approved

Dr. LRR. Lebethe
Acting DDG: Clinical Service

Date: 03/12/15

Annexure 4: Permission Letter from City of Johannesburg District Research Committee

GAUTENG PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

Joburg
a world class African city

JOHANNESBURG HEALTH DISTRICT

Enquiries:
Hillbrow CHC Administration Building, Klein Street
Hillbrow, Johannesburg
E-mail:
CoralF@joburg.org.za
Johannesburg.research@gmail.com

10 December 2015

Professor Marius Smuts
Nutrition
Potchefstroom Campus
E-mail: Marius.Smuts@nwu.ac.za

Dear Professor Smuts,

Re: ***Nutrition in Pregnancy and Early Development: The NuPED Study***

Your application dated 8 December 2015 refers. The District Research Committee has reviewed your application. This letter serves as an in-principle approval to access the Districts Health facilities (mentioned below) for the above project subject to following conditions:

- The facility to be visited: Florida, Bosmont, Sophiatown and Zandspruit Clinics
- The research can only commence after you submit an ethics clearance certificate from a recognized institution.
- Please contact the relevant RHDD prior to your visit to the facilities

Region	Regional Health Manager	Contact No.	Cell phone
B	Ms Paulinah Maepa	011 718 9656	082 551 5804
C	Mr. Tebogo Motsepe	011 761 0248	083 421 9405

- You will report to the Facility Manager before initiating the study.
- Participants' rights and confidentiality will be maintained all the time.
- No resources (Financial, material and human resources) from the above facilities will be used for the study. Neither the District nor the facility will incur any additional cost for this study.
- The study will comply with Publicly Financed Research and Development Act, 2008 (Act 51 of 2008) and its related Regulations.
- You will submit a copy (electronic and hard copy) of your final report. In addition, you will submit a six-monthly progress report to the District Research Committee. Your supervisor and University of South Africa will ensure that these reports are being submitted timeously to the District Research Committee.
- The District must be acknowledged in all the reports/publications generated from the research and a copy of these reports/publications must be submitted to the District Research Committee.

We reserve our right to withdraw our approval, if you breach any of the conditions mentioned above.

Please feel free to contact us, if you have any further queries. On behalf of the District Research Committee, we would like to thank you for choosing our District to conduct such an important study.

Regards,

Dr R Bismilla
Executive Director
City of Johannesburg
Date: 10/12/15

Annexure 5: Permission letter from the clinical Manager of Rahima Moosa Mother and Child Hospital

GAUTENG PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

RAHIMA MOOSA MOTHER AND CHILD HOSPITAL

RAHIMA MOOSA MOTHER AND CHILD HOSPITAL
Enquiries: Dr E Hank
Tel: (011) 470 9030/9031
Fax: (011) 477 4117
Email : Edward.Hank@gauteng.gov.za

Ms Elize Symington

Centre of Excellence for Nutrition

North-West University (Potchefstroom Campus)

6 July 2015

Re: Nutrition in Pregnancy and Early Development: The NuPED study

Dear Ms Symington

Permission is granted for you to conduct the research as indicated in your request as per the title above.

The terms under which this permission is granted is contained in the Researcher Declaration form that you signed. Failure to comply with these conditions will result in the withdrawal of such permission.

Note that it is imperative that you notify the hospital of the actual start and end dates of your study by notifying Karen Marshall by email (Karen.Marshall@wits.ac.za).

Should the study commence more than 12 months from receipt of this letter then the Researcher Declaratio form needs to be re-signed prior to commencement of the research. Your are strongly advised to keep a signed copy of the declaration form so as to ensure that the terms of this agreement are complied with at all times.

Yours sincerely,

Clinical Manager

ADDRESS: cnr. FUEL & OUDSTHOORN STREET CORONATIONVILLE 2093 / PRIVATE BAG X20 NEWCLARE 2112 JHB

Annexure 6: Permission letter from the Head of Department: Obstetrics and Gynaecology

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT

To: Head of Departm ent Obstetrics and Gynaecology
Rahima Moosa M other and Child Hospital

From: Prof Marius Smu s, Project Head
Centre of Excelle ce for Nutrition,N orth-West University, Potchefstroom
018 299 2086; M rius.Smuts@nwu.ac.za

3 September 2015

Permission letter for c nducting research in the Department of Obstetrics and Gynaecology

Nutrition du ing Pregnancy and Early Development: The NuPED study

With reference to previo s conversations and the attached permission letter by Dr E Hank, clinical manager of the hospital we are kindly requesting your permission for conducting the research project in your departme l.

The aim of the research project is to assess dietary intake and nutritional status of urban South African pregnant women and to determine associations with birth outcomes, maternal health and offspring health. Using a ongitudinal observational research design, pregnant women (<16 weeks gestation) (min. n=250) will be recruited from primary healthcare clinics in Johannesburg and followed up at RMMCH. Dietary intake and nutrient status will be assessed at <16, 24 and 36 weeks gestation. At birth, maternal and neonatal health will be assessed. The following data will be obtained from medical re ords in your department:

- Medical history (p arity; gravity, previously or currently diagnosed hypertension, diabetes, TB and HIV; ba terial vaginosis; early symptoms of pregnancy such as nausea and vomiting; smoking number of births, etc.)
- Blood pressure
- Ultrasound screen data
- Glucose tolerance test results

Kindly note that nursing staff will draw blood samples (additional tubes to be supplied by the research team) and anal sed by external laboratories. Urine samples will be collected as per standard operating proced ures, however, fieldworkers will take aliquots for research purposes.

The research team (tr ined fieldworkers) will be obtaining the socio-demographic data, anthropometrical measure ments, diet history and general health questionnaires.

On behalf of the research eam, we are herewith requesting your permission for conducting the research project in your de partment and obtaining the abovementioned data.

Prof Marius Smuts

Head: Department O&G

APPROVED
Outcome

29.9.15
Date

Annexure 7: Informed consent form

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

Health Research Ethics Committee
Faculty of Health Sciences
NORTH-WEST University
(Potchefstroom Campus)
2016 -04- 26

HREC Stamp

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM FOR ASSESSMENTS DURING PREGNANCY AND BIRTH

TITLE OF THE RESEARCH PROJECT:
Nutrition during Pregnancy and Early Development: The NuPED study

REFERENCE NUMBERS: *NWU-00186-15-S1; M150968*

PRINCIPAL INVESTIGATOR: *Prof Marius Smuts*

ADDRESS: *School of Physiology, Nutrition and Consumer Sciences, Potchefstroom Campus, Building G16, Room 157*

CONTACT NUMBER: *018 299 2086 / 082 451 0486*

Good day

You are invited to take part in a research project. Please take some time to read the information about this project. Please ask the researcher any questions if you do not fully understand. Your participation is ***entirely voluntary*** *and you are free to say no. If you say no, this will not affect you negatively in any way. You are also free to withdraw from the study at any point, even if you agreed to take part at first.*

This study has been approved by the ***Health Research Ethics Committee of the Faculty of Health Sciences of the North-West University (NWU-00186-15-S1) and the Human Research Ethics Committee of the University of Witwatersrand (M150968)*** *and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki and the ethical guidelines of the National Health Research Ethics Council. It might be necessary for the research ethics committee members or relevant authorities to inspect the research records. This study will be used by several students to obtain further academic qualifications.*

What is this research study all about?

A good diet during pregnancy is important for the healthy growth of the baby. A mother's diet can affect her baby's health. It is not very clear how different eating habits affect the baby's health.

The goals of this research are to describe the food intake and health of pregnant women throughout pregnancy and to determine if it relates to the babies' health.

Therefore, we would like to measure your diet (food intake) and health at early pregnancy, mid-pregnancy and late pregnancy. When you give birth, we will also measure how healthy your baby is. We may approach you after the study to further investigate the development and well-being of your baby, but then we will ask you to provide new consent. At least two hundred and fifty women will be included in this study.

Some of the blood that we will collect from you and the cord between you and your baby after birth will be used to look at some factors that you inherited from your parents and your baby inherited from you (genetic factors, such as DNA and RNA). Genetic factors are like a manual that tells your body how to work. Sometimes there are differences or changes that cause people to react differently to nutrients. We want to investigate these genetic changes to better understand how this works. We promise that all genetic tests and experiments will only focus on genetic factors to do with nutrient usage in the body and related to your and your baby's health.

Why have you been invited to participate?

You have been invited to take part because you are attending the antenatal clinic today.

You have also complied with the following inclusion criteria:

- *You are a pregnant woman, born in South Africa, Lesotho, Swaziland, Zimbabwe, Botswana or Namibia.*
- *You are planning to deliver your baby at Rahima Moosa Mother and Child Hospital.*
- *You are able to communicate effectively in English, Afrikaans, Sotho, Xhosa or Zulu.*

You will be excluded if you:

- *Are more than 18 weeks pregnant since we need to know your status in early pregnancy.*
- *Are younger than 18 years of age or older than 39 years of age since age may influence the health of the baby.*
- *Are stating that you are using illicit drugs since this may influence the growth of the baby.*
- *Are carrying a multiple pregnancy, such as twins or triplets, since these babies are usually born smaller.*
- *Have a known lifestyle disease such as diabetes, kidney disease, high blood cholesterol or high blood pressure or using medication for any of these, since this may influence the health of the baby.*
- *Have a known infectious disease such as tuberculosis or hepatitis or using medication for any of these, since this may influence the health of the baby.*
- *Have a known serious illness such as cancer, lupus or psychosis or using medication for any of these, since this may influence the health of the baby.*
- *Are a smoker, or have been smoking in the past year since this influences the growth of the baby.*
- *Note: You will not be excluded from the study if you have HIV, but will be asked if we can include your HIV status in our data.*

What will your responsibilities be?

If you agree to take part in the study, you will be expected to:

- *From here on have all your antenatal visits at Rahima Moosa Mother and Child hospital and not here at the clinic. You will be refunded for your travelling costs.*
- *Attend Rahima Moosa Mother and Child hospital next week, at 22 weeks pregnancy and at 36 weeks pregnancy for this research. The doctors or nurses may request you to attend other days as well for other medical reasons. Dates will be given to you for each visit.*
- *Answer questions about your age, education and living conditions only today.*
- *Answer questions about your diet and supplement use at each visit, as well as phone calls before and after the following visits.*
- *Answer questions about your general health, mood, allergy symptoms and medication usage at each visit.*
- *Indicate on a checklist how healthy you feel every day.*
- *Let us do some body measurements at each visit. We will only measure your weight by asking you to stand on a scale, your standing height against a height measure and your upper arm circumference with a tape measure.*
- *Get an ultrasound screen at each scheduled visit to the hospital.*
- *Give a urine sample at each visit.*
- *Give a blood sample at each visit. A total amount of 42ml (about three tablespoons full) will be drawn from your arm.*
- *Let us take your blood pressure at each visit.*
- *Do a diabetes test at around 24 weeks pregnancy. You have to fast from 10pm the previous night. The next morning you will be asked to drink a sweet drink at the hospital laboratory. Your blood sugar levels will be tested several times.*
- *Go to Rahima Moosa Mother and Child Hospital admissions when you feel labour pains. The nurse will then do some body measurements if possible.*
- *Allow us to take some body measurements of your newborn baby, such as weight, height and head circumference.*
- *Allow us to take some blood of the cord between you and your baby after the baby has been born and after the cord has been cut.*
- *Allow us to use your and your baby's medical records to check your health.*

Will you benefit from taking part in this research?

The direct benefits for you as a participant will be that you will receive the normal medical care from a gynaecologist and hospital staff. You will receive additional medical tests, such as an ultrasound screen and diabetes test. These services are not available at the clinic. You will receive immediate feedback on the measurements where results are available on the same day, such as blood pressure and the ultrasound screen. If there are any concerns, you can discuss this with the nurse or other medical professions. They can support you with the appropriate medical care.

The indirect benefit will be that you help us understand the dietary habits and health of pregnant women in South Africa and how that affects the health of their babies. By understanding more about this, we can help government to create policies that can address the health of South African pregnant women better.

Are there risks involved in you taking part in this research?
Most of the measurements that will be performed won't harm or hurt you in any way, but you might experience the following:

1. *If you give permission to a blood sample, you might feel uncomfortable or scared. This will only last for a short while. We want to make sure that you are not hurt in any way and therefore the qualified professional will draw the blood from your arm. She will talk to you and explain to you everything that she is going to do.*
2. *You may be concerned that the researchers will be testing your HIV status. The research team will not test your blood for HIV. The clinic nurse may test your blood for HIV as part of routine antenatal care. We do ask you permission that we get the result of this test which is transferred to your study number, thus it is anonymously used further on.*
3. *During the body measurements you will be asked to remove some of your clothes keeping on only your underwear or light clothing. This might make you feel uncomfortable or shy. To help you feel less shy and uncomfortable, only females will take these measurements. Also, the area where these measurements will be done will be private and closed off. This means that no one else will be able to see you. Only the person that will take the measurements and someone to help her will be with you.*
4. *When an ultrasound screen of your baby is taken, a clear gel will be squirted onto your belly. This will feel cold, but can do no harm. The medical professional conducting the ultrasound screen will talk you through the process.*
5. *Being part of such a big research study can be frightening and overwhelming. To prevent us from wasting your time and to make sure that you know where to go and what to do, there will be people available at all times to help you and show you where you have to go every time.*
6. *For the diabetes test, you will be asked not to eat or drink anything from 22:00 (ten o'clock) the night before. You will only be allowed to drink water. You should also not eat any breakfast on the morning of the study and not drink coffee, tea, juice or cold drink. Not eating or drinking anything might make you feel uncomfortable or light headed (dizzy or faint). When you arrive at around 7:00 on the day of your booking, the laboratory staff will give you a sweet sugary drink. This may taste too sweet for you, so the laboratory staff will give you diluted lemon juice to combat the sweetness. Your blood sugar levels will be tested first with a finger prick and by drawing about 3 ml blood from your arm at 1 and 2 hours after drinking the sugar drink. You will be provided with a food parcel to eat after the test.*
7. *Doing all of the measurements on the days of the research study, will take most of the day. This might make you feel very tired. You will be provided with refreshments to eat and drink during the day.*
8. *It is important that you indicate whether you have any food allergies. This will help the research team when providing meals to participants on research days.*

There are more benefits than dangers or risks when you take part in the study.

What will happen in the unlikely event of some harm/form of discomfort occurring as a direct result of you taking part in this research study?
Please let us know if you experience any physical or emotional discomfort during or after participating in the study and we will make appropriate arrangements for you to talk to a medical doctor or psychologist.

Who will have access to the data?
We will handle all your information as confidential as possible by allocating a study code to you and your baby when he/she is born. All samples will be labelled with this code and only the principal investigator and co-principal investigator will have access to the records containing your name. Only the researchers will work with your data. Data will be kept safe and secure by locking hard copies in locked cabinets at the clinic, until your baby is born. Thereafter, these documents will be kept secure in locked cabinets in the researcher's office and for electronic data it will be password protected. Reporting of findings will be anonymous.

What will happen with the data/samples?
Blood samples that will be sent overseas for laboratory analysis will be destroyed once all the pre-defined analyses have been completed. Blood and urine samples being analysed at North-West University will be stored for 7 years after completion of the study. Data will be stored for 15 years. There is the possibility that blood samples and data might be analysed by other researchers over time for the purpose as explained to you.
There is enough money to do the study and perform the most important analyses but some of the tests are very expensive and will only be done once more funding is obtained.

Will you be paid to take part in this study and are there any costs involved?
No, you will not be paid to take part in the study but your expenses for travelling to Rahima Moosa Mother and Child Hospital will be paid for study visits at <18, 22 and 36 weeks. At each visit you will receive a R5 cell phone voucher to enable you to make a call to the researchers or fieldworkers if you need to. Furthermore, you will be provided with a snack/lunch pack every two hours during assessments at <18, 22 and 36 weeks. You will receive a gift hamper to a value of R150 with goods for your baby as a token of appreciation.
Thus, if you take part there will be no costs involved for you.

Is there anything else that you should know or do?
- *You can contact Prof. Marius Smuts at 018 299 2086 / 082 451 0486 or Elize Symington at 072 218 2184 if you have any further queries or encounter any problems.*
- *You can contact the Health Research Ethics Committee of North-West University via Mrs Carolien van Zyl at 018 299 2089; carolien.vanzyl@nwu.ac.za if you have any concerns or complaints that have not been adequately addressed by the researcher.*
- *You can also contact the Health Research Ethics Committee of the University of Witwatersrand via:*
  - *Prof Peter Cleaton-Jones, Chairperson of HREC (Medical) Tel: 011 717 2301 Email: peter.cleaton-jones1@wits.ac.za*
  - *Ms Zanele Ndlovu, HREC (Medical) Secretariat, Tel: 011 717 1252/2700/ 1234, Email: Zanele.ndlovu@wits.ac.za*
- *You will receive a copy of this information and consent form for your own records.*

How will you know about the findings?
We will give you immediate feedback of results that we determine during the study, such as blood pressure and diabetes tests. However, take note that it will take time to perform the other analyses and that the results will only be available after several months. Once the study is completed and all the results are available, we will distribute the information to the clinics where you will attend for baby clinics. Should we find an abnormal value during our analyses that needs medical attention, we will inform you and the medical staff immediately for the necessary medical treatment.

Few questions (*to be completed by the person obtaining the consent*):

Did the participant understand the following questions?

	YES	NO
If you take part in the study, where will your follow-up antenatal visits be done?		
Will taking part in the study cost you any money?		
If you take part in the study, from where will we take blood from your new born baby?		

Declaration by participant

By signing below, I …….. agree to take part in a research study entitled: *Nutrition during Pregnancy and Early Development: The NuPED study.* I declare that:

- I have read this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.
- I understand and agree that blood samples from me and from the umbilical cord may be sent outside South Africa for laboratory analysis.

Do you have any food allergies?

No ☐

Yes ☐ Which allergies? _____

Do you give permission that some of your blood samples may be analysed outside of South Africa?

Yes, I give permission ☐

No, I don't give permission ☐

Do you give permission that we may collect your genetic material?

Yes, I give permission to collect my genetic material ☐

No, I don't give permission to collect my genetic material ☐

Do you give permission that we may collect your baby's genetic material from the cord blood?

Yes, I give permission to collect my baby's genetic material ☐

No, I don't give permission to collect my baby's genetic material ☐

Do you give permission that the researchers have access to your HIV test results from the clinic?

Yes, I give permission ☐

No, I don't give permission ☐

Do you give permission to have access to your and your baby's medical records at hospital or clinic?

Yes, I give permission ☐

No, I don't give permission ☐

Do you give permission for the researchers to contact you after the birth of your baby for possible follow-up tests?

Yes, I give permission ☐

No, I don't give permission ☐

Do you give permission that other researchers use the blood samples and data at a later stage?

Yes, I give permission ☐

No, I don't give permission ☐

Signed at (*place*) ..... on (*date*) ..... 20....

....

Signature of participant **Signature of witness**

Declaration by person obtaining consent:

I *(name)* …… declare that:

- I explained the information in this document to ……
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use an interpreter.

Signed at (*place*) ……. on (*date*) …… 20....

.....	.....
Signature of investigator/fieldworker	**Signature of witness**

.....
Signature of researcher

Annexure 8: Published format of Chapter 3

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BMC Pregnancy and Childbirth

STUDY PROTOCOL Open Access

Nutrition during pregnancy and early development (NuPED) in urban South Africa: a study protocol for a prospective cohort

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Abstract

Background: Adequate nutrition during pregnancy is important to ensure optimal birth outcomes, maternal health and offspring development. However, little is known about the dietary intake and nutritional status of pregnant women residing in urban South Africa. Therefore, the Nutrition during Pregnancy and Early Development (NuPED) cohort study was initiated to assess early nutrition-related exposures predictive of early childhood development in urban South Africa.

Methods: The aims of this prospective cohort study are: 1) to assess dietary intake and nutritional status of urban pregnant women in Johannesburg, South Africa, and 2) to determine associations with birth outcomes, measures of maternal health, as well as measures of offspring health and development. Pregnant women (< 18 weeks' gestation) ($n = 250$) are being recruited from primary healthcare clinics in Johannesburg and are followed-up at a provincial hospital. Participants' dietary intake and nutrient status (focus on micronutrients and fatty acids) are assessed at < 18, 22 and 36 weeks' gestation. Additional assessments during pregnancy include anthropometric and blood pressure measurements, obstetric ultrasound screens, and assessments of food security, maternal fatigue, prenatal depression, allergy, immune function, morbidity and gestational diabetes. At birth, maternal and neonatal health is assessed and an umbilical cord blood sample collected. Maternal and offspring health is followed-up at 6 weeks, as well as at 6, ≈7.5 and 12 months after birth. Follow-up assessments of mothers include anthropometric measures, diet history, nutrient status, blood pressure, breast milk composition, and measures of postnatal depression and fatigue. Follow-up assessments of the offspring include feeding practices, nutrient status, measures of growth, psychomotor, socio-emotional and immune development, morbidity, allergy, as well as analysis of the gut microbiome and the epigenome.

Discussion: Ensuring adequate nutrition during pregnancy is one of the key actions endorsed by the South African Government to promote optimal early childhood development in an effort to eradicate poverty. The results from this study may serve as a basis for the development of context-specific nutritional interventions which can improve birth outcomes and long-term quality of life of the mother and her offspring.

Keywords: Maternal health, Maternal diet, Nutrition, Pregnancy, Birth outcomes, Early development, DOHaD

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Background

Recent estimates indicate that 250 million children in low- and middle income countries are at risk of not reaching their developmental potential [1]. This is worrisome as suboptimal childhood development is associated with poorer adult health, well-being and productivity – leading to an intergenerational cycle of poverty. As the trajectories of physical and mental health later in life are determined fundamentally during the first 1000 days of life, both the World Health Assembly Nutrition Targets and the Sustainable Developmental Goals call for action to, among others, improve maternal, infant and young child nutrition in an effort to ensure sustainable social and economic progress.

Maternal health and nutrition gained heightened attention three decades ago with the publication of the developmental origins of health and disease (DOHaD) hypothesis. Barker and Osmond [2] proposed that the cardiovascular disease they observed in an adult population from England and Wales was at least partly associated with poor early nutrition, and specifically undernutrition in utero [3]. Undernourishment in utero can stress the foetus in ways that permanently affect physiological growth and development, and can be described as a reprogramming of the foetus's developing phenotype [4]. Apart from the long-term health consequences, severe growth-restricted foetuses are at increased risk of stillbirth, and the live births have an increased risk of neonatal death, morbidity and permanent deficits in growth and neurocognitive development [5, 6].

Several maternal nutritional factors have been investigated in relation to adverse pregnancy outcomes, as well as offspring health and development [7–9]. The nutrients most studied during pregnancy include B-vitamins (particularly folic acid), vitamin D, iron, long-chain polyunsaturated fatty acids (particularly n-3 fatty acids) and iodine [8, 10]. However, adequate maternal intakes of zinc and vitamin A may also be important for optimal pregnancy outcomes, as well as for maternal and offspring health [11–13]. Furthermore, better overall diet quality has been associated with a lower risk for maternal perinatal depression and gestational weight gain, which in turn are risk factors for suboptimal offspring development [14–16].

The health of the adult South African population is a concern. South Africa has large economic disparities and 20% of the population are living in extreme poverty, indicating they cannot afford the minimum required food intake [17]. During a national survey in 2012, approximately 40% of the population were reported to have a monotonous diet based mainly on starches [18]. The country is undergoing a rapid nutrition transition characterised by changes in dietary patterns and nutrient intake alongside urbanisation [19, 20], which has resulted in a growing double-burden of under- and over-nutrition [20, 21]. Hence, not surprisingly, 31% and 13% of South African women of reproductive age are anaemic [22] and vitamin A deficient [18], respectively, while 68% of women are overweight or obese, and 46% hypertensive [22]. The effects are also seen in children, with 27% of under-fives being stunted [22]. Both stunting and poverty are known risk factors for poor child development [23]. Maternal short stature is, in turn, a risk factor for birth complications [24] – illustrating the intergenerational effect of poor nutrition.

Ensuring adequate nutrition during pregnancy is one of the key actions endorsed by the South African Government to promote optimal early childhood development in an effort to eradicate poverty [25]. The *Guidelines for maternity care in South Africa* [26] therefore recommend routine nutritional assessment – such as measuring mid-upper arm circumference and haemoglobin levels – and daily supplementation of 200 mg ferrous sulphate, 1000 mg calcium and 5 mg folic acid. However, studies show that the majority of South African women only seek or get access to public antenatal care in their second trimester of pregnancy [27–30], which might be too late for the routine supplementation programme or other interventions to be effective.

Very little is known about the diet and nutritional status of pregnant women in South Africa, specifically residing in urban areas. Furthermore, understanding the associations of maternal diet and nutritional status during pregnancy with birth outcomes, as well as offspring health and development in the South African population will form the basis for the development of context-specific nutrition interventions that may improve birth outcomes and long-term quality of life of the mother and her offspring. Consequently, the *Nutrition during Pregnancy and Early Development (NuPED)* cohort study was initiated to investigate nutritional status during pregnancy and assess early nutrition-related exposures predictive of early childhood development in urban South Africa.

Aims of the study

The aims of the NuPED study are 1) to assess dietary intake and nutritional status of urban pregnant women in Johannesburg, South Africa, and 2) to determine associations with birth outcomes, measures of maternal health, as well as measures of offspring health and development. A simplistic conceptual framework showing the modifiable and non-modifiable exposure variables, as well as the outcome variables that will be determined to achieve the aims, is shown in Fig. 1.

Methods/design

Study design

This prospective cohort study follows 250 women throughout pregnancy to birth, and their infants up to

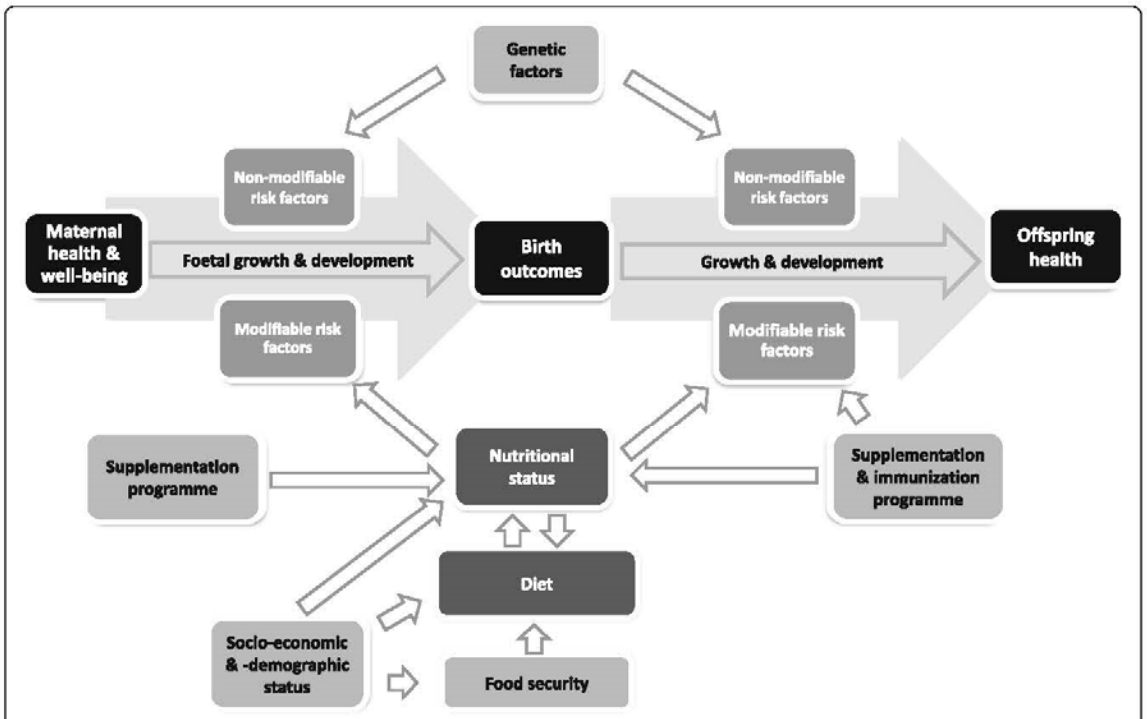

Fig. 1 Simplistic conceptual framework of exposure and outcome variables in the NuPED study. The study investigates the indicated variables from early pregnancy to infants up to 12 months. The variables fall mainly in the modifiable risk factors category with the core interest being nutritional status. The outcome variables include birth outcomes such as birth weight and gestational age as well as postnatal growth and psychomotor development

12 months of age. Briefly, data are collected early pregnancy, mid-pregnancy, late pregnancy and at birth. Postnatal assessments focus mainly on offspring health and development at 6 weeks, 6 months, 7.5 months (6 months + 6 weeks) and 12 months' postnatal age.

Recruitment of participants started on 7 March 2016 and completion of data collection is expected in June 2019.

Setting

The study is situated in Johannesburg, the largest city in South Africa. Recruitment of study participants takes place in two of the seven municipal regions of the city from which four primary health care clinics were identified. These clinics fall in the catchment area of Rahima Moosa Mother and Child Hospital (RMMCH). RMMCH is a provincial hospital focusing on maternal and paediatric healthcare, delivering more than 10,000 babies annually. Pregnancy data are collected at the antenatal care (ANC) clinic of RMMCH in addition to routine care. Birth data are collected in the relevant wards at RMMCH. Postnatal data are collected at the Empilweni Services and Research Unit (ESRU) at RMMCH. The execution of the study is coordinated by the Centre of Excellence for Nutrition of the North-West University.

Study population

The study population is urban pregnant women attending ANC at either one of four selected primary health care clinics or at the ANC clinic of the hospital. Women interested to partake in the study are screened according to inclusion and exclusion criteria, and referred to RMMCH ANC clinic for signing informed consent and data collection if eligible.

The inclusion criteria applied during recruitment screening are: 1) Confirmed pregnancy and planning to deliver her baby at RMMCH; 2) < 18 weeks' gestational age; 3) Born in South Africa, Lesotho, Swaziland, Zimbabwe, Botswana or Namibia and has been living in South Africa for at least 12 months; 4) Able to communicate effectively in one of the following languages: English, Afrikaans, Sotho, Zulu or Xhosa.

The exclusion criteria are 1) < 18 and > 39 years; 2) Multiple pregnancy; 3) Using illicit drugs (self-confessed); 4) Smoking (current and/or in past year); 5) Known non-communicable disease (NCD) namely diabetes, renal disease, high cholesterol, and hypertension; 6)

Known infectious disease namely tuberculosis and hepatitis; 7) Known serious illness namely cancer, lupus or psychosis.

Even though women with infectious disease are excluded, women who are HIV positive are still included. Due to the high prevalence of HIV in the country (36% of women aged 30–34 years [31]), their inclusion will make generalisation to the wider South African population a possibility.

Recruitment and consent procedures

Consecutive sampling is applied, thus, all accessible pregnant women at the recruitment sites may form part of the sample, if they meet the inclusion and exclusion criteria, arrive at study site on their booked date and sign informed consent. All pregnant women in the waiting areas of the ANC clinics are informed about the study. Those interested, receive a study information leaflet and are screened for eligibility individually in a private space upon which a booking date is supplied if eligible. The informed consent form is given to the participant to read, consider and discuss with her partner and/or family. Upon arrival at RMMCH on the booked date, the trained fieldworkers explain the informed consent form in local languages and all are given the opportunity to ask questions. All are assured that participation is voluntary and that participation or non-participation in the study will not affect their clinical care. All research participants provide written informed consent before data collection. Written informed consent is again obtained before infant assessments at 6 weeks postnatally.

Data collection

Figure 2 summarises the measurements and time points of data collection throughout the project. There are eight data collection time points (here forth referred to as phases). All data are collected either by health professionals on site or by trained fieldworkers. Phase 1 data are collected at < 18 weeks' gestation (as confirmed by

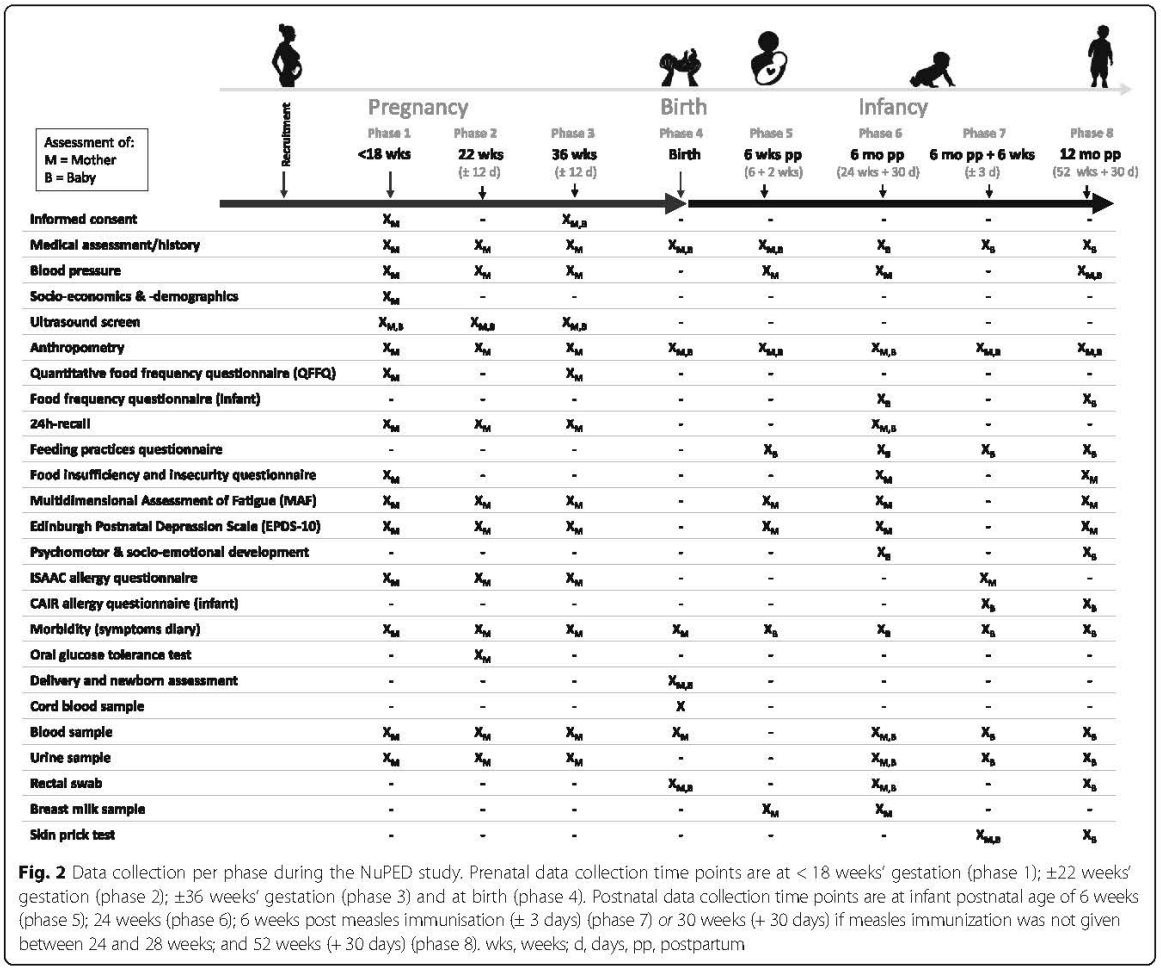

Fig. 2 Data collection per phase during the NuPED study. Prenatal data collection time points are at < 18 weeks' gestation (phase 1); ±22 weeks' gestation (phase 2); ±36 weeks' gestation (phase 3) and at birth (phase 4). Postnatal data collection time points are at infant postnatal age of 6 weeks (phase 5); 24 weeks (phase 6); 6 weeks post measles immunisation (± 3 days) (phase 7) *or* 30 weeks (+ 30 days) if measles immunization was not given between 24 and 28 weeks; and 52 weeks (+ 30 days) (phase 8). wks, weeks; d, days, pp, postpartum

obstetric ultrasound). These data will supply information on the nutritional status of women early in their pregnancy. It is important to note that in Johannesburg only 45% of women access ANC before 20 weeks gestation and only 23% in their first trimester (as reported from other urban areas) [30]. Thus, for practical purposes, the *early pregnancy window* was set at < 18 weeks' gestation. Phase 2 data are collected at 22 weeks' gestation (window ±12 days) when anomaly ultrasounds are typically scheduled. Phase 3 data are collected at 36 weeks' gestation (window ±12 days). Study midwifes and/or fieldworkers collect birth data (phase 4) within a window of 12 h. Postnatal data are collected at 6 weeks (+ 14 days) (phase 5); 24 weeks (+ 30 days) (phase 6); 6 weeks post measles immunisation (± 3 days) (phase 7) *or* 30 weeks (+ 30 days) if measles immunization was not given between 24 and 28 weeks; and 52 weeks (+ 30 days) (phase 8) postnatal age. The purpose of the phase 7 data collection point specifically is to assess measles immunoglobulin G (IgG) as marker of response to immunisation at 6 months and immune function.

Maternal socio-economics and -demographics

Socio-economic and -demographic data are collected at phase 1 by means of a structured interview. Data include date and country of birth, marital status, educational level, home language, employment status, household income, number of members in the household, and beneficiaries of social grants. Lastly, living standards data are obtained to allow classification according to the Living Standards Measure (LSM) developed by the South African Audience Reference Foundation (SAARF) [32]. This measure is widely used in South Africa to describe the socio-economic status of the population [33].

Maternal household food insufficiency and insecurity

The level of the participating women's household food insufficiency and insecurity is assessed at phase 1 in pregnancy and again at phases 6 and 8 postnatally. In a structured interview, women are asked questions on food insecurity and child hunger using the Community Childhood Hunger Identification Project (CCHIP) index [34] that was also used to determine the status of food security in previous national surveys in South Africa [35]. Furthermore, women were asked a validated, single-item question on food insufficiency –"*How many days in the past week have you gone hungry? By this I mean days when you felt you didn't have enough to eat.*"–that was previously used to determine food insufficiency in pregnant women in South Africa [36].

Maternal dietary intake

Maternal dietary intake data are obtained by means of two dietary assessment methods, namely the 24-h recall (24-HR) and a quantified food frequency questionnaire (QFFQ). Both methods are interviewer administered by using standardised probing questions [37]. Standard measuring equipment, common size containers (e.g. cups, bowls and glasses) as well as two- and three-dimensional food models are being used to assist in quantifying portion sizes.

A single 24-HR, which obtains details about nutritional supplement use as well, is administered at phases 1, 2 and 3 in pregnancy, as well as at 6 months postnatally (phase 6). Each participant is requested to recall all foods and drinks consumed the previous day from when she woke up until the next day the same time. The recall is done chronologically unless the participant wishes to recall randomly. The purpose of the single 24-HR is to describe the average intake of the group [38]. All supplement use, as well as food cravings and aversions, are additionally recorded daily by participants on a calendar.

The second dietary assessment method, the QFFQ, is completed at phases 1 and 3. It was validated for the population in the Transition and Health during Urbanisation of South Africans (THUSA) study [39] and its reproducibility was proven [40, 41]. It was also used previously to assess individual and total omega-3 and omega-6 fatty acid intake in a rural and urban South-African population [42]. This QFFQ includes a list of typically consumed foods and minor changes were made to the questionnaire according to vernacular used by the study population in that particular area. Participants are asked according to the ~140 food items listed in the QFFQ, the type/brand, cooking methods, frequency and the amount of all food and drink consumed in the past 4 weeks.

For the QFFQ data, portion sizes are converted to grams per week per food item, by two registered dietitians/nutritionists. For the 24-HR all portion sizes are converted to grams per day per food item. The resources to assist with this include the Condensed Food Composition Tables for South Africa [43] and the South African Medical Research Council (SAMRC) Food Quantities Manual [44].

Maternal anthropometric measurements

Maternal weight and mid-upperarm circumference (MUAC) are obtained at each phase (1 to 8), and height only at phase 1 and 5. All measurements are done twice and recorded to the nearest 0.05 kg for weight, 0.1 cm for MUAC and height. Standardised methods of the International Society for the Advancement of Kinanthropometry [45] are used with a calibrated digital scale for weight, a mobile stadiometer for height; and non-stretchable metal measuring tape for MUAC.

Maternal medical assessment and history

Medical history is obtained at each prenatal visit (phases 1 to 3) by means of participant responses and inspection

of medical files. Information includes medication use (including vaccines), HIV status, obstetric history, hospital admission during pregnancy, use of alcohol, and exposure to passive smoking. At the first postnatal visit (phase 6) a follow-up is made on the maternal medical history at birth. Blood pressure is measured at each prenatal visit (phases 1 to 3) as well as postnatally (phases 5, 6 and 8) according to international guidelines [46] using calibrated equipment. Appropriate sized cuffs are used for obese participants.

Standard procedures are used for a 2-h 75 g oral glucose tolerance test (OGTT) between 24 and 28 weeks gestation to determine development of gestational diabetes mellitus [47].

Maternal morbidity

Maternal morbidity symptoms are assessed from enrolment to birth using a daily calendar. Mothers are instructed on how to complete the calendar and to return completed calendars at each visit. The infectious morbidity symptoms assessed are fever, headache, diarrhoea, nasal discharge and coughing. Other possible pregnancy-related symptoms included are constipation, nausea, vomiting, extreme tiredness and heartburn. Any medication and supplementation use is also recorded daily.

Maternal allergy assessment

The International Study for Asthma and Allergies in Childhood (ISAAC) questionnaire [48] is used to assess allergy symptoms in maternal participants at phases 1 to 3 during pregnancy, and 7 postnatally. Additionally, skin prick tests for common allergens are used at phase 7 postnatally to assess sensitisation [49]. The questionnaire is designed to assess rhinitis, asthma and eczema in children and has been used successfully in an older black population in South Africa [50]. A positive score on any of these three symptoms indicates an allergic phenotype.

Maternal skin prick tests are performed by a medical doctor according to the procedure described in the Allergy Society of South Africa's position statement on skin prick testing [49]. In mothers, sensitisation to a house dust mite mixture including Dermatophagoides farinae, as well as German cockroach, mould mixture, cat and dog dander, maize pollen, Bermuda grass and *Quercus robur* (English oak), Eucalyptus, Cypressus arizonica (Arizona cypress), *Platanus hybrida* (London plane) and Acacia trees are measured. A diagnosis of 1) sensitised with clinical symptoms, 2) sensitised and clinically tolerant, 3) sensitised and unknown clinical reactivity, 4) not sensitised with clinical symptoms, 5) unknown sensitization with clinical symptoms or 6) not sensitized with no clinical symptoms is made. The mother is given medical advice and referred if necessary.

Maternal depression and fatigue

Perinatal depression is assessed at phases 1 to 3 during pregnancy and at phases 5, 6 and 8 postnatally using the Edinburgh Postnatal Depression Scale (EPDS). The EPDS is a 10-item scale assessing depressive symptoms experienced in the past 7 days [51], which has been validated for assessing perinatal depression in African settings, including South Africa [52]. Maternal fatigue is assessed at the same time points using the Multidimensional Assessment of Fatigue (MAF) scale, which was shown to be a reliable and valid measure of fatigue in pregnant and postpartum women [53]. Both questionnaires are interviewer administered.

Foetal ultrasonography: Gestational age and foetal growth

Foetal ultrasonography examination is carried out by an obstetrician at the first data collection time point to confirm gestation. Estimation of foetal crown rump length and/or biparietal diameter or femur length between 6 and 18 weeks' gestation indicates an accuracy within 5–7 days [54]. Foetal crown-rump length is used to determine gestational age of participants in their first trimester [55]. For participants in their second trimester, a combination of multiple biometric parameters (biparietal diameter, head circumference, abdominal circumference, and femur length) are used to determine gestational age [55]. Ultrasound is also used to determine the number of foetuses and confirm foetal movement, as well as foetal growth at 22 and 36 weeks' gestation (phases 2 and 3).

Birth and neonatal assessments

Maternal data collected at birth (phase 4) are obtained from maternal medical files and include hospital admission and discharge dates and times, mode of delivery, induction/augmentation of delivery, type of anaesthetic or pain relief if any, rapid plasma reagin (RPR) status (indicative of syphilis infection), HIV status, rhesus negative status and presence of maternal diabetes mellitus. If the delivery is induced or caesarean section conducted, the reason for this intervention is obtained. The study nurses obtain maternal weight before birth with a calibrated digital scale.

Neonatal data collected at birth from the medical file include date and time of birth, gender, Apgar score (at 1 and 5 min) [56], vital signs, medical interventions required, foetal distress and presence of meconium stained liquor. Four identically trained study nurses obtain newborn anthropometry (weight, midarm circumference (MAC), crown-heel length (CHL), head circumference (HC) and thoracic circumference (TC)) within 12 h of birth [57]. If the measurements cannot be taken by the study nurses, hospital records are used to obtain anthropometrical data (using the same calibrated infant scale).

Newborn weight is measured with a calibrated digital infant scale to the nearest 10 g. In order to minimize intra-observer variability all circumferences and CHL are measured with an inelastic tape to the nearest 0.5 cm (metal measuring tape not used to prevent possible lacerations). CHL is measured by placing the newborn in supine position on the tape measure on a flat surface with all limbs extended and measurement taken from vertex to heel of foot, with foot held in a perpendicular position to the leg.

Infant dietary intake and feeding practices

Data on infant feeding practices are collected at each postnatal phase (5 to 8). Mothers are asked how soon after birth the infant was breastfed, if the infant is currently being breastfed and if not, the duration of breastfeeding. All mothers are asked details about any other food or drink (including infant formula, medicine and supplements) given to the infant.

An unquantified food frequency questionnaire for the infant is administered at phases 6 and 8 for qualitative assessment (types and frequency) of milk and complementary feeding at 6 and 12 months postnatally. An adapted questionnaire previously used in the South African context is used [58]. Frequency of the type of food eaten during the past month can be reported by the mother as *every day*; *most days* (not every day, but at least 4 times per week); *once a week* (less than 4 times per week, but at least once per week) or *never*.

A single 24-HR for the infant is administered at phase 6 for quantitative assessment (macro- and micronutrient intakes) of intakes at 6 months postnatally. Similar methods and aids are used as described for maternal dietary intake. However, smaller bowls and different sizes of small spoons are used for infant dietary intake to ease realistic reporting for the mother. Also, emphasis is placed on dished food not eaten and the amount left in the bowl to ensure actual intake is reported.

Infant anthropometric measurements

Infant growth is assessed at each postnatal visit. Before measurements are taken, the infant is assessed for presence of oedema. The infant is weighed on a calibrated scale with minimum of clothing, namely only a vest, and without a nappy; and recorded to the nearest 5 g.

Recumbent length of the infants is taken by means of an infantometer to the nearest 0.1 cm. All foot and headwear is removed before measurements are taken. The measurement is taken with the infant lying on his/her back on the infantometer, legs extended with the head and foot board making contact with the infant.

Infant medical assessments

A medical doctor performs a general and physical medical assessment of infants at each postnatal visit. The infant assessment includes HIV status history and a general, ear, nose and throat, respiratory, cardiovascular, abdominal and neurological examination as well as any current complaints. It also includes a medical plan for the infant.

Infant morbidity

Infant morbidity assessment is performed at each postnatal visit by a medical doctor with a structured questionnaire. A morbidity calendar, kept daily by the mother/caregiver, is used as reference. The calendar and symptoms are explained to the mother at birth and each postnatal visit up to phase 7, whereby each new section of the calendar is handed to the mother for return at the next visit. Symptoms assessed are fever, diarrhoea, vomiting, nasal discharge, coughing, diaper and other rash. Any unscheduled visits to a medical facility and medicine given to the infant are also recorded. The medical doctor diagnoses and determines the duration of each morbidity event using the structured questionnaire with reference to the morbidity calendar.

Infant allergy assessment

A medical doctor assesses the allergy phenotype and sensitisation of infants with the Childhood Allergy and Immunology Research (CAIR) questionnaire and skin prick tests at phases 7 and 8 postnatally. The CAIR questionnaire was developed by the School of Paediatrics and Child Health of the University of Western Australia and is designed to assess asthma, rhinitis and eczema in infants.

Infant skin prick tests are performed at phases 7 and 8 by a medical doctor according to the procedure described in the Allergy Society of South Africa's position statement on skin prick testing [49]. Skin prick tests to determine sensitisation to common allergens are common medical practice in infants older than 4 months [59, 60]. In infants, sensitization to a house dust mite mixture including Dermatophagoides farinae, German cockroach, mould mixture, cat and dog dander, maize pollen, Bermuda grass, chicken egg, cow's milk, cod fish, peanuts, wheat and soy bean flour and potato are measured. A diagnosis of 1) sensitised with clinical symptoms, 2) sensitised and clinically tolerant, 3) sensitised and unknown clinical reactivity, 4) not sensitised with clinical symptoms, 5) unknown sensitization with clinical symptoms or 6) not sensitized with no clinical symptoms is made. The infant's medical plan is managed accordingly.

Infant immune response

The infant's IgG response to measles immunisation is assessed at phase 7, which is 6 weeks after measles immunisation was administered at the study site. The measles immunisation in South Africa forms part of the National Expanded Programme for Immunisation and permission to administer it at the study site has been

granted by the Department of Health of Gauteng Province and the City of Johannesburg. Response to an immunisation is regarded as a good marker to measure immune function in vivo [61] and the response will be in the log phase 6 weeks after immunisation, which is the most sensitive stage to measure differences in response among infants.

Infant psychomotor and socio-emotional development

Psychomotor and socio-emotional development of the offspring is being assessed using the Protocol for Child Monitoring –Infant version (PCM-I), which combines both parental report and direct observation by trained assessors to provide a comprehensive evaluation of a child's motor skills, cognition, language, personal and socio-emotional development [62]. The PCM-I consists of items derived from: 1) the Kilifi Developmental Inventory (KDI) [63], previously used by the investigators to determine psychomotor development in an infant population in South Africa [64], 2) the Developmental Milestone Checklist (DMC-II) [65, 66], and 3) the Profile of Social-Emotional Development (PSED), which is based in part on the Brief Infant/Toddler Social Emotional Assessment [67].

Biological sample collection

Venous blood (42 ml) is drawn from the participating women into labelled EDTA-coated, serum and trace element free evacuated tubes at phases 1–4 during pregnancy and at phase 6 postnatally. At birth (phase 4), umbilical cord blood samples are taken immediately after the separation of the newborn from the umbilical cord and before placental delivery into labelled EDTA-coated, serum and trace element free evacuated tubes. Venous blood from the infant (3 ml) is drawn at phases 6, 7 and 8. Dry blood spots are collected on filter paper cards (Whatman, Inc) immediately after blood collection (maternal, cord and infant). The filter paper cards are allowed to dry at room temperature for 24 h, placed in ziplock bags with desiccants, and stored at − 20 °C until analysis. In case venous blood cannot be obtained from the infants, capillary blood is being collected by foot venepuncture.

Venous blood is processed within 1 h after blood draw; plasma/serum separated and red blood cells washed twice with normal saline. Buffy coats are stored in 1:1 vol: vol RNA later (Ambion).

Midstream spot urine samples (5 ml) are collected from the participating women at phases 1, 2 and 3 during pregnancy and at phase 6 postnatally into a urine collection cup. From the infants, a 2–5 ml urine sample is collected at phases 6, 7 and 8 using adhesive paediatric urine collection bags. Urine samples are transferred into labelled microtubes and stored at − 20 °C within 4 h.

Breast milk samples (fore-milk) are collected from lactating mothers at phases 5 and 6 as described previously [68].

Rectal swabs (FLOQSwab, COPAN) are collected from both the mother and the baby at phases 4 and 6 of data collection. The mucosal microbe sample is taken approximately ±3 cm into the anal canal, beyond the anal verge. After collection the cotton bud end of the swab with the collected sample is immediately preserved in RNAlater (Ambion) and stored at − 20 °C.

Biological samples are processed on site and stored at − 20 °C for a maximum of 14 days. Thereafter, frozen samples are transported to the North-West University for storage at − 80 °C until analysis. Storage temperature is monitored and logged for the entire duration of the study.

Biochemical analyses

Haemoglobin is determined on site in whole blood (20 μL) using HemoCue (Hb 201+, Ängelholm, Sweden). The iron status indices, ferritin and transferrin receptor, as well as the vitamin A status indicator retinol binding protein will be determined using the Q-Plex™ Human Micronutrient Array (7-plex, Quansys Bioscience, Logan, UT, USA) [69]. This multiplex immune-assay also includes the acute phase proteins C-reactive protein (CRP) and alpha1-acid glycoprotein (AGP), as well as the malaria marker HRP2 and thyroglobulin, which is a marker of iodine status. Urinary iodine concentrations are determined in spot urine samples using a modification of the Sandell-Kolthoff reaction with spectrophotometric detection [70]. Vitamin A and E status will be determined using high pressure liquid chromatography (HPLC) and ultraviolet light detection [71]. Vitamin D status will be determined by measuring total 25-Hydroxyvitamin D [25(OH)D] concentrations in serum using liquid chromatography tandem mass spectrometry (LCMSMS) [72]. Fatty acids in red blood cell total phospholipids are determined using gas chromatography tandem mass spectrometry (GCMSMS) [73]. Zinc concentrations are determined in serum using atomic absorption spectrometry [74].

Thyroid hormones (thyroid stimulating hormone, thyroglobulin, total thyroxine) will be determined in whole blood spots by using electrochemiluminescence immunoassays. Lipid-derived immune modulators will be determined with LCMSMS [75]. Cytokines and hepcidin will be determined using ELISA.

Kynurenine pathway metabolites (mediates interactions between immunological and neurological function) will be determined using LCMSMS [76]. Brain-derived neurotrophic factor (BDNF) as a potential marker of neuronal growth and differentiation will be determined using ELISA [77].

Gut microbiome profiling will be done by isolating microbial DNA from the collected rectal swabs using Qiagen Stool minikit and analysing the 16S rRNA genes DNA sequences on the Ion Torrent 16S metagenomics solution offered by ThermoFisher Scientific.

Targeted epigenetic marks, specifically DNA methylation signatures, will also be assessed in the context of the primary and secondary outcomes of this study. Gene specific DNA methylation will be assessed using the Qiagen EpiTech system. Both, the EpiTect Methyl II Signature and EpiTect Methyl II Complete PCR Arrays (Qiagen) will be considered.

Targeted genotyping of genes of interest in the context of fatty acid, lipid and micronutrient metabolism will also be investigated following the Ion AmpliSeq Targeted Sequencing approach using the Ion *Chef*™ and Ion *S5*™ Systems (ThermoFisher).

Data management and analysis

Sample size calculation

The number of participants to sample has been calculated using the G*Power 3.1.9.2 statistical programme [78]. The statistical calculation involved is the linear multiple regression: fixed model, single regression coefficient. The calculation was based on a small effect size F^2 of 0.05; probability of error (alpha) of 5%; a power of 80% and ten predictors on the birth outcome "low birth weight". The result was that 196 participants will be required. Taking into consideration that participants may opt out of the project (at 25% rate), it is calculated that a minimum of 245 participants should be recruited. We intended to recruit a minimum of 250 participants. However, should the researchers be able to obtain additional funding, additional participants may be included.

Data management

Data are managed by two dedicated data managers. Raw data are captured and saved in password protected Microsoft Access documents with passwords known only to the operator responsible for data imputing and the principal investigators. A second person checks 20% of all the captured data randomly and notes and corrects any errors. If there are more than 5% errors, respective data are re-captured. The final version of the database will be stored under protected zipped files. Data are collected on dual core electronic archives with automatic backup. Information of the single datasets are stored using anonymous IDs. The document linking anonymous IDs to participants will be collected and stored separately.

Dietary data are captured in Microsoft Excel (Microsoft Corporation, Washington, USA) and all electronic entries are double checked by a registered dietitian for the correct food code and a reasonable amount reported. Analyses will be done by the SAMRC by linking data to the most recent food composition database. Data will then be screened by means of range checks and for outliers in total energy, protein, fat, vitamin A and vitamin C intake.

Data analysis

Overall, data processing and statistical analysis are performed using the SAS statistical package (SAS, Cary, NC, USA). Analysis of baseline (phase 1) data will be conducted to describe the nutritional status and basic socio-economic characteristics of the pregnant women. Data will be tested for outliers and normality by means of Q-Q plots and histogram visual inspection. Test for normality will be performed by the Shapiro-Wilk test. Normally distributed data will be expressed as means ± SD; non-normally distributed data will be expressed as medians (25th percentile, 75th percentile).

Data will be analysed cross-sectionally to determine associations between variables at each time point by using appropriate statistical methods (e.g. multiple linear regression analysis, ANCOVA, logistic regression analysis), adjusting for potential covariates.

Data will be analysed prospectively to determine associations between variables at different time points (longitudinally) by using appropriate statistical methods (e.g. linear mixed effects models), adjusting for potential time-dependent and static covariates.

Data will also be analysed retrospectively in matched-control sub-studies by determining associations between observed outcomes and variables collected at previous time points.

The level of significance will be set at $P < 0.05$.

Discussion

The importance of perinatal nutrition and its role in offspring health, is recognised [79]. Nutrition during pregnancy is an important factor associated with both maternal and infant health outcomes [80]. To date, however, South African public health nutrition interventions for pregnant women are limited to folic-iron and calcium supplementation, while it is highly likely that the diet of pregnant women living in South Africa is lacking vital micronutrients and essential fatty acids beyond those supplied, or is even containing excessive amounts of specific micro- and macro-nutrients. In order to advocate evidence-based healthcare policy and practice, the identification of nutrient deficiencies and poor eating patterns of pregnant women in South Africa, which are associated with adverse neonatal outcomes and delayed early offspring development, is imperative.

Little is known about the dietary behaviour and nutritional status of pregnant women living in South Africa. To the best of our knowledge, this is the first South African study focusing on the assessment of both maternal

dietary intake and nutritional status in women pre- and postnatally and to investigate associations with outcomes of maternal and infant health. This study is novel due to the comprehensive set of nutrition related data and indicators of maternal and infant health being obtained in a South African setting. Therefore, this explorative project will contribute to identifying factors that may be targeted in future pre- and/or post-conception maternal interventions for optimal offspring development and possibly reduction in adult NCD risk.

This study also has its challenges. The inclusion and exclusion criteria for participants were designed as such to obtain a sample of generally healthy women, who are able to speak the local language and who could be followed-up from early pregnancy until the infants are 12 months old. South African statistics show that only 52% of women attend antenatal care before 20 weeks' gestation [30], thus access to women early in pregnancy is restricted and limits enrolment into the study. Furthermore, many healthcare facilities in Johannesburg serve a predominantly migrant population [81] posing a challenge for longitudinal data collection. Additionally, these women may choose to eat traditional foods [82] that do not form part of the South African Food Database for nutritional analysis; and may not be able to speak a local language [83] hampering detailed reporting during dietary assessments. Thus, migrating women or those unable to speak local languages could not be included in the study.

A limitation of the study is that women who fit the inclusion criteria are invited to join the study by visiting the data collection site at an agreed date. Having this option of attending may contribute to self-selection bias. Our recruitment data to date indicate that of those invited to take part in the study; only approximately 50% arrive at the data collection site on the booked date.

Even so, this study will provide a comprehensive and unique database from an urban South African setting which will allow for cross-sectional, prospective and retrospective analyses to describe the nutritional status and dietary intake of pregnant women, and to determine associations with health outcome measures. These results will supply context to intervention studies with the aim to improve maternal as well as offspring health in South Africa.

Abbreviations

24-HR: 24-h recall; AGP: Alpha1-acid glycoprotein; ANC: Antenatal care; BDNF: Brain-derived neurotrophic factor; CHL: Crown-heel length; CRP: C-reactive protein; DOHaD: Developmental Origins of Health and Disease; ELISA: Enzyme-linked immunosorbent assay; ESRU: Empilweni Services and Research Unit; GC-MS/MS: Gas chromatography-mass spectrometry; HC: Head circumference; HPLC: High-performance liquid chromatography; HRP2: Histidine-rich protein 2; MAC: Mid-arm circumference; MUAC: Mid-upper arm circumference; NCD: Non-communicable disease; NuPED study: Nutrition during pregnancy and early development study; OGTT: Oral glucose tolerance test; QFFQ: Quantified food frequency questionnaire; RMMCH: Rahima Moosa Mother and Child Hospital; RPR: Rapid plasma reagin; SD: Standard deviation; TC: Thoracic circumference

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Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analysed for the purposes of this protocol article.

Authors' contributions

EAS, JB, LM, LZ and CMS conceptualised and designed the study. EAS, JB, LM and LZ are implementing the research. LM and LZ are responsible for laboratory analyses. JB, LM and RC are responsible for data capturing and analyses. EAS drafted the paper with equal contributions from JB, LM, LZ and CMS. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Human Research Ethics Committees of the North-West University, Potchefstroom (NWU-00186-15-A1 and NWU-00049-16-A1) and the University of Witwatersrand, Johannesburg (M150968 and M161045) provided ethical approval for the study. Permission to conduct the research in the relevant clinical setting was obtained from the Gauteng Health Department, City of Johannesburg District Research Committee and the Clinical Manager of Rahima Moosa Mother and Child Hospital.

All participants sign written informed consent before data collection.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Annexure 9: Language editing certificate

DECLARATION

I, C Vorster (ID: 710924 0034 084), Language editor and Translator and member of the South African Translators' Institute (SATI member number 1003172), herewith declare that I did the language editing of a study, written by Ms E Symington (student number 12135445) from the North-West University.

Title of the study: Intake and status of fatty acids and iron during pregnancy in association with birth outcomes in urban black South African women

C Vorster

31 May 2019

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Date

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Annexure 10: Chapter 4 – Accepted manuscript

Maternal iron-deficiency is associated with premature birth and higher birth weight despite routine antenatal iron supplementation in an urban South African setting: the NuPED prospective study

Short title:

Maternal iron-deficiency is associated with premature birth and higher birth weight despite routine antenatal

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Abstract

Background: Recent studies are suggesting a U-shaped relationship between antenatal iron exposure and birth outcomes. Little is known about the iron status and associated birth outcomes of pregnant women in South Africa. Our aim was to assess iron status at early, mid- and late pregnancy, and to determine associations with gestational age and birth weight in women in Johannesburg, South Africa.

Methods: In this prospective study of 250 pregnant women, we measured haemoglobin, biomarkers of iron status and inflammation at <18, 22 and 36 weeks of gestation, plus birth weight and gestational age at delivery. Associations of anaemia and iron status with birth outcomes were determined using regression models adjusted for confounders.

Results: At enrolment, the prevalence of anaemia, iron depletion (ID) and iron deficiency erythropoiesis (IDE) was 29%, 15% and 15%, respectively, and increased significantly with pregnancy progression. Anaemia and ID at 22 weeks, as well as IDE at 36 weeks were associated with higher birth weight (β=135.4; 95% CI: 4.8, 266.1 and β=205.4; 95% CI: 45.6, 365.1 and β=178.0; 95% CI: 47.3, 308.7, respectively). Women in the lowest ferritin quartile at 22 weeks gave birth to babies weighing 312 g (95% CI: 94.8, 528.8) more than those in the highest quartile. In contrast, IDE at 22 weeks was associated with a higher risk for premature birth (OR: 3.57, 95% CI: 1.24, 10.34) and women in lower haemoglobin quartiles at <18 weeks had a shorter gestation by 7 days (β=-6.9, 95% CI: -13.3, -0.6) compared to those in the highest quartile.

Conclusion: Anaemia, ID and IDE prevalence increased during pregnancy despite routine iron supplementation. ID and anaemia at mid-pregnancy were associated with higher birth weight, while IDE was associated with premature birth. These results suggest that current antenatal screening and supplementation practices in South Africa need to be revisited.

Keywords

Pregnancy; iron deficiency; iron supplementation; gestation; birth weight

Introduction

Iron deficiency anaemia (IDA) is the result of prolonged iron depletion and affects a third of the world's population [1]. Pregnancy is a period of increased physiological iron requirements, and a deficiency may have variable effects on pregnancy, maternal and child health outcomes depending on the severity and time point of occurrence [2]. Preconception IDA has been associated with reduced infant growth and increased risk of adverse pregnancy outcomes [3], while IDA in the third trimester has been associated with poorer mental development of the child [4]. More recently, U-shaped associations of maternal haemoglobin and serum ferritin concentrations with risk for low birth weight and preterm birth have received heightened attention [5,6].

Data on iron intake and status of pregnant women in South Africa are sparse. National data on anaemia and iron status are available for women of reproductive age only. The South Africa Demographic and Health Survey conducted in 2016 estimated that 33% of women of reproductive age (15 – 49 years) are anaemic [7]. In the 2012 South African National Health and Nutrition Examination Survey (SANHANES) conducted among younger women (15 to 35 years) the prevalence of anaemia, iron depletion (ID) and IDA was 23%, 16% and 10%, respectively [8]. From these national data and a systematic review [9], approximately 23% to 33% of South African women are expected to enter pregnancy anaemic, and about half of these cases may be attributed to iron deficiency.

The South African Government is addressing iron deficiency in several ways. Firstly, the Government introduced mandatory fortification of maize meal and wheat flour with eight micronutrients, including iron (35 mg electrolytic iron per kg), in 2003 [10]. Secondly, and more specifically to improve status in pregnant women, the *Guidelines for maternity care in South Africa* [11] recommend routine nutritional assessment as well as daily supplementation with 200 mg ferrous sulphate (±65 mg of elemental iron), 1000 mg calcium and 5 mg folic acid. Regimes in South Africa may differ per province and pregnant women in Johannesburg (current study setting) receive 170 mg ferrous sulphate (±55 mg elemental iron) with calcium and folic acid. Supplementation is supplied to all

pregnant women irrespective of individual iron or anaemia status. However, the effectiveness of routine antenatal iron supplementation in preventing anaemia and iron deficiency during pregnancy has not been evaluated nationally. A cross-sectional study at a regional hospital in Durban, South Africa, reported an anaemia (haemoblogin <11 g/dL) prevalence of 43% in 2000 pregnant women between 34 and 36 weeks of gestation, despite receiving routine iron supplements [12].

Determining haemoglobin concentrations forms part of the routine nutritional assessment during antenatal care, while iron status is only further investigated if referred by a physician. Since haemoglobin is not a sensitive marker of iron status, the iron status of pregnant women in South Africa is not well described. Thus, the current study was motivated by the limited evidence on the effectiveness of routine iron supplementation in iron replete pregnant women [13] as well as the limited data available on pregnant women in South Africa. Therefore, the aim of our study was to assess iron status at early, mid- and late pregnancy, and to determine associations with both birth weight and gestational age in urban pregnant women in Johannesburg, South Africa. In an effort to explain the observed iron status, we additionally assessed iron intake during early pregnancy.

Materials and methods

Study design and participants

This study formed part of the *Nutrition during Pregnancy and Early Development* (NuPED) study, which is a prospective study conducted in South Africa's largest city, Johannesburg. The NuPED study protocol has been published previously [14]. Briefly, generally healthy pregnant women were recruited from primary healthcare clinics in Johannesburg between March 2016 and November 2017. Women were eligible for inclusion if they were aged 18 – 39 years, <18 weeks of gestation with singleton pregnancies, proficient in local languages, born in South Africa or neighbouring countries, and if they have been residing in Johannesburg for at least 12 months. Women were excluded if they reported using illicit drugs, were smoking, or had been diagnosed with a non-communicable disease

(namely diabetes, renal disease, high cholesterol, and hypertension), an infectious disease (namely tuberculosis and hepatitis), or a serious illness (namely cancer, lupus or psychosis). Due to South Africa's high prevalence of HIV infection (36% of women aged 30-34 years [15]), women who were HIV positive were included in the study in order for it to be a better representation of the general population. The volunteering women who agreed to participate were followed-up at the antenatal clinic of an academic hospital until June 2018. Data were collected at early pregnancy (<18 weeks of gestation), mid-pregnancy (±22 weeks), late pregnancy (±36 weeks) and at birth.

Outcome measurements

The primary outcome measures were birth weight and gestational age at birth. At birth, four trained study nurses obtained neonatal weight (to the nearest 10g) using calibrated digital infant scales within 12 hours of birth [16]. In case the study nurse could not obtain the birth weight herself, it was obtained from the medical record (measured using the same calibrated scales). Low birth weight (LBW) was defined as birth weight <2500 g [17]. Date and time of birth were recorded from maternal records. Women who delivered elsewhere were followed-up telephonically to obtain baby's date of birth and sex of the baby. Gestational age at birth was calculated in days using gestational age determined at the first visit, which was before 18 weeks gestation (minimum -maximum range: 6-17 weeks), by means of foetal ultrasonography examination using international recommendations [18]. Preterm birth was defined as birth <37 + 0 weeks of gestation (259 days) [19].

Exposure measurements

Dietary and supplemented iron intake

Maternal dietary intake data were obtained at the first visit (<18 weeks of gestation) by means of an interviewer administered quantified food frequency questionnaire (QFFQ) using standardised probing questions [20]. The QFFQ was validated for a previous South African study [21], and its

reproducibility was proven in similar study populations [22,23]. Women were asked according to the ~140 food items listed in the QFFQ, cooking methods, the type/brand, frequency and the amount of all food and beverages consumed in the past four weeks. To assist in portion size quantification, standard measuring equipment, two- and three-dimensional food models and common size containers (e.g. cups, bowls and glasses) were used. Three registered dietitians/nutritionists converted reported intakes to grams per week per food item using the Condensed Food Composition Tables for South Africa [24] and the South African Medical Research Council (SAMRC) Food Quantities Manual [25]. Analyses were done by the SAMRC by linking dietary intake data to the most recent food composition database to determine total daily dietary iron intake levels. The database includes the iron content values of fortified foods as per the food fortification programme. The Estimated Average Requirement (EAR) cut-point method was used to determine the proportion of subjects with intake below the EAR, indicative of inadequate intake of iron in this population.

Supplement use was determined from participants' daily recorded supplement use (yes/no) on a supplied calendar from enrolment until birth. In addition, at each visit, the women were asked the type/brand, frequency and the amount of all dietary supplements used in the past week, taking into consideration supplementation supplied as part of routine care as well as store bought supplements. From these data, average daily iron intake from routine supplements and total supplements during pregnancy were calculated. Percentage compliance with routine supplementation was calculated as total reported routine supplemented iron intake divided by total routine iron supplied X 100. Routine iron supplementation included 55 mg elemental iron per day provided as 170 mg dried ferrous sulphate.

Haematological biomarkers

Maternal venous blood was drawn into labelled EDTA-coated and serum evacuated tubes at each visit during pregnancy. Haemoglobin concentrations were determined in whole blood (20µL) using calibrated HemoCue haemoglobin meters (Hb 201+, Ängelholm, Sweden). Haemoglobin values were

adjusted for altitude as Johannesburg is located at 1753 meters above sea level [1]. Anaemia was defined as haemoglobin <11 g/dL at <18 weeks of gestation and haemoglobin <10.5 g/dL for mid- and late pregnancy based on cut-offs per trimester [26,27]. In addition, for the purpose of comparability, the prevalence of anaemia is reported according to the WHO [1] haemoglobin cut-off (<11 g/dL) throughout pregnancy. In cases where severe anaemia (haemoglobin <7 g/dL) was detected, the women were referred to the medical doctor on site and treated according to maternity care guidelines [11]. The treatment entailed higher doses of oral iron supplementation and these cases were therefore retained in analyses. In this study no women received parenteral iron therapy or blood transfusion.

Serum was separated within 1h after blood draw and stored at -20 °C for a maximum of 14 days until transportation for storage at -80 °C until analysis. Ferritin and soluble transferrin receptor (sTfR) concentrations were determined using the Q-Plex™ Human Micronutrient Array (7-plex, Quansys Bioscience, Logan, UT, USA) [28]. This fully quantitative chemiluminescent multiplex assay also includes the acute phase proteins C-reactive protein (CRP) and α_1-acid glycoprotein (AGP). Ferritin concentrations were adjusted for inflammation using the correction factors recommended by Thurnham et al. [29]. Iron depletion (ID) was defined as adjusted ferritin <15 µg/L [30]. Iron deficient erythropoiesis (IDE) was defined as sTfR >8.3 mg/L [31]. Iron deficiency anaemia (IDA) was defined as ferritin <15 µg/L plus haemoglobin <11 g/dL [1].

Covariates

Socio-economic and -demographic data, including maternal age and living standards measurements (reflective of socio-economic status) [32], were collected at the first visit early in pregnancy by means of an interviewer-administered questionnaire. Maternal anthropometric measurements (height and weight) were obtained using standardised methods from the International Society for the Advancement of Kinanthropometry [33] at each study visit. To determine body mass index (BMI) weight (kg) was divided by height (m) squared. An obstetrician conducted foetal ultrasonography

examination to confirm gestational age and singleton pregnancy at the first visit [34,35]. A 2-hour 75 g oral glucose tolerance test was performed between 24 and 28 weeks of gestation using standard procedures [36]. Medical files were inspected to obtain data on maternal medical history, including parity, HIV status, mode of delivery, labour induction, as well as sex of the baby. During analyses, women were considered HIV positive irrespective of date of HIV contraction (prior to or during pregnancy).

Statistical methods

Sample size calculation was done using the G*Power 3.1.9.2 statistical programme [37]. The calculation was based on multiple linear regression analysis (fixed model, single regression coefficient); a small effect size F^2 of 0.05; probability of error (alpha) of 5%; a power of 80% and 10 predictors with birth weight as outcome. The result indicated a required sample size of 196 pregnant women. Considering an attrition rate of 25%, a minimum of 245 women were required. The sample size for this study was 250.

Data processing and statistical analysis of data were performed using SPSS version 25 (SPSS Inc, Chicago, IL, USA). Raw data were captured in Microsoft Access (Microsoft Corporation, Washington, USA) and 20% of all captured data were randomly checked for correctness. Dietary data were captured in Microsoft Excel (Microsoft Corporation, Washington, USA) and all electronic entries were double checked for the correct food code and a realistic amount captured.

Data were tested for outliers and normality by means of Q-Q plots, histograms and Shapiro-Wilk test. Normally distributed data are expressed as means ± SD; non-normally distributed data are expressed as medians (25th percentile - 75th percentile), except Table 2 which displays medians with minimum – maximum ranges. Descriptive statistics were conducted to describe iron intake at early pregnancy. To examine the longitudinal trajectory of the iron status parameters with pregnancy progression, median concentrations were determined at each visit.

Univariable analyses per outcome were performed using Mann-Whitney U-test for continuous variables and Chi-square test for categorical variables. To test for significance of change in haematological biomarkers (haemoglobin, ferritin, sTfR, CRP and AGP) over time we used the 2-tailed paired *t* test. For the significance of change in proportions for the conditions (anaemia, ID, IDE, IDA and inflammation) over time we used the McNemar test. Next we used logistic regression analyses to investigate the relationship between the exposure (haemoglobin, ferritin, sTfR) and outcome variables (low birth weight and preterm birth) as binary outcomes with odds ratios (OR) and 95% confidence intervals (CI). Multiple linear regression analyses were conducted for continuous outcome variables (birth weight in grams and gestational age at birth in days). The β coefficient was reported with 95% CIs. In both regression analyses, 3 models were applied and different sets of covariates for the two outcome variables. For birth weight, model 1 adjusted for maternal age, gestational age at birth and sex of the baby. Model 2 included the covariates of model 1 plus parity and socio-economic status. Model 3 included the covariates of models 1 and 2 plus HIV status, maternal BMI at enrolment and glucose tolerance. For gestational age at birth, model 1 adjusted for maternal age, baby sex and delivery intervention (induction or caesarean section). Model 2 adjusted additionally for parity and socio-economic status. Model 3 adjusted in addition to models 1 and 2 for HIV status, maternal BMI at enrolment and glucose tolerance. Lastly, univariate comparisons were done between quartiles of each iron biomarker adjusted for the same covariates as with the regression analyses. *P* values of <0.05 were considered significant.

Ethical considerations

During recruitment, an informed consent form was supplied to potentially eligible women who were interested in being part of the study. Written informed consent was obtained at the first visit from all the women before data collection. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by both the Human Research Ethics Committees of the North-West University, Potchefstroom (NWU-00186-15-

A1 and NWU-00049-16-A1) and the University of the Witwatersrand, Johannesburg (M150968 and M161045). The Gauteng Health Department, City of Johannesburg District Research Committee and Clinical Manager of Rahima Moosa Mother and Child Hospital gave permission to conduct research at the indicated clinical setting.

Results

Participant characteristics and birth outcomes

A total of 595 potentially eligible women residing in Johannesburg, South Africa, were recruited and invited to take part in the study of which 313 volunteered (53%) and signed written informed consent. After screening, 63 women were excluded based on inclusion and exclusion criteria. In total, 250 women were enrolled and completed data collection at baseline (<18 weeks of gestation). Of these, 232 completed follow-ups to delivery: eleven women were lost to follow-up and seven had a miscarriage/intra-uterine foetal death. These 18 cases were included in cross-sectional analysis of data at enrolment, thus all 250 women were included when enrolment data were reported. One entry for birth data was excluded in regression analyses due to early emergency c-section (28 weeks of gestation). There was one maternal fatality shortly after delivery. Several women (n=29) delivered their babies elsewhere and therefore, birth weight data were missing (see S1 Table). At enrolment there were no significant differences in participant demographic characteristics between women with and without birth weight data (n=40). There was, however, a significant difference in the anaemia and iron status at enrolment of women with and without birth weight data. Women without birth weight data were significantly more anaemic and had a lower iron status at enrolment than women with birth weight data.

Characteristics of pregnant women at enrolment (<18 weeks of gestation) in the total study group, as well as by birth outcome, are shown in Table 1. Most of the women were of black-African descent (88%) with a median age of 27 (IQR: 24-32) years and gestation of 14 (12-16) weeks at enrolment.

A quarter of the women (25%) were born in Zimbabwe, although the majority was born in South Africa (72%). Fifty-eight percent of women have completed secondary school and 23% post-school education. Many women (40%) were unmarried/single and 59% had an LSM score indicative of middle-class living standards. The median BMI (26.3 [23.0-30.6] kg/m^2) at enrolment was above a healthy range with 33% of women being overweight and 28% obese. More than a quarter of the women were HIV positive (26%). Thirty percent were nulliparous. At enrolment, the only significant difference between women who ultimately delivered premature and non-premature babies were their CRP status. When comparing women who delivered LBW vs non-LBW babies, those who gave birth to LBW babies had a significantly lower BMI at enrolment (24.9 [21.1-26.5] vs 27.1 [23.3-30.8] kg/m^2) and fewer women had increased CRP ($p<0.01$). The median birth weight was 3050 (2324-3380) grams and 14% (n=29) of the babies were born with LBW (<2500 g). The median gestational age at birth was 274 (266-282) days and 11% (n=26) of babies were born preterm (<259 days).

Table 1: Characteristics of pregnant women from the NuPED study at enrolment (<18 weeks of gestation) by birth outcome

Characteristics	**Total sample (n=250)*** *Median (IQR) or n (%)*	**LBW, 14% (n=29)**	**Non-LBW, 86% (n=174)**	***p*†**	**Preterm, 11% (n=26)**	**Term, 89% (n=207)**	***p*†**
Age (years)	27 (24-32)	27 (23-32)	28 (24-32)	0.69	28 (22-34)	27 (24-32)	0.83
Gestational age (weeks)	14 (12-16)	14 (11-17)	14 (12-16)	0.99	14 (10-16)	14 (12-16)	0.49
BMI (kg/m^2)	26.3 (23.0-30.6)	24.9 (21.1-26.5)	27.1 (23.3-30.8)	**0.02**	25.3 (21.7-28.4)	26.5 (23.3-30.7)	0.10
Underweight (<18.5 kg/m^2)	8 (3)	4 (14)	3 (2)		0	7 (3)	
Normal weight (18.5-24.9 kg/m^2)	89 (36)	11 (38)	61 (35)	**<0.01**	13 (50)	71 (35)	0.26
Overweight (25-29.9 kg/m^2)	81 (33)	9 (31)	58 (34)		9 (35)	68 (33)	
Obese (≥30 kg/m^2)	71 (28)	5 (17)	51 (29)		5 (15)	60 (29)	
Ethnicity							
Black African	219 (88)	23 (79)	153 (88)		22 (88)	182 (88)	
Mixed ancestry	28 (11)	5 (17)	21 (12)	**0.04**	3 (12)	24 (12)	0.94
White	1 (<1)	-	-		-	-	
Indian	1 (<1)	1 (3)	0		0	1 (1)	
Country of birth							
South Africa	172 (72)	23 (85)	117 (70)		19 (79)	139 (70)	
Zimbabwe	60 (25)	4 (15)	47 (28)	0.42	5 (21)	54 (27)	0.73
Lesotho	4 (2)	0	2 (1)		0	4 (2)	
Swaziland	3 (1)	0	1 (1)		0	2 (1)	
Living Standards Measure (LSM)							
Low (LSM 1-4)	17 (7)	2 (7)	10 (6)		1 (4)	14 (7)	
Medium (LSM 5-7)	148 (59)	16 (55)	104 (60)	0.89	17(62)	124 (60)	0.85
High (LSM 8-10)	85 (34)	11 (38)	60 (35)		9 (35)	69 (33)	
Marital status							
Unmarried/single	100 (40)	18 (62)	66 (38)		14 (56)	79 (38)	
Married	68 (27)	5 (17)	46 (26)		4 (16)	60 (29)	
Divorced/Separated	2 (1)	1 (3)	1 (1)	0.06	0	2 (1)	0.36
Living together	57 (23)	3 (10)	44 (25)		4 (16)	49 (24)	
Traditional marriage[#]	22 (9)	2 (7)	17 (10)		3 (12)	17 (8)	
Highest level of education							
Primary school or less	9 (4)	1 (3)	4 (2)		0	9 (4)	
Grade 8 – 10	37 (15)	4 (14)	23 (13)	0.94	6 (24)	27 (13)	0.28
Grade 11 – 12	145 (58)	16 (55)	105 (60)		12 (48)	125 (60)	
Post-school education	58 (23)	8 (28)	42 (24)		7 (28)	46 (22)	
Parity							
Nulliparous	74 (30)	14 (48)	47 (27)		10 (39)	57 (28)	
Primiparous	88 (35)	5 (17)	65 (37)	0.07	7 (27)	76 (37)	0.66
Multiparous	88 (35)	10 (35)	62 (36)		9 (36)	73 (36)	
HIV status							
Positive	64 (26)	6 (21)	43 (25)	0.64	7 (27)	53 (26)	0.89
Negative	186 (74)	23 (79)	131 (75)		19 (73)	154 (74)	
Inflammatory status							

Elevated CRP (>5 mg/L)	149 (60)	10 (35)	110 (63)	**<0.01**	10 (39)	130 (63)	**0.02**
Elevated AGP (>1 g/L)	28 (11)	3 (10)	22 (13)	0.73	3 (12)	24 (12)	0.99

LBW: Low birth weight; IQR: interquartile range; CRP: C-reactive protein; AGP: α_1-acid glycoprotein; LSM: Living Standards Measure

Data are presented as n (%) for categorical variables and median (IQR) for continuous variables.

† Mann-Whitney-U test for continuous variables, and Chi-square test for categorical variables.

* n-values are equal to 250 for LSM, highest level of education, parity and HIV status; 239 for Country of birth; and 249 for all other variables.

#: Traditional marriage, recognised under South African customary law, is entered between parties based on tradition which does not require the approval of an officiator for validation. It is also different from civil marriage in that a polygamous marriage is permissible [38].

Maternal dietary and supplemented iron intake

The results on dietary iron intake at early pregnancy and supplemented iron intake throughout pregnancy are displayed in Table 2. Median (minimum – maximum range) maternal dietary iron intake as reported at <18 weeks of gestation was 19.1 (4.6-46.1) mg per day from foods, which included fortified foods. There was no significant difference in dietary iron intake between the anaemic and non-anaemic women (p=0.45) nor between the ID and non-ID women (p=0.24). Most women (62%) consumed less iron than the Estimated Average Requirement (EAR) (22 mg/day) for women during pregnancy, and two women (1%) had a dietary iron intake above the upper limit (UL) (45 mg) from foods [39]. The estimated median percentage compliance to routine iron supplementation during the course of pregnancy was 100% (0-100), and the median supplemented iron intake of 55 (0-110) mg/day is reflective of routine iron supplementation. Women reported to buy nutritional supplements in addition to routine iron supplementation which is reflected in the maximum value of total supplemental iron intake of 125 mg elemental iron per day. There was no significant difference in mean supplemental iron intake or supplementation compliance between the anaemic and non-anaemic women (p=0.74 and p=0.83); while there was a significant difference in reported routine supplement intake between the ID and non-ID (p=0.04) women at 36 weeks of gestation.

Table 2: Dietary iron intake at early pregnancy (<18 weeks of gestation) and supplemental iron intake throughout pregnancy

	All women		Anaemic		Non-anaemic			ID		Non-ID		
	n (%)	Median (Min-Max)	n (%)	Median (Min-Max)	n (%)	Median (Min-Max)	$p^{\dagger *}$	n (%)	Median (Min-Max)	n (%)	Median (Min-Max)	$p^{\dagger *}$
Dietary iron intake at early pregnancy, mg/day	250	19.1 (4.6-46.1)	70	19.5 (6.9-43.2)	173	18.7 (4.6-46.1)	0.45	37	19.5 (8.6-39.8)	213	18.9 (4.6-46.1)	0.24
< EAR (<22 mg/d)	155 (62)		41 (59)		111 (64)			23 (62)		132 (62)		
Between EAR and UL	93 (37)		29 (41)		60 (35)			14 (38)		79 (37)		
>UL (>45 mg/d)	2 (1)		0		2 (1)			0		2 (1)		
Reported supplemental iron intake at 36 weeks of gestation:												
Routine supplemental iron intake, mg/day	227	55 (0-110)	63	55 (0-110)	158	55 (0-110)	0.74	34	55 (0-55)	193	55 (0-110)	**0.04**
% Compliance	227	100 (0-100)	63	100 (0-100)	158	100 (0-100)	0.83	34	100 (0-100)	193	100 (0-100)	0.06
Total supplemental iron intake (routine + store-bought) mg/day	227	55 (0-125)	63	55 (8-110)	158	55 (8-125)	0.96	32	55 (8-73)	190	55 (8-125)	0.07

EAR: Estimated Average Requirement; UL: Tolerable Upper Intake Level

Anaemic: Haemoglobin <11 g/dL; ID: Ferritin <15 µg/L

Data are presented as median (minimum-maximum) for continuous variables and n (%) for categorical variables.

***** Nonparametric tests for independent samples were used for comparing continuous variables.

Haematological outcomes with pregnancy progression

Table 3 shows the haematological biomarkers relevant to iron and inflammatory status with pregnancy progression as measured at the three time points. Fig 1 illustrates the anaemia, ID, IDE and IDA trajectory with pregnancy progression. It should be noted that even though 232 women completed follow-up visits up to birth, only 199 blood samples were available for the 36 weeks visit. This is due to 27 women giving birth prematurely (before this visit) and six samples not available for analyses. At enrolment, there were only 3 cases with severe anaemia (haemoglobin <7 g/dL). The median haemoglobin concentration at early pregnancy was 11.7 (10.8-12.7) g/dL and declined to 11.2 (10.1-12.1) g/dL at the second visit (p<0.001) and plateaued to the end of pregnancy (11.2 [10.1-12.1] g/dL) (p=0.88). Anaemia prevalence (using the WHO cut-off: haemoglobin <11 g/dL throughout pregnancy) increased from 29% to 44% (p<0.001) and 45% (p=0.99) at the three time points, respectively. However, when using the more conservative haemoglobin cut-off of <10.5 g/dL for the second and third time points, the anaemia prevalence of 29% at early pregnancy remained unchanged at the second (32%; p=0.70) and third (34%; p=0.69) visit. Median adjusted ferritin concentrations declined gradually and significantly from 47.6 (21.3-98.7) μg/L to 31.7 (17.8-58.6) μg/L and 20.8 (13.9-40.1) μg/L at the three time points, respectively. ID prevalence increased from 15% to 19% (p=0.06) and 33% (p=0.001). The prevalence of IDA (using WHO haemoglobin cut-off) increased from 9% to 14% (p=0.08) and 23% (p=0.008), while the prevalence of IDA using the lower haemoglobin cut-off changed from 9% to 11% (p=0.68) and 19% (p=0.01). Median sTfR concentrations increased significantly from 4.8 (3.8-6.6) mg/L at <18 weeks to 6.8 (5.4-8.3) and 8.1 (6.5-10.5) mg/L with pregnancy progression. Consequently, IDE prevalence increased significantly from 15% to 25% and 47% during pregnancy. Both median CRP and AGP concentrations declined significantly with pregnancy progression. At enrolment, 60% of participants had elevated CRP (>5 mg/L) and 11% had elevated AGP (>1 g/L) (Table 1).

Table 3: Haematological biomarkers of iron status at three time points in pregnant women residing in Johannesburg, South Africa

Biomarker	<18 weeks Median (IQR)	22 weeks Median (IQR)	36 weeks Median (IQR)	*p*† (<18 & 22 weeks)	*p*† (22 & 36 weeks)
n	250	232	199		
Haemoglobin, g/dL	11.7 (10.8-12.7)	11.2 (10.1-12.1)	11.2 (10.1-12.1)	**<0.001**	0.88
Serum ferritin, µg/L	47.6 (21.3-98.7)	31.7 (17.8-58.6)	20.8 (13.9-40.1)	**<0.001**	**<0.001**
Serum sTfR, mg/L	4.8 (3.8-6.6)	6.8 (5.4-8.3)	8.1 (6.5-10.5)	**<0.001**	**<0.001**
Serum CRP, mg/L	6.5 (3.1-14.4)	6.6 (3.1-12.8)	4.8 (2.8-9.8)	0.06	**<0.001**
Serum AGP, g/L	0.75 (0.63-0.86)	0.61 (0.53-0.74)	0.55 (0.47-0.65)	**<0.001**	**<0.001**

sTfR: soluble transferrin receptor; CRP: C-reactive protein; AGP: α_1-acid glycoprotein.

Data are presented as medians (interquartile range [IQR]) for continuous variables

† Wilcoxon nonparametric tests were used for comparing continuous variables between <18 and 22 weeks and between 22 and 36 weeks of gestation.

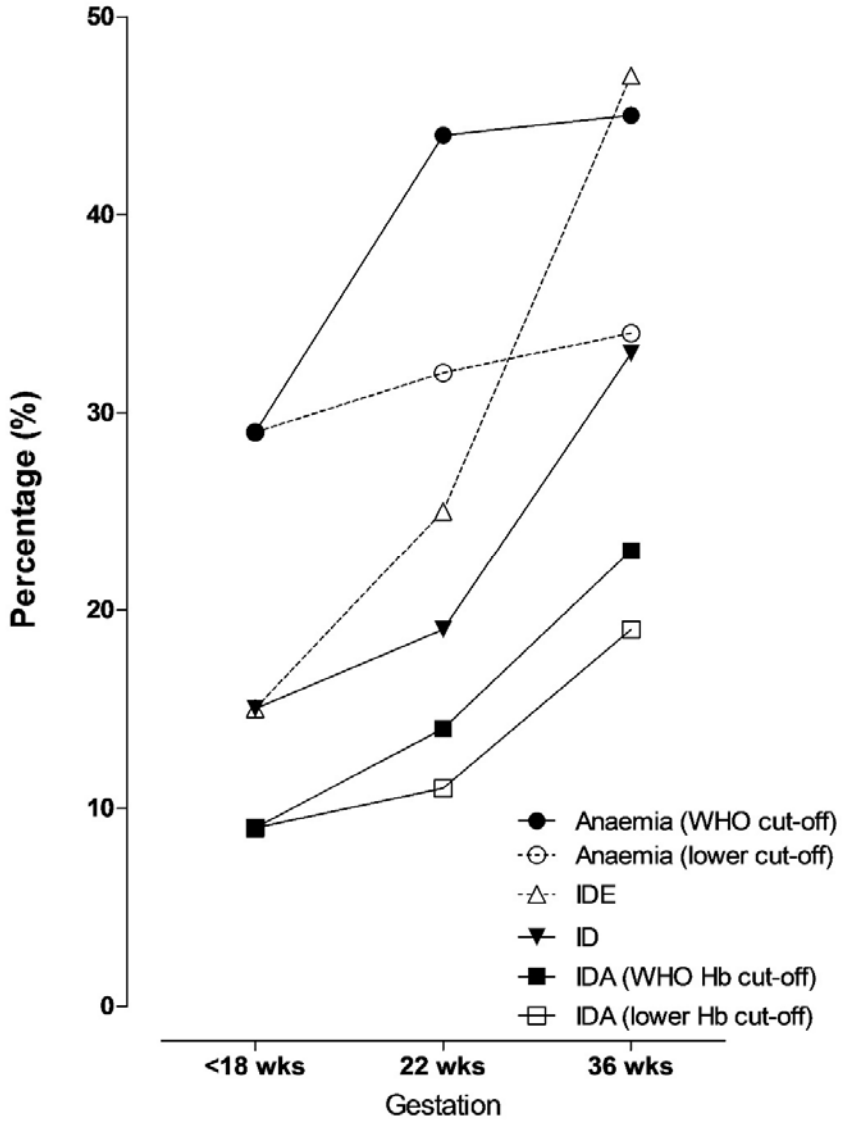

Fig 1: Iron and anaemia status trajectory over the course of pregnancy in women residing in Johannesburg, South Africa. ID – iron depletion; IDE – iron deficiency erythropoiesis; IDA – iron deficiency anaemia; Hb – haemoglobin; wks - weeks. Anaemia (WHO cut-off): haemoglobin <11 g/dL); Anaemia (lower cut-off): haemoglobin <10.5 g/dL at 22 and 36 weeks of gestation; IDE: sTfR >8.3 mg/L ID: Ferritin <15 µg/L; IDA (WHO Hb cut-off): ferritin <15 µg/L plus haemoglobin <11 g/dL; IDA (lower Hb cut-off): ferritin <15 µg/L plus haemoglobin <10.5 g/dL at 22 and 36 weeks of gestation.

Associations of iron and anaemia status with birth outcomes

Results from the logistic regression analyses of the associations of maternal iron status with LBW as well as preterm birth as binary outcomes are shown in Table 4. The only significant association found was that women who were IDE at 22 weeks of gestation had 3.6 times the risk of giving birth prematurely (OR: 3.57, 95% CI: 1.24, 10.34) as indicated in the fully adjusted model.

Table 4: Associations between maternal iron status and birth outcomes (LBW and premature birth) (binary logistic regression, odds ratios and 95% confidence intervals)

		Associations with LBW*								
		Model 1 (n=186)			**Model 2** (n=186)			**Model 3** (n=156)		
	Predictor	**OR**	**95% CI**	***p***	**OR**	**95% CI**	***p***	**OR**	**95% CI**	***p***
<18 weeks	**Anaemia**, Hb <11 g/dL	0.40	0.10, 1.61	0.20	0.38	0.09, 1.64	0.20	0.33	0.06, 1.79	0.20
	ID, Fer <15 µg/L	0.27	0.04, 2.01	0.20	0.25	0.03, 1.93	1.83	0.99	0.13, 7.16	0.98
	IDE, sTfR >8.3 mg/L	0.18	0.02, 1.64	0.13	0.15	0.02, 1.36	0.09	0.56	0.05, 6.23	0.64
22 weeks	**Anaemia**, Hb <11 g/dL	0.50	0.15, 1.65	0.26	0.47	0.14, 1.65	0.24	0.59	0.13, 2.67	0.49
	Anaemia, Hb <10.5 g/dL	0.39	0.10, 1.62	0.20	0.35	0.08, 1.49	0.15	0.59	0.12, 2.95	0.52
	ID, Fer <15 µg/L	0.32	0.05, 1.89	0.21	0.31	0.05, 1.93	0.21	0.35	0.4, 3.29	0.36
	IDE, sTfR >8.3 mg/L	0.64	0.15, 2.66	0.53	0.57	0.13, 2.49	0.45	0.89	0.22, 5.65	0.89
36 weeks	**Anaemia**, Hb <11 g/dL	1.04	0.31, 3.49	0.95	1.27	0.36, 4.5	0.71	2.47	0.44, 13.83	0.30
	Anaemia, Hb <10.5 g/dL	1.37	0.39, 4.78	0.62	1.82	0.49, 6.83	0.37	1.93	0.36, 10.40	0.45
	ID, Fer <15 µg/L	1.52	0.44, 5.24	0.51	1.52	0.43, 5.37	0.51	0.98	0.13, 7.16	0.98
	IDE, sTfR >8.3 mg/L	0.93	0.28, 3.06	0.90	0.97	0.28, 3.32	0.96	0.90	0.16, 5.05	0.91
		Associations with premature birth[#]								
		Model 1 (n=187)			**Model 2** (n=187)			**Model 3** (n=154)		
<18 weeks	**Anaemia**, Hb <11 g/dL	1.22	0.46, 3.26	0.69	1.21	0.45, 3.23	0.71	1.59	0.50, 5.04	0.43
	ID, Fer <15 µg/L	1.81	0.60, 5.54	0.30	1.75	0.56, 5.46	0.33	2.44	0.67, 8.90	0.18
	IDE, sTfR >8.3 mg/L	1.10	0.70, 1.73	0.67	0.75	0.20, 2.79	0.67	0.98	2.3, 4.12	0.99
22 weeks	**Anaemia**, Hb <11 g/dL	0.51	0.19, 1.34	0.17	0.48	0.18, 1.30	0.15	0.60	0.20, 1.84	0.37
	Anaemia, Hb <10.5 g/dL	0.94	0.35, 2.52	0.89	0.92	0.34, 2.51	0.87	1.07	0.34, 3.30	0.91
	ID, Fer <15 µg/L	0.89	0.28, 2.89	0.85	0.88	0.27, 2.86	0.83	1.34	0.37, 4.82	0.66
	IDE, sTfR >8.3 mg/L	2.46	0.99, 6.12	0.05	2.46	0.99, 6.14	0.05	3.57	1.24, 10.34	**0.02**
36 weeks	**Anaemia**, Hb <11 g/dL	0.66	0.16, 2.81	0.58	0.62	0.14, 2.74	0.52	0.55	0.09, 3.44	0.52
	Anaemia, Hb <10.5 g/dL	1.06	0.25, 4.55	0.94	1.03	0.23, 4.62	0.97	1.06	0.16, 6.92	0.94
	ID, Fer <15 µg/L	1.04	0.24, 4.43	0.96	1.01	0.23, 4.39	0.97	0.35	0.04, 3.21	0.35
	IDE, sTfR >8.3 mg/L	1.61	0.41, 6.32	0.50	1.58	0.38, 6.58	0.53	2.05	0.38, 11.09	0.40

Hb: haemoglobin; Fer: serum ferritin; LBW: low birth weight; ID: iron depletion; IDE: iron deficiency erythropoiesis; sTfR: soluble transferrin receptor; OR: odds ratio; CI: confidence interval

* Model 1 for low birth weight adjusted for: maternal age, gestational age at birth and sex of the baby; model 2 adjusted in addition to model 1: parity and living standards measure (socio-economic status); model 3 adjusted in addition to models 1 and 2: HIV status, maternal BMI at enrolment and glucose tolerance.

Model 1 for premature birth adjusted for: maternal age, baby sex and delivery intervention (induction or caesarean section); models 2 and 3 adjusted for the same additional factors as for low birth weight.

p values of <0.05 were considered significant.

Table 5 shows the results from the multiple linear regression analyses on the associations of iron and anaemia status with birth weight (in grams) and gestational age at birth (in days) as outcomes. In the fully adjusted model, anaemia (haemoglobin <10.5 g/dL) at 22 weeks of gestation was associated with a 207 g higher birth weight in neonates (β=207.0; 95% CI: 70.5, 343.6). When anaemia was defined according to the WHO haemoglobin cut-off (<11 g/dL), anaemia was associated with a 135g higher birth weight in neonates (β=135.4; 95% CI: 4.8, 266.1). Similarly, ID at 22 weeks was associated with a 205g higher birth weight (β=205.4; 95% CI: 45.6, 365.1). IDE at 36 weeks was associated with a 178 g higher birth weight (β= 178.0; 95% CI: 47.2, 308.7).

Anaemia defined using the WHO cut-off (haemoglobin <11 g/dL) at 22 weeks of gestation was associated with an increase in gestational age by 5 days (β=4.82; 95% CI: 0.39, 9.24). This association did not hold when the lower haemoglobin cut-off was used (haemoglobin <10.5 g/dL).

Table 5: Associations between maternal iron status and birth outcomes (birth weight and gestational age at birth) (multivariable linear regression, β-values and 95% confidence intervals)

Birth weight (n=203)*

		Model 1			Model 2			Model 3		
	Predictor	**β**	**95% CI**	***P***	**β**	**95% CI**	***P***	**β**	**95% CI**	***P***
<18 weeks	**Anaemia**, Hb <11 g/dL	18.2	-126.1, 162.6	0.80	24.0	-120.2, 168.2	0.74	63.5	-87.4, 214.4	0.41
	ID, Fer <15 µg/L	-10.3	-194.3, 173.8	0.91	22.2	-163.2, 207.5	0.81	42.0	-152.1, 236.0	0.67
	IDE, sTfR >8.3 mg/L	150.3	-29.3, 326.9	0.10	183.9	3.8, 363.9	0.05	128.7	-60.5, 317.9	0.18
22 weeks	**Anaemia**, Hb <11 g/dL	104.0	-18.1, 226.1	0.09	145.0	21.2, 268.9	**0.02**	135.4	4.8, 266.1	**0.04**
	Anaemia, Hb <10.5 g/dL	177.1	48.0, 306.2	**<0.01**	213.7	84.5, 342.8	**<0.01**	207.0	70.5, 343.6	**<0.01**
	ID, Fer <15 µg/L	210.2	47.0, 373.3	**0.01**	190.0	38.0, 342.1	**0.02**	205.4	45.6, 365.1	**0.01**
	IDE, sTfR >8.3 mg/L	40.1	-109.4, 189.7	0.59	52.3	-97.0. 201.6	0.49	58.1	-97.0, 213.3	0.46
36 weeks	**Anaemia**, Hb <11 g/dL	-45.5	-183.0, 92.1	0.52	-24.9	-166.4, 116.7	0.73	25.6	-120.9, 172.0	0.73
	Anaemia, Hb <10.5 g/dL	13.3	-131.8, 158.3	0.86	33.8	-113.6, 181.1	0.65	105.9	-42.8, 254.6	0.16
	ID, Fer <15 µg/L	37.6	-97.1, 172.2	0.58	48.9	-85.3, 183.0	0.47	75.7	-61.0, 212.5	0.28
	IDE, sTfR >8.3 mg/L	199.2	-5.7, 244.1	**0.06**	164.22	35.0, 293.4	**0.01**	178.0	47.2, 308.7	**<0.01**

Gestational age at birth (n=233)#

		Model 1			Model 2			Model 3		
<18 weeks	**Anaemia**, Hb <11 g/dL	1.34	-3.20, 5.87	0.56	1.34	-3.24, 5.91	0.57	1.39	-3.65, 6.43	0.59
	ID, Fer <15 µg/L	-3.27	-9.03, 2.50	0.27	-3.68	-9.54, 2.18	0.22	-3.40	-9.84, 3.05	0.30
	IDE, sTfR >8.3 mg/L	0.66	-504, 6.35	0.82	0.35	-5.43, 6.14	0.90	1.56	-4.80, 7.91	0.63
22 weeks	**Anaemia**, Hb <11 g/dL	4.65	0.73, 8.58	**0.02**	4.57	0.51, 8.63	**0.03**	4.82	0.39, 9.24	**0.03**
	Anaemia, Hb <10.5 g/dL	2.23	-2.25, 6.70	0.33	1.98	-2.60, 6.55	0.40	3.07	-1.97, 8.11	0.23
	ID, Fer <15 µg/L	0.53	-4.70, 5.76	0.84	0.01	-0.03, 0.04	0.79	1.14	-4.64, 6.91	0.70
	IDE, sTfR >8.3 mg/L	-2.45	-7.16, 2.26	0.31	-2.56	-7.31, 2.19	0.29	-2.90	-8.07, 2.28	0.27
36 weeks	**Anaemia**, Hb <11 g/dL	-0.49	-4.88, 3.89	0.82	-0.70	-5.26, 3.85	0.76	2.50	-5.33, 4.50	0.87
	Anaemia, Hb <10.5 g/dL	0.07	-4.55, 4.68	0.98	-0.08	-4.82, 4.66	0.97	-0.80	-5.83, 4.22	0.75
	ID, Fer < 15 µg/L	2.56	-2.03, 7.16	0.27	2.50	-2.15, 7.14	0.29	2.26	-2.61, 7.12	0.36
	IDE, sTfR >8.3 mg/L	-1.01	-5.30, 3.30	0.64	-1.36	-5.90, 3.17	0.55	-0.33	-5.11, 4.46	0.89

Hb: haemoglobin; Fer: serum ferritin; ID: iron depletion; IDE: iron deficiency erythropoiesis; sTfR: soluble transferrin receptor; CRP: C-reactive protein; AGP: α_1-acid glycoprotein; CI: confidence interval

* Model 1 for birth weight adjusted for: maternal age, gestational age at birth and sex of the baby; model 2 adjusted in addition to model 1: parity and living standards measure (socio-economic status); model 3 adjusted in addition to models 1 and 2: HIV status, maternal BMI at enrolment and glucose tolerance.

Model 1 for gestational age at birth adjusted for: maternal age, baby sex and delivery intervention (induction or caesarean section); models 2 and 3 adjusted for the same additional factors as for birth weight.

p values of <0.05 were considered significant.

To further investigate the association of low and high concentrations of iron biomarkers with birth outcomes, haematological biomarker concentrations were divided in quartiles, and univariate comparisons of birth weight and gestational age conducted in adjusted models as shown in Table 6. There was a significant difference between the second lowest (haemoglobin: 10.8-11.7 g/dL) and the highest quartile (haemoglobin >12.7 g/dL) of haemoglobin at <18 weeks of gestation with gestational age at birth. Women in the second lowest haemoglobin quartile at early pregnancy had a significantly shorter gestation by 7 days (β=-6.9, 95% CI: -13.3, -0.6) when compared to women in the highest quartile. In addition, we found significant differences in birth weight between quartiles of serum ferritin concentrations at 22 weeks and 36 weeks of gestation. Women in the lowest ferritin quartile (<17.8 µg/L) at 22 weeks gave birth to babies weighing 311.8 g (95% CI: 94.8, 528.8) more than those in the highest quartile (>58.6 µg/L). At 36 weeks of gestation, those in the lowest ferritin quartile (ferritin <13.85 µg/L) gave birth to babies weighing 410.3 g (95% CI: 184.3, 636.3) more than those in the highest quartile (ferritin >40.08 µg/L) (p<0.001). Women in the third ferritin quartile (ferritin 20.80-40.08 µg/L) at 36 weeks of gestation also had significantly heavier babies (276.2 g, 95% CI: 72.2, 480.2) than women in the highest quartile (>40.08 µg/L).

Table 6: Associations between quartiles of haemoglobin, ferritin and sTfR at three time points with birth weight and gestational age at birth (β-values and 95% confidence intervals)

Iron index	Median birth weight* (g)	n	β (95% CI)	*p*	Median gestational age[#] (days)	n	β (95% CI)	*p*
	<18 weeks				**<18 weeks**			
Haemoglobin (g/dL)				0.28				0.15
Quartile 1: <10.8	3058	35	-39.7 (-258.5, 179.1)	0.72	271	36	-3.7 (-10.3, 2.9)	0.27
Quartile 2: 10.8-11.7	3122	42	23.6 (-187.2, 234.4)	0.83	268	41	-6.9 (-13.3, -0.6)	**0.03**
Quartile 3: 11.8-12.7	2923	36	-174.4 (-392.0, 43.1)	0.12	273	37	-1.3 (-7.9, 5.3)	0.70
Quartile 4: >12.7	3098	40	1		275	39	1	
Ferritin (µg/L)				0.57				0.07
Quartile 1: <21.30	3151	37	123.2 (-93.4, 339.87)	0.26	267	38	-6.0 (-12.2, 0.25)	0.06
Quartile 2: 21.30-47.56	3019	43	-8.14 (-212.4, 196.1)	0.94	276	43	2.5 (-3.5, 8.5)	0.41
Quartile 3: 47.57-98.68	3079	34	52.1 (-163.5, 267.7)	0.63	273	33	-0.5 (-6.9, 5.9)	0.88
Quartile 4: >98.68	3027	42	1		273	42	1	
sTfR (mg/L)				0.64				0.99
Quartile 1: >6.63	3161	41	130.7 (-81.4, 342.8)	0.23	273	42	0.1 (-6.3, 6.4)	0.99
Quartile 2: 4.82-6.63	3094	39	29.6 (-189.4, 248.6)	0.79	272	38	0.6 (-6.0, 73)	0.85
Quartile 3: 3.76-4.81	3035	36	41.5 (-177.1, 260.4)	0.71	270	36	-0.3 (-6.9, 6.3)	0.92
Quartile 4: <3.76	2967	40	1		274	40	1	
	22 weeks				**22 weeks**			
Haemoglobin (g/dL)				0.52				0.72
Quartile 1: <10.8	3163	38	137.9 (-79.7, 355.42)	0.21	274	40	2.4 (-4.2, 9.0)	0.47
Quartile 2: 10.8-11.2	3068	41	12.6 (-171.9, 257.1)	0.70	272	41	0.4 (-6.1, 6.8)	0.91
Quartile 3:11.3-12.1	3007	36	-18.17 (-229.4, 193.1)	0.87	270	35	-1.5 (-7.9, 4.9)	0.64
Quartile 4: >12.1	3025	41	1		272	40	1	
Ferritin (µg/L)				**0.045**				0.60
Quartile 1: <17.78	3248	33	311.8 (94.8, 528.8)	**0.005**	270	35	-4.2 (-10.8, 2.5)	0.22
Quartile 2: 17.78-31.66	3041	41	104.8 (-104.0, 313.6)	0.32	271	41	-3.6 (-10.1, 2.8)	0.27
Quartile 3: 31.67-58.63	3067	40	131.2 (-73.8, 336.1)	0.21	272	39	-2.9 (-9.2, 3.5)	0.37
Quartile 4: >58.63	2936	40	1		275	39	1	
sTfR (mg/L)				0.35				0.37
Quartile 1: >8.33	2962	36	-109.2 (-330.1, 111.8)	0.33	268	37	-4.7 (-11.3, 1.9)	0.10
Quartile 2: 6.85-8.33	3044	39	-180.6 (-394.5, 33.33)	0.10	273	39	0.6 (-5.9, 7.1)	0.86
Quartile 3: 5.36-6.84	3025	42	-156.2 (-366.8, 54.34)	0.15	273	41	-1.0 (-7.01, 0.2)	0.76
Quartile 4: <5.36	3231	37			275	37	1	
	36 weeks				**36 weeks**			
Haemoglobin (g/dL)				0.99				0.75
Quartile 1: <10.03	3169	31	-0.6 (-242.4, 241.2)	1.00	275	32	-2.1 (-7.2, 3.0)	0.41
Quartile 2: 10.03-11.2	3140	36	-29.0 (-255.1, 197.1)	0.80	275	34	-2.1 (-7.0, 2.8)	0.40
Quartile 3:11.3-12.1	3143	35	-26.1 (-246.4, 194.3)	0.82	274	35	-2.3 (-7.0, 2.4)	0.34

Quartile 4: >12.1	3169	39	1		277	39	1	
Ferritin (µg/L)				**0.004**				0.57
Quartile 1: <13.85	3346	30	410.3 (184.3, 636.3)	**<0.001**	277	31	1.4 (-3.7, 6.5)	0.58
Quartile 2: 13.85-20.79	3129	33	193.7 (-20.7, 408.0)	0.08	273	32	-2.1 (-7.0, 2.7)	0.39
Quartile 3: 20.80-40.08	3212	46	276.2 (72.2, 480.2)	**0.008**	275	46	-0.6 (-5.2, 3.9)	0.79
Quartile 4: >40.08	2936	35	1		275	34	1	
sTfR (mg/L)				0.17				0.84
Quartile 1: >10.53	3300	30	156.1 (-71.8, 384.1)	0.18	275	30	0.8 (-4.2, 5.8)	0.75
Quartile 2: 8.06-10.53	3096	36	-60.1 (-272.3, 152.1)	0.58	274	35	-1.4 (-6.0, 3.2)	0.56
Quartile 3: 6.45-8.05	3102	41	-78.0 (-283.7, 127.6)	0.45	275	41	-0.3 (-4.8, 4.1)	0.88
Quartile 4: <6.45	3149	37	1		276	37	1	

*Birth weight adjusted for maternal age, gestational age at birth, sex of the baby, parity, living standards measure (socio-economic status); HIV status, maternal BMI at enrolment and glucose tolerance.

#Gestational age adjusted for maternal age, delivery intervention, sex of the baby, parity, living standards measure (socio-economic status); HIV status, maternal BMI at enrolment and glucose tolerance

Discussion

In this prospective study of pregnant women residing in Johannesburg, South Africa, the prevalence of anaemia, ID and IDE increased despite iron supplementation forming part of routine antenatal care. We found that ID and anaemia at mid-pregnancy, as well as IDE at late-pregnancy were associated with higher birth weight. In contrast, women with IDE at mid-pregnancy had a 3.6 times higher risk of giving birth prematurely and women with a lower haemoglobin at early pregnancy gave birth significantly earlier than those in the highest haemoglobin quartile.

In this sample of generally healthy, non-smoking, singleton pregnancies from an urban area of South Africa, we observed a similar iron deficiency prevalence during early pregnancy as in women of reproductive age participating in previous national surveys [7,8,40]. More than a quarter (29%) of the women were anaemic at early pregnancy, while 15%, 15% and 9% of women were ID, IDE and IDA, respectively. The WHO recommends routine daily iron supplementation (30-60 mg elemental iron) plus folic acid for all pregnant women to cover increased iron requirements. Furthermore, in settings where at least 40% of pregnant women have haemoglobin concentrations <11 g/dL , a daily dose of 60 mg elemental iron should be preferred over a lower dose [1,41,42]. Even though the prevalence of anaemia is less than 40% in South African women of reproductive age, the recommendation is to supplement all pregnant women with 60 mg elemental iron daily (in conjunction with folic acid and calcium) [11]. In our setting, all pregnant women receive 55 mg elemental iron (170 mg dried ferrous sulphate) daily with folic acid and calcium. Compliance to routine supplementation was high in our study (median of 100%) and some women reported purchasing supplements from shops in addition to the routine regime. Even so, we found significant declines in iron status with pregnancy progression. Two cohort studies in West African countries with routine iron supplementation for pregnant women observed a similar decline in iron status during pregnancy [43,44]. The sharp decline in haemoglobin concentrations that we

observed in our sample of women at mid-pregnancy can be explained by maternal red blood cell mass and plasma volume expansion leading to haemodilution [45].

It is also known that serum ferritin concentrations gradually decline with pregnancy progression. While haemodilution may explain this phenomenon, it has been suggested that declines in serum ferritin concentrations may reflect iron mobilisation from stores to cover increased requirements for red blood cell production, as well as placental transfer to the foetus [46]. In our sample of pregnant women, 47% had elevated sTfR concentrations by late pregnancy. It is unclear why these women receiving iron supplements experienced such a marked increase in sTfR concentrations. Increased sTfR expression is reflective of erythropoietic activity, typically expected with red blood cell mass expansion in pregnancy [47]. In addition to increased need, the women in this study showed significant reductions in ferritin concentrations with pregnancy progression and depleted iron stores could be one reason for increased sTfR [48]. However, increased sTfR is also strongly associated with functional tissue iron deficiency, indicating that iron cannot be mobilised for erythropoiesis despite adequate iron stores [49]. Elevated concentrations of the hormone hepcidin, which is the main regulator of systemic iron homeostasis [46], may explain this observation. With sufficient systemic iron (thus in iron-replete cases), hepcidin concentrations increase, which in turn reduce the release of iron from enterocytes, macrophages and hepatocytes. Conversely, production of hepcidin is suppressed during iron deficiency to allow release of iron from stores and to increase dietary iron absorption. Recent studies showed that hepcidin is actively reduced during the second and third trimesters of pregnancy to support increased iron requirements [44]. However, hepatocyte hepcidin production increases with inflammation irrespective of iron status [46]. More than half of the women enrolled in our study (n=149; 60%) entered the study with elevated CRP (>5 mg/L) concentrations, indicating a high prevalence of acute and sub-clinical inflammation. African ethnicity has been associated with higher circulating CRP concentrations [50]. In addition, this sample of pregnant women had a high prevalence of overweight and obesity

(33% and 28%, respectively), as well as HIV infection (26%), which may have contributed to a more inflammatory state. These factors may have led to an increase in hepcidin concentrations, and consequently to a reduction in iron absorption and release from hepatic stores. In addition, high intakes of calcium (1 g calcium supplementation/day to all pregnant women in South Africa) have been shown to inhibit iron absorption [51]. The *Guidelines for maternity care South Africa* [11] indicates that calcium "is best taken 4 hours before or after iron supplements". However, it is not known how well this recommendation is implemented. This context may explain the increasing prevalence of ID, IDE and IDA with pregnancy progression in an iron supplemented population.

In our sample, the mean daily iron intake (19 mg, 4.6-46.1 mg) at early pregnancy was approximately 3 mg less than the EAR for pregnant women (22 mg/day), but sufficient to meet the EAR for non-pregnant women (8.1 mg/day) [39]. This supports the current recommendation that iron should be supplemented during pregnancy, but arguably at a lower dose in this setting. Women's iron requirements differ depending on stage of pregnancy. Prior to pregnancy, women's iron requirements are higher than for men due to menstruation. In the first trimester of pregnancy iron requirements are less than prior to pregnancy due to cessation of menses [46], while requirements increase drastically from the second trimester due to blood volume expansion and increased erythropoietic activity. Therefore, if reported dietary intakes during early pregnancy are reflective of dietary intakes prior to pregnancy, it is likely that most women achieved the recommended iron intake prior to pregnancy. This may explain the relatively low prevalence of ID at enrolment and highlights the need for exploring other determinants of anaemia [52] in women, such as other micronutrient deficiencies and/or inflammation.

In our sample, anaemia and ID at mid-pregnancy were associated with a 207 g and 205 g higher birth weight, respectively. Consistently, IDE at late pregnancy was associated with a 178 g higher birth weight. When comparing quartiles of ferritin concentration at mid-pregnancy, women in the lowest quartile (ferritin <17.78 μg/L) gave birth to significantly heavier babies (312 g) than women

in the highest quartile (ferritin >58.63 μg/L). To our knowledge, we are the second study in an African setting (with routine iron supplementation [43]) to find this association. In the cohort of pregnant women in Papua New Guinea (n=279), malaria infection was common and the prevalence of anaemia (haemoglobin <11 g/dL) at enrolment (±25 weeks of gestation) very high (95%). Lower ferritin concentrations at enrolment were associated with higher mean birth weights, and iron deficient women gave birth to 230 g heavier newborns when compared to iron-replete women. The authors indicated that only 7% and 12% of the association was mediated through placental and peripheral malaria infection, respectively, demonstrating an association between iron deficiency and higher birth weight through Malaria-independent mechanisms. Similar associations have been found elsewhere. A Chinese cohort study (n=511) of non-anaemic pregnant women receiving iron supplements as part of routine antenatal care also found a significantly higher birth weight in the lowest compared to the highest ferritin quartile [53]. Furthermore, an Indian cohort (n=1196) (non-anaemic with supplementation) showed similar results with the highest tertile of supplemental iron intake associated with low birth weight [54].

The observed inverse associations between maternal iron status and birth weight in settings of routine iron supplementation can be interpreted from two viewpoints. Firstly, the association between ID, anaemia and IDE with higher birth weight could be an indication that antenatal iron supplementation is protective in iron depleted women, resulting in improved foetal growth [55]. Systematic reviews on the efficacy of antenatal iron supplementation versus placebo have shown significant reductions in anaemia and iron deficiency at term. Evidence for a beneficial effect on birth outcomes is, however, less clear [56–58]. The most recent Cochrane review indicated that there is low quality evidence for iron supplementation reducing the risk for low birth weight (RR 0.84; 95% CI: 0.69, 1.03; 11 studies) [57]. In contrast, there is emerging evidence of a U-shape association of iron status and haemoglobin with birth weight [5,6]. Thus, the second viewpoint is from the right side of this U-shape association, i.e. high iron intakes being associated with lower

birth weight. Routine iron supplementation in a mixed population of deficient and replete women may contribute to mixed results. In our sample, iron supplementation may have had a negative impact on foetal growth in iron-replete women. Hwang and colleagues [59] found that excessive maternal iron intake at mid-pregnancy was associated with reduced foetal growth in a South Korean cohort (n=337). The foetuses of women in the third tertile of total iron intake had smaller outcomes in biparietal diameter, abdominal circumference and femur length at mid-pregnancy. Even though this South Korean cohort demonstrated lower than recommended iron intake from foods, supplementation contributed to intakes above the Tolerable Upper Intake Level (45 mg). Other studies indicate that iron supplementation in iron-replete women is associated with adverse maternal and foetal outcomes [54,60], although results are inconsistent [61]. When considering the median ferritin and haemoglobin concentrations as well as the prevalence of ID and anaemia at early pregnancy in our sample, it is apparent that most women were iron-replete while receiving iron supplementation. We speculate that provision of supplemental iron in these iron-replete women may have had negative consequences to the mother, which may have compromised foetal growth. These negative consequences may include oxidative damage and haemoconcentration which consequently result in impaired placental perfusion and thus foetal growth [58,62]. There is evidence that unabsorbed supplemental iron reaching the colon can negatively alter gut microbiome composition and increase gut inflammation [63]. This supports the notion that strategies to prevent iron deficiency during pregnancy should consider the inflammation burden in the target population, and take relevant actions to reduce inflammation in an effort to improve iron absorption and utilisation.

When we assessed the predictors for gestational age, associations were different than for birth weight. Firstly, lower haemoglobin in early pregnancy was associated with shorter gestation which supports previous research [64]. It is suspected that these women entered pregnancy with anaemia since the quartiles associated with the shorter gestation reflects pre-pregnancy anaemia, i.e.

haemoglobin <12 g/dL, and haemodilution only peaks late in second trimester or early third trimester [45]. Secondly, as expected, poor iron status increased the risk for premature birth. IDE at mid-pregnancy quadrupled the risk for premature birth. It would be expected that in a supplemented sample the risk would be attenuated in the ID group [57], however, as explained above, elevated sTfR concentrations could be a consequence of inflammation causing iron not to be mobilised.

The key strength of this study was that data were collected prospectively with multiple variables and data collection points across pregnancy. In addition, the iron biomarkers assessed allowed for a complete description of iron status. Previous studies may not have considered concomitant inflammation when interpreting iron status data, however, our analyses included CRP and AGP, which were used to adjust ferritin concentrations accordingly [29]. The analyses were strengthened due to assessment and inclusion of several confounders.

The study limitations should be considered when interpreting the results. Due to the observational study design, conclusions on causality of observed relationships are not possible. However, our findings may generate hypotheses for further investigation, given the consistency of results. An additional limitation is self-selection bias at recruitment since women were recruited at primary healthcare clinics and then volunteered and agreed to participate at a different setting. Lost to follow-up resulted in missing birth weight data, which may have skewed results. Lastly, the sample was of relatively small size and not representative of the general population. Women were selected to be non-smoking, generally healthy and presented at primary healthcare clinics somewhat earlier than the typical 20 weeks of gestation [65].

In conclusion, we found an increase in ID, IDE and IDA with pregnancy progression despite routine iron supplementation in an urban South African setting. We observed an inverse association between maternal iron status and birth weight, while IDE at mid-pregnancy increased the risk for premature birth. These results add to the raising concern on the consequences of iron

supplementation in iron-replete pregnant women. Nonetheless, there is no question that ID and anaemia should be prevented in pregnancy. However, the challenge remains on how to do so safely in a public health setting. Considering that South Africa has a well-implemented food fortification programme, high prevalence of inflammation, possible influence of antenatal calcium supplements on iron absorption, as well as the known risks associated with both low and high iron exposure, we recommend that the current antenatal supplementation regime in South Africa be revisited.

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Supplementary table 1: Characteristics and iron status of pregnant women from the NuPED study at enrolment (<18 weeks of gestation) by birth weight data availability¥

Characteristics at enrolment	**Birth weight data obtained 84% (n=203)**	**Birth weight data missing 16% (n=40)**	***p*†**	**Total sample (n=250)*** *Median (IQR) or n (%)*
Age (years)	28 (24-32)	27 (24-30)	0.45	27 (24-32)
Gestational age (weeks)	14 (12-16)	14 (13-16)	0.20	14 (12-16)
BMI (kg/m^2) (n=242)	26.3 (23.1-30.6)	26.5 (22.2-33.7)	0.98	26.3 (23.0-30.6)
Underweight (<18.5 kg/m^2)	7 (4)	0	0.62	8 (3)
Normal weight (18.5-24.9 kg/m^2)	72 (36)	15 (38)		89 (36)
Overweight (25-29.9 kg/m^2)	67 (33)	12 (30)		81 (33)
Obese (≥30 kg/m^2)	56 (28)	13 (33)		71 (28)
Ethnicity (n=242)				
Black African	176 (87)	36 (92)	0.07	219 (88)
Mixed ancestry	27 (13)	2 (5)		28 (11)
White	1 (1)	0		1 (<1)
Indian	0	1 (3)		1 (<1)
Country of birth (n=232)				
South Africa	140 (72)	27 (71)	0.15	172 (72)
Zimbabwe	51 (86)	8 (14)		60 (25)
Lesotho	2 (1)	2 (5)		4 (2)
Swaziland	1 (1)	1 (3)		3 (1)
Living Standards Measure (LSM) (n=243)				
Low (LSM 1-4)	12 (6)	4 (10)	0.63	17 (7)
Medium (LSM 5-7)	120 (59)	23 (58)		148 (59)
High (LSM 8-10)	71 (35)	13 (33)		85 (34)
Marital status (n=242)				
Unmarried/single	84 (41)	12 (31)	0.29	100 (40)
Married	51 (25)	16 (41)		68 (27)
Divorced/Separated	2 (1)	0		2 (1)
Living together	47 (23)	9 (23)		57 (23)
Traditional marriage#	19 (8)	2 (5)		22 (9)
Highest level of education (n=242)				
Primary school or less	5 (3)	4 (10)	0.17	9 (4)
Grade 8 – 10	27 (13)	8 (21)		37 (15)
Grade 11 – 12	121 (60)	20 (51)		145 (58)
Post-school education	50 (25)	8 (18)		58 (23)
Parity (n=243)				
Nulliparous	61 (30)	10 (25)	0.88	74 (30)
Primiparous	70 (35)	15 (38)		88 (35)
Multiparous	72 (35)	15 (38)		88 (35)
HIV status (n=243)				
Positive	49 (24)	13 (33)	0.27	64 (26)
Negative	154 (76)	27 (67)		186 (74)
Inflammatory status (n=243)				
Normal CRP	83 (41)	14 (35)	0.49	149 (60)
Elevated CRP (>5 mg/L)	120 (59)	26 (65)		
Normal AGP	178 (88)	38 (95)	0.18	28 (11)
Elevated AGP (>1 g/L)	25 (12)	2 (5)		
Anaemia (n=236)				
Normal haemoglobin	146 (75)	22 (55)	**0.01**	173 (71)
Anaemic (Hb <11g/dL)	50 (25)	18 (45)		70 (29)
Iron stores (n=243)				
Normal serum ferritin	179 (88)	28 (70)	**<0.01**	213 (85)
Iron depleted (Fer <15 µg/L)	24 (12)	12 (30)		37 (15)
Iron deficiency erythropoiesis (n=243)				
Normal sTfR	179 (85)	33 (83)	0.66	212 (85)
Increased sTfR (sTfR >8.3 mg/L)	30 (15)	7 (17)		38 (15)

IQR: interquartile range; CRP: C-reactive protein; AGP: α_1-acid glycoprotein; LSM: Living Standards Measure; Hb: Haemoglobin; Fer: ferritin; sTfR: soluble transferrin receptor.

Data are presented as n (%) for categorical variables and median (IQR) for continuous variables.

¥ Women who had a miscarriage or intrauterine foetal death (IUFD) also had missing birth weight data (n=7), but were not considered in this table.

† Mann-Whitney-U test for continuous variables, and Chi-square test for categorical variables.

* n-values are equal to 250 for LSM, highest level of education, parity, HIV status, iron stores and iron deficiency erythropoiesis; 243 for anaemia; 239 for Country of birth; and 249 for all other variables.

#: Traditional marriage, recognised under South African customary law, is entered between parties based on tradition which does not require the approval of an officiator for validation. It is also different from civil marriage in that a polygamous marriage is permissible.

Annexure 11: Questionnaires used for exposure and outcome variables

NuPED

Nutrition during Pregnancy and Early Development

Quantitative Food Frequency Questionnaire

						Name of fieldworker:

Participant number *Phase*

Today's date: | 2 | 0 | 1 | | | | | | **Day of the week:**

year *month* *day*

Please think carefully about the food and drink you have consumed during the ***PAST MONTH*** (four weeks). We have divided the foods into different groups for example all the porridges and cereals together. I will go through a list of food groups and drinks with you and I would like you to tell me:

- Which foods you eat in each of the different food groups
- How the food is prepared
- How much of the food you eat at a time
- How many times a day you eat it and if you do not eat it everyday, how many times a week or a month you eat it.

To help you to describe the amount of a food you eat, I will show you pictures of different amounts of the food as well as other food models, containers, etc.

There are no right or wrong answers.

Everything you tell me is confidential. Only your subject number appears on the form.

Is there anything you want to ask now?

Are you willing to go on with the questions?

Before we start I would like to find out what type of margarine, oil and milk you **USUALLY** use in your home.

1. What type of **MARGARINE** do you USUALLY use in your home? Give brand name if possible. Mark O☐
   - ☐ Tub/Soft margarine (brand name) _____
   - ☐ Brick/Hard margarine (brand name) _____
   - ☐ I don't know
   - ☐ Do not use margarine in home
   - ☐ Butter (brand name) _____

2. What type of **OIL** do you USUALLY use in the preparation of food in your home? Mark ONE.
   - ☐ Sunflower oil (give brand name)_____
   - ☐ Canola oil (give brand name) _____
   - ☐ Olive oil (give brand name) _____
   - ☐ Other (give brand name) _____
   - ☐ Oil previously used _____
   - ☐ I don't know
   - ☐ Do not use OIL ever in the home

3. What type of **MILK** do you USUALLY use in your home? Mark only ONE
   - ☐ Full cream milk / Fresh cow's milk/ Box milk full cream
   - ☐ Low fat milk / 2% milk / Box low fat or 2% milk
   - ☐ Fat free milk / Skim milk / Box fat free or skim milk
   - ☐ Powder milk (eg Elite; give brand name) _____
   - ☐ I don't know
   - ☐ Do not use milk

4. What type of **CREAMER** do you USUALLY use in your home?
   - ☐ Cremora, Ellis Brown, Coffee Mate, Tea Mate etc
   - ☐ Cremora Lite
   - ☐ I don't know
   - ☐ Do not use creamer

QUANTIFIED FOOD FREQUENCY QUESTIONNAIRE

INSTRUCTIONS: Circle the subject's answer. Fill in the amount and times eaten in the appropriate columns.

I shall now ask you about the type and the amount of food you have been eating in the **LAST MONTH**. Please tell if you eat the food, how much you eat and how often you eat it. We shall start with maize meal porridge.

In the last **four weeks**, did you eat…?

MAIZE MEAL, COOKED PORRIDGES AND BREAKFAST CEREALS								
FOOD	**DESCRIPTION**	**AMOUNT**	**TIMES EATEN Complete one column**				**CODE**	**AMOUNT / WEEK**
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Maize-meal porridge	Stiff (pap)						4401	
	Soft porridge (slappap)						4400	
	Crumbly (phutu)						4402	
Sour porridge (Tini)	Maize meal						9829	
	Mabella						9827	
	Other:							
Mabella	Stiff						3437	
	Soft							
Oats							3239	
Tastee wheat	Soft						3240	
Other cooked porridge	Type							
Morvite	Soft						9804	
Breakfast cereals	All bran flakes						3242	
	Corn flakes plain						3243	
	Weetbix						3244	
	Rice crispies plain						3252	
	Other:							

If yes, in the last **four weeks**, how often did you eat the food?

Do you pour milk on your maize meal (e.g. stiff, phutu soft porridge), cooked porridge or cereal?

Ye 1 No 2

If yes, what type of milk (whole fresh, sour, 1%, fat free, milk blend, etc) _____
If no, go directly to the "sugar" section.

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	Weekly Times/ week	Monthly Times/ month	No		
If yes, how much milk	Whole milk/full cream milk/ fresh cow's milk						2718	
	Maas/sour milk						2787	
	Low fat / 2% milk						2772	
	Fat free / skim milk						2775	
	Other							

Do you put sugar on your porridge or cereal? 1 Ye 2 No

If no, go directly to the next question "do you put anything else in your porridge?".

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	Weekly Times/ week	Monthly Times/ month	No		
If yes, how much sugar WHITE or BROWN	Cooked porridge						3989	
	Cereal						3989	
	Other porridge / cereal						3989	
	Other							

Do you put anything else in your porridge? 1 Ye 2 No

If yes, what? _____ How much? _____

OTHER STARCH

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Samp	Bought Self ground						3250	
Samp and beans	Give ratio of samp:beans						3402 (1:1)	
Samp and other (e.g. peanuts)	Give ratio of samp:other Specify other:						3250 (samp)	
Rice	White						3247	
	Brown						3315	
	Maize Rice						3250	
	Any fat added?							
Pasta	Macaroni, plain						3262	
	Spaghetti, plain						3262	
	Spaghetti, canned in tomato sauce						3258	
	Macaroni & cheese Cheese: Milk: Fat:							
	Other specify							
Pizza	Home made: Specify topping						3353 (base+ch +tom+oliv)	
	Bought: Specify topping						3353 (base+ch +tom+oliv)	

You are being very helpful. Can I now ask you about meat?

CHICKEN, MEAT, FISH

How many times do you eat meat (beef, mutton, pork, chicken, fish) per week?

_____

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Chicken	Meat & skin, boiled						2926	
	Meat without skin, boiled						2963	
	Meat & skin, roasted/ grilled						2925	
	Meat without skin, roasted/ grilled						2950	
	Kentucky / Chicken Licken (Fried in batter/crumbs)						3018	
	Nando's						2925	
	Other							
Chicken stew	With potato and onion WITH skin						9813	
	With tomato and onion WITH skin						2985	
	With vegetables WITH skin						3005	
	With tomato and onion NO skin						4379	
	With vegetables NO skin						4378	
Chicken BONE stew	With potato, onion and tomato						9814	
	Other							
Chicken feet	Nothing added						2997	
	Stew with potato, onion and tomato						9815	
Chicken head							2999	
Chicken offal	Stew with tomato and onion and sunflower oil						9816	
	Liver, cooked						2970	
	Other							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
RED MEAT	**How do you like your meat?** With fat **OR** Fat trimmed							
Red meat BEEF	BRISKET, boiled/fried without added fat						4363	
	BRISKET, fried in added fat Type of fat:						4363	
	Beef, stewed with cabbage						3006	
	Beef, stewed with potato, onion and tomato						9817	
	Beef, stewed with vegetables						3020	
	Mince (lean/ topside), nothing added						2921	
	Mince (regular), nothing added						4363	
	Mince, tomato & onion added						2987	
	Beef **BONE** stew with potato and onion and oil						9819	
	Other							
MUTTON	Meat, with fat, cooked						2947	
	Mutton, no fat, cooked						3036	
	Mutton, chop, grilled						2927	
	Mutton, stewed with vegetables						2916	
	Other							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Beef/mutton Offal	Offal, cooked						3003	
	Stewed with vegetables							
	Liver, beef, fried/cooked						2920	
	Liver, sheep, fried/cooked						2955	
	Kidney, beef, cooked						2923	
	Kidney, sheep, cooked						2956	
	Brain, sheep, cooked						2952	
	Lung, beef, cooked						3019	
	Lung, sheep, cooked						4337	
	“Gemaldes” (lung & fat)						9809	
	Heart, beef, cooked						2968	
	Heart, sheep, cooked						2969	
	Other							
Goat meat	Grilled/roasted/cooked						4281	
	Stewed with vegetables							
	Other							
Venison/ Wild buck							2913	
Horse/Donkey							9807	
Rabbit							4327	
Other type of meat	Specify							

What type of vegetables is usually put into meat stews?

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Wors / Sausage	Beef & pork, boerewors						2931	
Bacon							2906	
Patties	Beef, fried						2984	
	Chicken, fried						3011	
Cold meats AND Processed meats	Polony						2919	
	Ham						2967	
	Vienna						2936	
	Frankfurter, beef & pork						2937	
	Frankfurter/Sausage, chicken						3012	
	Russian/Salami						2948	
	Other							
Canned meat	Bully beef, plain						2940	
	Bully beef with potato & onion & oil						2994	
	Other							
Meat pie **BOUGHT Or HOMEMADE**	Beef						2939	
	Steak and kidney						2957	
	Sausage roll						2939	
	Cornish						2953	
	Chicken						2954	
	Other							
Hamburger	Bought						9818	
	Other							
Biltong	Beef (with fat OR without fat)						3021	
Dried wors Dried sausage	Beef						2949	

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Dried beans	Baked beans in tomato sauce						3176	
	Bean salad / Sousbone						3174	
	Soup with dried beans, beef and vegetables						3145	
	Sugar beans, cooked						3205	
	Other							
Lentils	Whole, cooked						3203	
	Lentil soup with beef and vegetables						3153	
Soya products eg. Imana, Knorr, Jileleke, Toppers	Cooked						3196	
	Soup/Gravy made with soya products						9831	
	Stewed with extra potato, onion and tomato						9830	
	Other							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Pilchards in tomato sauce or chilli or brine	Whole						3102	
	Mashed with fried onion						3102 (70%) 3730 (30%)	
	With tomato and onion						9820	
	Other							
Fish	Hake, fried with batter/crumbs in sunflower oil						3072	
	Hake, fried in sunflower oil						3060	
	Hake, steamed						4373	
	Moddervis / Yellow fish* fried in oil						3084	
	Moddervis / Yellow fish baked with onion (NO oil added)						3089	
	Other							
Other canned fish	Tuna in oil						3056	
	Sardines in oil						3104	
	Sardines in tomato sauce						3087	
	Other							
Fish cakes	Bought: Fried						3080	
	Home made with potato, fried in sunflower oil						3098	
Fish fingers	Bought (baked)						3081	
Eggs	Boiled/poached						2867	
	Scrambled (full cream milk & brick margarine)						2890	
	Scrambled (NO milk, ONLY oil added)						2869	
	Scrambled (NO oil, ONLY full cream milk)						2872	
	Fried in oil						2869	
	Fried in brick margarine						2877	
	Other							

Moddervis/ yellow fish is a more fatty fish than hake.

VEGETABLES

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Cabbage	**How do you cook cabbage?**							
	Boiled, nothing added						3756	
	Boiled with potato and onion and sunflower **oil**						3815	
	Boiled with potato and onion and **brick margarine**						3813	
	Fried in **oil**						3812	
	Fried in **brick margarine**						3810	
	Boiled with potato, onion and tomato and oil						9821	
	Raw with nothing added						3704	
	Other							
Spinach or morogo or beetroot leaves or other green leafy	**How do you cook spinach?**							
	Boiled, nothing added						3913	
	Boiled with **oil** added							
	Boiled with **brick margarine** added						3898	
	Boiled with **tub margarine** added						3899	
	Boiled with potato, onion and tomato and oil						9822	
	Other							
Tomato and onion gravy	With oil						9823	
	Without fat, without sugar						3925	
	Canned						4192	
	Thickened with packet soup powder						9832	
	Other							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Pumpkin (yellow) Butternut Hubbard squash Table Queen Etc	Boiled, nothing added						4164	
	Boiled with sugar only (NO fat)						3728	
	Boiled with **brick margarine** & sugar						3893	
	Boiled with **tub margarine** and sugar						9833	
	Boiled with **oil** and sugar						9828	
	Other							
Carrots	Boiled, nothing added						3757	
	Boiled with **oil** added							
	Boiled with **brick margarine** added						3816	
	Boiled with **tub margarine** added						3817	
	Boiled with **sugar** only						3818	
	Boiled with **oil** and sugar							
	Boiled with **brick margarine** and sugar						3819	
	Boiled with **tub margarine** and sugar						3820	
	Boiled with potato, onion and sunflower **oil**						3824	
	Boiled with potato, onion and **brick margarine**						3822	
	Boiled with potato, onion and **tub margarine**							
	Chakalaka						9812	
	Raw, nothing added						3709	
	Other							
Mealies/ Sweet corn	On cob – fat added **Fat:**						3725	
	On cob – no fat added						3725	
	Creamed sweet corn / canned						3726	
	Whole kernel/canned						3942	
	Whole kernel, frozen, boiled						4132	
	Other							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Beetroot	Salad						3699	
	Boiled, nothing added						3698	
How do you cook potatoes?								
Potatoes	Boiled/baked with skin						4155	
	Boiled/baked without skin						3737	
	Boiled with sunflower **oil** added						3873	
	Boiled with **brick margarine** added						3867	
	Boiled with **tub margarine** added						3868	
	Mashed with **whole milk** and **brick margarine**						3876	
	Mashed with **whole milk** and **oil**							
	Roasted in beef fat						3878	
	Roasted in sunflower oil						3979	
	French fries (chips) / Fried potatoes						3740	
	Other							
Sweet potatoes	**How do you cook sweet potatoes?**							
	Boiled/baked with skin						3748	
	Boiled/baked without skin						3903	
	Boiled with **sugar** and **oil** added						9834	
	Boiled with **sugar** and **brick margarine** added						3749	
	Other							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Broccoli	Boiled						3701	
	Raw						3702	
Cauliflower	Boiled						3716	
Green beans	Boiled, nothing added						3696	
	Cooked with potato, onion and sunflower **oil**						3794	
	Cooked with potato, onion and **brick margarine**						3792	
	Other							
Mixed vegetables	Canned						4264	
	Frozen, boiled (carrot, corn, peas, green beans)						3727	
	Frozen, boiled (carrot, cauliflower, green beans)						4265	
	Other							
Salad vegetables	Mixed salad: tomato, lettuce and cucumber (no dressing)						3921	
	Raw tomato						3750	
	Cucumber, raw						4119	
	Coleslaw (cabbage) (mayonnaise)						3705	
	Coleslaw (cabbage) (commercial)						3707	
	Potato salad (mayonnaise)						3928	
	Baked bean salad						9824	
	Other salad vegetables							
Mayonnaise / salad dressing	Mayonnaise						3488	
	Vinegar, oil						3487	
	Low oil salad dressing						3505	
	Salad cream						3489	
	Other: Specify							
Other vegetables (specify prep)								

Now we come to fruit

FRUIT

Do you like fruit? Yes 1 No 2

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Apples							3592	
Banana							3540	
Pears							3582	
Oranges							3560	
Naartjie							3558	
Grapes							3550	
Peaches	Fresh						3565	
	Canned						3567	
Apricots	Fresh						3534	
	Canned						3535	
Mangoes							3556	
Guavas	Fresh						3551	
	Canned						3553	
Watermelon	Fresh						3576	
Fruit salad	Fresh						3588	
	Canned						3580	
Fig (Vye)							3544	
Avocado							3656	
Wild fruit/berries	Specify type							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Dried fruit	Apple, dried, raw						3600	
	Peach, dried, raw						3568	
	Mixed fruit, dried, raw						3593	
	Mixed fruit, dried and cooked with sugar						3590	
	Fruit roll, dried (all types)						3655	
	Other							
Other fruit	_____ ___ _____ ___							

Let me ask you about ***Custard***.

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Custard	Homemade, full cream milk or fresh cow's milk						2716	
	Homemade, lowfat milk						2779	
	Homemade, skim milk						2717	
	Commercial eg Ultramel						2716	
	Other							
Custard with other food (e.g. with jelly, fruit salad, baked pudding)								

BREAD AND BREAD SPREADS

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Bread / Bread rolls	White						3210	
	Brown						3211	
	Whole wheat						3212	
Do you spread anything on the bread?			Always		Sometimes		Never	
Margarine	**What brand do you have at home now?**							
	Tub, regular						3496	
	Tub, medium fat						9806	
	Tub, light/low fat						3524	
	Brick, regular						3484	
	Brick, medium fat						9805	
	Brick, lite/low fat						3528	
	Other							
Peanut butter							3485	
Jam/syrup/ honey							3985	
Marmite / Fray bentos / Oxo							4058	
Fish/meat paste							3109	
Cheese	Cheddar						2722	
	Gouda						2723	
	Other							
Sandwich spread							3522	
Achaar							3117	
Other spreads	Specify							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Dumpling	White flour						9835	
	Whole wheat flour						3212	
Vetkoek	White flour						3257	
	Whole wheat flour						3324	
Provita, crackers, etc	Provita						3235	
	Cream crackers						3230	
	Other savoury biscuits like Bacon kips, wheat crackers, etc						3331	

DRINKS

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Tea	English (normal)						4038	
	Rooibos						4054	
Coffee							4037	
White sugar	Tea						3989	
	Coffee						3989	
Brown sugar	Tea						4005	
	Coffee						4005	
Milk per cup of TEA	**Do you use milk in your TEA? If no, go to milk in coffee.** Ye / No **If YES, What type of milk do you use in TEA?**							
	Fresh / long life whole/full cream						2718	
	Fresh/long life: 2%/low fat						2772	
	Fresh/long life: fat free / skim milk						2775	
	Creamer/whitener like Ellis Brown / Cremora						2751	
	Cremora **Lite**							
	Condensed milk						2714	
	Evaporated milk						2715	
	Other							
	None							
Milk per cup of COFFEE	**Do you use milk in your COFFEE? COFFEE? If no, go to milk as such.** Ye / No **If YES, What type of milk do you use in**							
	Fresh/long life: whole/full						2718	
	Fresh/long life: 2%/low fat						2772	
	Fresh/long life: fat free						2775	
	Creamer/whitener like Ellis Brown						2751	
	Cremora **Lite**							
	Condensed milk						2714	
	Evaporated milk						2715	
	Other							
	None							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Milk as such	**What type of milk do you drink milk as such?**							
	Fresh/long life: whole / full cream milk						2718	
	Fresh/long life: 2% milk / low fat milk						2772	
	Fresh/long life: fat free / skim milk						2775	
	Condensed milk						2714	
	Sour/maas						2787	
	Other							
Milk drinks	Flavoured milk						2774	
	Milo made with full cream milk						2735	
	Milo made with skim milk						2747	
	Drinking chocolate made with water						4287	
	Other							
Yoghurt	Drinking yoghurt low fat						2756	
	Plain low fat						2734	
	Low fat sweetened with fruit						2732	
Squash	Sweet O						4027	
	Six O							
	Oros/Lecol – with sugar or other						3982	
	- artificially sweetener						3990	
	KoolAid (powder mixed with water)						4027	
	Other							
Fizzy drinks Coke, fanta, etc	Sweetened						3981	
	Diet							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Fruit juice	Fresh/Liquifruit/Ceres						2866	
	Tropica (Dairy –fruit juice mix)						2791	
	Other							
Mageu/Motogo							4056	
Home brew beer							4039	
Beer							4031	
Cider	Sweet						4057	
Spirits Eg Brandy, gin, vodka, whisky, cane, etc							4035	
Wine red							4033	
Wine White							4033	
Other specify								
WATER	Tap, borehole, dam, river, etc						4042	
	Bottled						4042	

SNACKS AND SWEETS

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Potato crisps							3417	
Peanuts	Raw						4285	
	Roasted						3458	
Other nuts								
Savoury snack e.g. Fritos, Doritos, Cheese curls, Niknaks							3267	
Raisins							3552	
Peanuts and raisins								
Chocolates	Milk chocolate, plain						3987	
	Kit Kat/ Tex (with wafers) etc						4024	
	Chocolate coated bars like Bar One, TV bar, etc						3997	
	Other							
Popcorn	Plain						3332	
	Sugar-coated/candied						3359	
Candies/Sweets	Sugus, gums, hard sweets, etc						4000	
Toffees / Fudge / caramels							3991	
Biscuits/cookies	Homemade, plain						3233	
	Commercial, plain						3216	
	Commercial, with filling						3217	
	Other							
Cakes	Butter cake, homemade with **whole milk** and **brick margarine NO icing**						3288	
	Chocolate cake, homemade with **whole milk and brick margarine NO icing**						3289	
	Icing for cake made with **brick margarine**						4014	
	Other							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Tarts	Apple tart with a batter made with whole milk and brick margarine						3327	
	Other							
Scones	Plain made with whole milk and brick margarine						3237	
	Other							
Muffin	Bran						3407	
	Plain						3408	
	Other							
Rusks	Buttermilk, commercial						3329	
	Homemade, white						3222	
	Other							
Savouries	Sausage rolls, small						2939	
	Samoosas: Meat filling						3355	
	Samoosas: Vegetable filling						3414	
	Biscuits eg bacon kips						3331	
	Other							

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Jelly	Jelly						3983	
	Custard added made with whole milk	Yes/No					2716	
	Other							
Baked pudding	Baked in a syrup						3312	
	Baked without a syrup						3429	
	Custard added made with whole milk	Yes/No					2716	
	Other							
Instant pudding	Made with whole milk						3266	
	Made with low fat milk						3395	
	Other							
Ice cream	Regular						3483	
	Soft serve						3518	
	Other							
Sorbet							3491	
Other specify								

SAUCES, GRAVIES AND CONDIMENTS

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		
Tomato sauce							3139	
Worcester sauce							4309	
Chutney							3168	
Pickles							3866	
White sauce	Made with whole milk and brick margarine						3142	
Packet soups	Dry powder (all types)						3158	
	Made with water (all types)						3165	
Gravy	Made from meat and thickened						3120	
Other								

WILD FRUITS, WILD BIRDS, ANIMALS OR INSECTS (hunted in rural areas or on farms)

FOOD	DESCRIPTION	AMOUNT	TIMES EATEN Complete one column				CODE	AMOUNT / WEEK
			Daily Times/ day	**Weekly** Times/ week	**Monthly** Times/ month	**No**		

MISCELLANEOUS: Please mention *ANY OTHER FOODS* used more than once/two times a week which we have NOT talked about

INDIGENOUS/TRADITIONAL FOODS/PLANTS/ANIMALS

Please tell me if you use any indigenous plants OR other indigenous foods like mopani worms, locusts ect to eat

PLEASE GIVE DETAILS

NuPED

Nutrition during Pregnancy and Early Development

24-hour recall dietary intake form

Fieldworker: _____

Participant nr: | | | | **Phase:** | | **Date:** | 2 | 0 | Y | Y | M | M | D | D |

1. What day was yesterday? (tick correct one)

Monday	*Tuesday*	*Wednesday*	*Thursday*	*Friday*	*Saturday*	*Sunday*

2. Would you describe the food that you ate yesterday as typical of your usual food intake?

Yes	1

No	2

Greetings!

Thank you for giving up your time to participate in this study. I hope you are enjoying it so far. Here we want to find out what people living in this area eat and drink. This information is important to know as it will tell us if people are eating enough and if they are healthy.

There are no right or wrong answers.

Everything you tell me is confidential. Only your subject number appears on the form.

Is there anything you want to ask now?

Are you willing to go on with the questions?

I want to first ask you a few general questions about your food intake, the preparation of food and the type of food that you use in your home. Answer the following few questions in terms of your habits in the **past month**.

Instruction

Tick the box with the subject's answer.

3. What type of pot do you usually use to prepare food in? (may answer more than one)

Iron pot …… ☐ **1**

Stainless steel pot …… ☐ **2**

Aluminium pot …… ☐ **3**

Glassware …… ☐ **4**

Other (specify) …… ☐ **5**

4. Do you eat maize meal porridge? Yes ☐ **1** No ☐ **2**

If YES, what type do you have at home now?

Brand name: _____

Don't know: _____ **2**

Grind self: _____ **3**

If brand name is given, do you usually use this brand? Yes ☐ **1** No ☐ **2** Don't know ☐ **3**

4.1 Where do you get your maize meal from? (may answer more than one)

Shop …… ☐ **1**

Employer …… ☐ **2**

Harvest and grind self …… ☐ **3**

Other (specify) …… ☐ **4**

Don't know …… ☐ **5**

5. Do you eat fat/margarine or use it in the preparation of food? Yes ☐ No ☐

If YES, what type do you have at home now?

Brand name: _____

Don't know: _____ **2**

If brand name is given, do you usually use this brand? Yes ☐ **1** No ☐ **2** Don't know ☐ **3**

6. Do you use oil in the preparation of food? Yes | No

If YES, what type do you have at home now?

Brand name: _____

Don't know: _____ **2**

If brand name is given, do you usually use this brand? Yes **1** | No **2** | Don't know **3**

6.1 Do you deep fry any food? Yes **1** | No **2**

What type of oil do you buy for deep frying?

Brand name: _____

Do you use the same oil more than once? Yes **1** | No **2**

If yes, how many times will you use the same oil? _____

7. What type of salt do you use?

Give brand names _____

Do you add salt to food while it is being cooked?	Always	Sometimes	Never	Don't know

Do you add salt to your food after it has been cooked?	Always	Sometimes	Never

Do you like salty foods e.g. salted peanuts, crisps, chips, *Fritos*, biltong, dried sausage, etc

Very much	Like it	Not at all

8. Supplement use:

8.1 Did you receive any vitamins or vitamins and minerals at the antenatal clinic?

Yes	1	No

Do you use any of the following?

	Name of product (Brand)* Type?	**Quantity of capsules/ pills at a time?**	**How many times/week**	**When did you start taking these?**
8.2 Vitamins and/or minerals from the **shop**				
8.3 Vitamins and/or minerals from **clinic**				
8.4 Tonics				
8.5 Health foods				
8.6 Body building preparations/shakes				
8.7 Dietary fibre supplement				
Other: Specify _____				

If the answer is "I don't know" request the woman to bring the supplement with to the next visit.

8.8 If you <u>are</u> using the supplements from the shop or clinic, do you take it with food or drink?

Yes	1	No

Specify:

Prompt for coffee, tea, juice, cereal, porridge (pap), bread, meat, chicken, green veg, milk, fruit

_____

_____

8.8.1 If yes, do you usually take the supplement before, during or after a meal?

Before	During	After

8.8.2 What is the typical meal you have when you take the supplement?

_____

_____

8.9 If you are <u>not</u> using the supplements from the clinic, please tell me why not?

_____

_____

Now I want to find out about everything you ate or drank yesterday, including water or food you pick from the veld. Please tell me everything you ate from the time you woke up yesterday up to the time you went to sleep. I will also ask you where you ate the food and how much you ate.

To help you to describe the amount of a food you eat, I will show you pictures and examples of different amounts of the food. Please say which picture or example is the closest to the amount you eat, or if it is smaller, between the sizes or bigger than the pictures.

After you woke up yesterday, when was the first time you ate yesterday?

Time	Place	Description of food and preparation method	Amount	Amount in gram (office)	Code (office)

Time	Place	Description of food and preparation method	Amount	Amount in gram (office)	Code (office)

Did you taste any foods during preparation?
Did you snack on anything during the day?

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Biological samples sheet

Y = Yes N = No NA = Not assessed

Participant nr: | | | |

Circle the appropriate response or write the value where necessary.

DATE: (DD/MM/YYYY)					
FIELDWORKER:					
	Phase 1 (<18 wks)	**Phase 2 (22 wks)**	**Phase 3 (36 wks)**	**Phase 4 (birth) MATERNAL**	**Phase 4 (birth) CORD**
Urine sample collected	YES / NO	YES / NO	YES / NO	*Not applicable*	*Not applicable*
Time collected				*Not applicable*	*Not applicable*
Glucose in urine (MCR)	Y / N / NA	Y / N / NA	Y / N / NA	*Not applicable*	*Not applicable*
Protein in urine (MCR)	Y / N / NA	Y / N / NA	Y / N / NA	*Not applicable*	*Not applicable*
Phlebotomist (Name):					
Time of blood collection					
Blood sample: *Nr of EDTA tubes (purple)*					
Blood sample: Nr of serum tubes (yellow)					
Blood sample: Nr of trace element tubes (blue)					
*GTT (at 24 – 28 wks gestation)** *Fasting:*	*Not applicable*		*Not applicable*	*Not applicable*	*Not applicable*
Glucose (60 min)	*Not applicable*		*Not applicable*	*Not applicable*	*Not applicable*
Glucose (120 min)	*Not applicable*		*Not applicable*	*Not applicable*	*Not applicable*
Laboratory assistant (Name):					
Blood spot on filter paper	YES / NO	YES / NO	YES / NO	YES / NO	YES / NO
Hb (HemoCue)	g/dL	g/dL	g/dL	g/dL	g/dL
Hb (MCR)	g/dL	g/dL	g/dL	g/dL	g/dL

GTT: Glucose Tolerance Test; MCR: Maternal Case Record; Hb: Haemoglobin

*Criteria for diagnosing Diabetes Mellitus (SEMDSA guidelines, 2009):

Patient with diabetic symptoms plus one of the following:
Random plasma glucose (*not part of NuPED data*): ≥11.1 mmol/L
Fasting plasma glucose: ≥7.0 mmol/L
2 hour glucose during 75 OGTT: ≥11.1 mmol/L

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Nutrition during Pregnancy and Early Development

Anthropometry - Neonatal

Participant nr: | | | | **Date:** | 2 | 0 | Y | Y | M | M | D | D | **Fieldworker:** _____

Gestational Age at birth (in weeks)			weeks		days		
Birth weight (g)	1st measurement						
	2nd measurement						
Crown-heel length (cm)	1st measurement			.			
	2nd measurement			.			
Mid-upper arm circumference (cm)	1st measurement			.			
	2nd measurement			.			
Head circumference (cm)	1st measurement			.			
	2nd measurement			.			
Thoracic circumference (cm)	1st measurement			.			
	2nd measurement			.			

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Newborn Assessment

Participant number: | | | | **Date:** 2 0 Y Y M M D D **Fieldworker:** _____

Birth date:	YYYY / MM / DD	Birth time:	H H : M M
Gender:	☐ Male ☐ Female	Gestational age:	_____ weeks/_____ days
Resuscitation:	☐ None ☐ Oxygen ☐ Mask ☐ Intubation		
Total Apgar Score (1min):		Total Apgar score (5min):	
Mode of delivery:	☐ NVD ☐ C/S ☐ Vacuum ☐ Forceps		
Problems with delivery:	…… ……		
Vitamin K administration	☐ Yes ☐ No		
Risk factors to baby: *Pregnancy*		*Treatment:*	
RPR positive	☐ No ☐ Yes		
RPR unknown	☐ No ☐ Yes		
Rhesus negative	☐ No ☐ Yes		
HIV positive	☐ No ☐ Yes		
HIV unknown	☐ No ☐ Yes		
Maternal diabetes	☐ No ☐ Yes		
Risk factors to baby: *Labour*		*Treatment:*	
MSL	☐ No ☐ Yes		
Foetal distress	☐ No ☐ Yes		
Preventative care:	☐ Polio ☐ BCG ☐ RtHC filled in		
Feeding at discharge?	☐ EBF ☐ EFF		
First examination of Neonate:			
Temperature:	☐ 36-37°C ☐ Hypothermic ☐ Hyperthermic		
Resp rate:	☐ 40 – 60 pm ☐ Fast ☐ Slow		
Apex beat:	☐ 120 – 160/min ☐ Tachycardia ☐ Bradycardia		
Any abnormalities or adverse events:			

"Better is the end of a thing than the beginning thereof:

and the patient in spirit is better than the proud in spirit."

~ Ecclesiastes, 7:8