The relationship of long-chain polyunsaturated fatty acid status with blood pressure in pregnant African women: The NuPED study

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“I can do all things through Christ who gives me strength” – Philippians 4:13

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ABSTRACT

Introduction

Globally, hypertensive disorders affect 10% of pregnancies and are the most common risk factors for maternal and perinatal morbidity and mortality. Between 2011 and 2013, 14.8 % of maternal mortalities were associated with hypertensive disorders in South Africa. Adequate intake and status of n-3 long chain polyunsaturated fatty acids (LCPUFA) have been linked to improved endothelial function and reduced blood pressure. However, the South African longitudinal Prospective Urban Rural Epidemiology (PURE) study has shown a positive association between plasma n-3 LCPUFA and hypertension and a negative association between n-6 LCPUFA and hypertension. In addition, in South Africa, pregnant women are supplemented routinely with 60 mg/d of elemental iron to prevent iron deficiency. This supplementation programme may lead to oxidative stress and increased inflammation, especially in women with sufficient iron status, increasing their risk to develop gestational hypertension. This study assessed the relationship of n-3 and n-6 LCPUFA status with blood pressure in pregnant African women receiving routine iron supplementation.

Methods

This was a longitudinal analysis where 250 pregnant women were recruited at <18 weeks gestation in clinics in Gauteng province, South Africa. Socio-demographic and dietary intake data were collected at <18 week's gestation. Anthropometric and medical history were collected, and blood pressure measured at <18, 22 and 36 week's gestation. Red blood cell phospholipid fatty acid composition, inflammation and iron status biomarkers were analysed at the three time points. Statistical analyses were performed using SPSS version 26 to assess the descriptive status of participants and determine the relationship between LCPUFA status and blood pressure.

Results

The compositions of arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and n-3 LCPUFA correlated positively with systolic blood pressure ($r=0.133$, $p=0.044$; $r=0.208$, $p=0.002$; $r=0.217$, $p=0.001$ and $r=0.213$, $p=0.001$) at 22 week's gestation. EPA, DHA and total n-3 LCPUFA correlated positively with diastolic blood pressure ($r=0.149$, $p=0.024$; $r=0.226$, $p=0.001$ and $r=0.210$, $p=0.001$) and EPA with pulse pressure ($r=0.133$, $p=0.045$) at 22 week's gestation. The n6/n3 LCPUFA ratio correlated negatively with both systolic blood pressure and diastolic blood pressure in week 2 of gestation ($r=-0.170$, $p=0.010$ and $r= -0.175$, $p=0.008$ respectively). Compositions of EPA, DHA, n-3 LCPUFA and the n6/n3 LCPUFA ratio (all $p<0.05$) were higher in the prehypertensive than in the normotensive group in the unadjusted and adjusted

linear regression models (adjusted for age, gestational age, parity, aspirin intake and mid-upper arm circumference (MUAC) and additionally for the respective individual or grouped fatty acid intake of the status marker which was assessed) at 22 week's gestation. There was no loss of significance when additionally adjusting for alpha-1-Acid glycoprotein, C-reactive protein, ferritin or transferrin receptor at <18 week's gestation.

Conclusion

The findings of this dissertation show a positive relationship between n-3 LCPUFA status and blood pressure in the context of routine iron supplementation during pregnancy in women of African descent. Notably, the relationship was independent of dietary n-3 LCPUFA intake, therefore, we hypothesise that the higher n-3 LCPUFA may be a genetically driven metabolic response to the physiological circumstances in this predominantly black African study population.

Key terms: blood pressure, inflammation, iron status, long-chain polyunsaturated fatty acid (LCPUFA) status, pregnant women.

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LIST OF ABREVIATIONS

AA	Arachidonic acid
AGP	α1-acid glycoprotein
ALA	α-linoleic acid
ANC	Antenatal care
BMI	body mass index
CDC	Center for Disease Control
CEN	Centre of Excellence for Nutrition
COJ	City of Johannesburg
CRP	C-reactive protein
DHA	Docosahexaenoic acid
DGLA	Dihomo-gamma-linolenic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
DoH	Department of Health
ELISA	enzyme-linked immunosorbent assay
FAME	Fatty Acid Methyl Ester
FAO	Food and Agricultural Organisation
GC-MS/MS	Gas chromatography-mass spectrometry
GLA	γ-linolenic acid
Hb	Haemoglobin
HIV	Human immunodeficiency virus
HREC	Health research ethics committee
IDA	Iron deficiency anaemia
ID	Iron deficiency
IL	Interleukin
LA:	Linoleic acid
LCPUFA	Long chain polyunsaturated fatty acid

MUAC	Mid-Upper Arm Circumference
MUFA	monounsaturated fatty acid
n	Number
n-3	Omega-3
n-6	Omega-6
NHANES	National Health and Nutrition Examination Survey
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Prostaglandin
PGI	Prostacyclin
PUFA	Polyunsaturated fatty acid
RMMCH	Rahima Moosa Mother and Child Hospital
MRC	Medical Research Council
NuPED	Nutrition during Pregnancy and Early Development
NWU	North-West University
NCDs	Non-communicable diseases
QFFQ	Quantified food frequency questionnaire
RBC	Red blood cell
SA	South Africa
SANHANES	South African National Health and Nutrition Examination Survey
SD	Standard deviation
SFA	Saturated fatty acid
sTfR	Soluble transferrin receptor
Tf	Transferrin
24-HR	24-hour recall
TX	Thromboxanes
WHO	World Health Organization

CHAPTER 1 INTRODUCTION

1.1 Problem statement

Hypertensive disorders are the most common risk factors for maternal and perinatal morbidity and mortality and affect 10% of pregnancies worldwide (Barton *et al.*,2001). Gestational hypertension is a major public health problem and if left unresolved could progress to preeclampsia (Wu *et al*., 2012; Barton *et al*., 2001).

An adequate omega-3 (n-3) long-chain polyunsaturated fatty acid (n-3 LCPUFA) status during pregnancy may be advantageous as it has inflammation-resolving and cardioprotective effects (Cabo *et al*., 2012; Innes & Calder, 2020). The mechanism through which the n-3 LCPUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) lower blood pressure is by lowering systemic vascular resistance and improving endothelial function, and possibly reducing inflammation (Innes & Calder, 2020).

In South Africa (SA), the Department of Health routinely supplements all pregnant women with a dose equal to 60 mg of elemental iron and 0.4 mg folic acid, regardless of their iron status, to prevent iron deficiency (DoH, 2015). Pregnancy is already a state of higher than normal oxidative stress, and a high dose routine iron supplementation may further increase oxidative stress and therefore cause a heightened inflammatory state, particularly in iron-sufficient women (Casanueva & Viteri, 2003; Wessling-Resnick, 2010).

Therefore, we will investigate whether LCPUFA status associates with blood pressure during pregnancy in African women living in urban SA and receiving routine iron supplementation, and if so, whether inflammation and/or iron status influence this relationship.

1.2 Background and rationale

1.2.1 Gestational hypertension

According to the World Health Organization (WHO), hypertension is a global public health issue and contributes to the burden of heart disease, stroke and kidney failure and premature mortality and disability. A report released by the WHO in 2013 shows that hypertension accounts for 43% and 51% of deaths worldwide due to heart disease and stroke respectively (WHO. 2012). Hypertensive disorders are the most common risk factor for maternal and perinatal morbidity and mortality and affect 10% of pregnancies (Barton *et al*., 2001).

In SA, hypertensive disorders accounted for 14.8% of maternal mortalities in the triennium 2011-2013, as reported in the sixth Saving Mothers Report (Saving Mothers, 2011-2013). A maternal mortality ratio of 24.01 per 100 000 live births from in-hospital cases was reported in the 2014 to 2016 triennium (Moodley, 2018). Gestational hypertension is defined as elevated maternal blood pressure of ≥140 mm Hg systolic or ≥90 mm Hg diastolic that occurs after 20 gestational weeks without the presence of proteinuria (Barton *et al*., 2001; Brown *et al*., 2001).

1.2.2 Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFA) are fatty acids that have two or more double bonds in their backbone. The two major groups of essential PUFA are n-3 and n-6 fatty acids. Alpha-linolenic acid (ALA) and linoleic acid (LA) are the simplest form of the n-3 and n-6 PUFA respectively (Shek *et al*., 2012). The human body does not have the capacity to synthesise certain PUFA termed essential PUFA, for this reason they have to be obtained from the diet. Humans lack the desaturase enzyme that can insert a double bond at the C-15 position of the fatty acid carbon chain and cannot synthesise the essential fatty acids LA or ALA *de novo*. EPA and DHA are synthesised in the human body from ALA, and AA from LA, at a low conversion rate of about 1% (Vesa *et al.*, 2008).

Major dietary sources of n-3 LCPUFA are fish oils, which are high in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), while ALA is found in seeds of canola, rape, perilla, walnuts and chia, and also in chloroplasts of leafy green vegetables (Adkins & Kelley, 2010; Rowghani *et al.*, 2007). The main sources of the major n-6 LCPUFA, arachidonic acid (AA), are meat, and AA is also synthesised from LA.

The sources of the essential n-6 PUFA LA, include sunflower, flaxseed, hempseeds, grapeseed and pumpkin seeds (Calder, 2015; Vesa *et al*., 2008).

The n-3 LCPUFA, DHA and EPA play a vital role in the structure of human cells, the central nervous system and the immune response (Greenberg *et al*., 2008; Malan *et al*., 2015). Fatty acids have various functions, which include the provision of fuel for energy, forming part of structural components of cell membranes, modification of protein structure, regulation of gene expression and acting as precursors to synthesise bioactive lipid mediators (Calder, 2008). The n-3 and n-6 LCPUFAs are vital constituents of phospholipids of all tissues with the essential function of promoting regular cell function, foetal and postnatal growth during pregnancy (Leghi & Muhlhausler, 2016). It is hence critical to sustain an adequate intake of fatty acids during pregnancy (Mennitti *et al*., 2015).

A recommendation was made by the European Commission with the International Society for the study of Fatty Acids and Lipids (ISSFAL) that pregnant and lactating women should consume at least 200 mg DHA per day, which could be met by consuming one to two portions of fish rich in n-3 fatty acids per week (recommended by Health Canada and the United States Dietary Guidelines Advisory Committee) (Jia *et al.*, 2015). The adequate consumption of n-3 PUFA, in particular EPA and DHA, have resulted in positive outcomes on metabolic diseases such as hypertension (Wang *et al.,* 2016).

1.2.3 Gestational hypertension and polyunsaturated fatty acids

(Innes & Calder, 2020; Harris *et al.* 2017) demonstrated the protective effects of n-3 LCPUFA by assessing the hazard ratio from 10 cohort studies and found a 15% reduced risk for fatal coronary heart disease for a high versus a low n-3 PUFA index (Harris *et al*.,2017). It has been hypothesised that the supplementation of n-3 LCPUFA during pregnancy may reduce the risk of preeclampsia, gestational hypertension, intra-uterine growth retardation and preterm delivery (Horvath, 2007). This could be due to improved vasodilatory effects and reduced constrictor effects in the vasculature, as well as reduced inflammation as more pro-resolving lipid mediators are produced from available n-3 LCPUFA (Cabo et al., 2012; Innes & Calder, 2020). Evidence indicates that DHA lowers blood pressure by improving endothelial function of the coronary arteries and aorta, regulation of calcium release in the smooth muscle cells, as well as regulating the renin-angiotensin-aldosterone system to be efficient in lowering both the systolic (SBP) and diastolic blood pressures (DBP) (Lorente -Cebrian, 2013). Several meta-analyses have shown EPA and DHA to lower both SBP and DBP by enhancing endothelial function to improve flow-mediated dilation and arterial compliance (Innes & Calder, 2020). Furthermore, EPA and DHA have been reported to lower inflammation by decreasing the production of the pro-inflammatory eicosanoids, such as the thromboxane B2 and leukotriene B4 (Jiang *et al*., 2016). Eicosapentaenoic acid and DHA also lower the blood concentrations of inflammatory markers, such as the acute-phase protein, C-reactive protein (CRP), and the pro-inflammatory cytokines, tumour necrosis factor (TNF)-α and interleukin (IL)-6 (Li *et al*.,2014). These effects are mediated by pro-resolving lipid mediators metabolised from the n-3 LCPUFA (Innes & Calder, 2020).

However, some observational studies have shown results that are positive and negative with regard to the association of n-3 LCPUFA with hypertension and gestational hypertension (Clausen *et al*., 2001; Olafsdottir *et al*., 2006; Lim *et al*., 2015; Zec *et al*., 2019). Furthermore, the randomised controlled trials conducted in Caucasian women from as early as two to 36 weeks of gestation showed no effect on blood pressure. Bulstra-Ramakers *et al.* (1995) did a study in the Netherlands on 63 women from two to 14 weeks of gestation till birth to observe the effect of 3g EPA administered daily on gestational hypertension. Findings showed one-third of the women

developed hypertension regardless of the supplementation. Similarly, another study by Onwude *et al.* (1995) found a relative risk (RR) of 0.88; 95% CI 0.47- 1.66 for developing gestational hypertension in 223 women from Leeds. They were followed from 24 weeks of pregnancy and administered 1.62g eicosapentaenoic acid and 1.08g docosahexaenoic acid daily and found no reduced risk of gestational hypertension after the supplementation regime (Onwude *et al.*, 1995). Salvig *et al.* (1996) demonstrated a tendency of 2.7g n-3 PUFAs to affect DBP. They found a lower number of women with DBP above 90mmHg in the fish oil group compared with the control group (Salvig *et al.*, 1996). Moreover, Barden *et al.* (2006) reported the treatment to have no effect on both SBP and DBP (Barden *et al.*, 2006). However, a dose-response relationship and duration of n-3 fatty acid supplementation required to decrease blood pressure is yet to be investigated (Salvig *et al.*, 1996). Studies in African populations are scarce. The available evidence on fatty acid status and blood pressure are in an adult African population and show contradictory results. In the Prospective Urban Rural Epidemiology (PURE) study among Black adults in SA, EPA was shown to be adversely associated with hypertension throughout the 10 years of follow up (Zec *et al*., 2019). Conversely, the n-6 LCPUFA, AA, had an inverse relationship with blood pressure in this population (Zec *et al.,* 2019). To our knowledge, there has been no study on fatty acid status and blood pressure during pregnancy in an African population residing in SA.

1.2.4 Routine iron supplementation during pregnancy and hypertension

Anaemia is a major public health problem with the WHO analysis reporting a worldwide prevalence of 42% in pregnant women in 2008 (Pasricha, 2014; Thejpal, 2015). Iron deficiency (ID) is the major cause of anaemia in SA, accounting for a minimum of 50% of all reported cases (Nojilana *et al*., 2007; Visser & Herselman, 2013). The South African Demographic and Health Survey reported a prevalence of 33% in women of reproductive age (15-49 years) as being anaemic in 2016 (NDoH, Stats SA, SAMRC & ICF, 2017).

However, recent studies conducted in SA on iron deficiency anaemia (IDA) concluded that IDA is a moderate public health problem compared to other African countries (Mwangi *et al*., 2017; Nojilana *et al*., 2007). The prevalence of anaemia and ID in South African children below the age of 5 was 10.5 % and 11.0 % respectively, and that of women of childbearing age was 23% and 15% respectively, as reported in the “Iron for Africa” report (Mwangi *et al*., 2017). The Establishing Adult Reference Intervals for Selected Analytes in SA study found a prevalence of 12.6% for anaemia in healthy males and females older than 18 years (Phatlhane *et al*., 2016). This prevalence is considered a minor public health problem according to the WHO/CDC cut off points (Phatlhane *et al*., 2016). Pregnant women of all ages require 27 mg/day to meet iron demands throughout pregnancy (Adu-Afarwuah *et al.*, 2017). In pregnancy, the WHO recommends a daily oral iron supplementation of 30 mg to 60 mg of elemental iron and 0.4 mg folic acid to prevent

anaemia during foetal development (WHO, 2012). Following this recommendation, the South African Department of Health's Integrated Nutrition Programme, supplements 200 mg ferrous sulphate tablets daily (equivalent to ~60 mg elemental iron) to all pregnant women through healthcare facilities from the second trimester. In addition, they are given daily 5 mg folic acid tablets, without determining iron status or anaemia first (DoH, 2015). This routine iron supplementation may, however, be harmful to the health of women with adequate iron stores, since excessive iron can lead to increased oxidative stress, causing lipid peroxidation through the generation of free radicals and also damage the Deoxyribonucleic acid (DNA) and protein structures (McArdle *et al.*, 2014). It has also been shown that a high iron status in pregnant women leads to oxidative stress, which in turn increases inflammation through nuclear factor-kappa B- (NF-κB-) mediated cell signalling pathways (Tan *et al*., 2018). Furthermore, a randomised control trial (RCT) in pregnant women with haemoglobin (Hb) ≥13.2 g/dL receiving 50 mg/d of iron from the second trimester found an association between iron excess and the risk of developing hypertension compared to the placebo group (Chen *et al*., 2019; Ziaei *et al*., 2007).

How n-3 and n-6 LCPUFA status during pregnancy associates with blood pressure in pregnant women of African descent receiving routine iron supplementation has, to our knowledge, not yet been described.

1.3 Research aim

The aim of this project was to assess the relationship of n-6 and n-3 LCPUFA with blood pressure in pregnant African women receiving routine iron supplementation in urban SA.

1.4 Research objectives

The objectives are, in pregnant women living in urban SA and receiving routine iron supplementation, to:

- characterise the red blood cell total phospholipid AA, total n-6 LCPUFA, EPA, DHA and total n-3 LCPUFA status at <18, 22 and 36 week's gestation;
- characterise the blood pressure at <18, 22 and 36 week's gestation;
- assess the association between AA, EPA, DHA and total n-6 and n-3 and LCPUFA compositions with blood pressure during pregnancy

and, if associations are found, to:

- assess whether n-3, n-6, essential PUFA and LCPUFA intake influence the association;
- assess whether iron status and/or inflammation influence the association.

1.5 Research team

The research team is described in Table 1-1.

Table 1-1: Research team expertise, skills and legal competencies

Partner name	Team member	Qualification, knowledge, experience, skills	Professional registration	Role and responsibility
North-West University	Prof. Marius Smuts	PhD Biochemistry, experience in fatty acids and cardiovascular outcomes	HPCSA: Nutritionist	Principal investigator of the main study in which this MSc was nested: Nutrition during Pregnancy and early development: the NuPED study
North-West University	Dr Linda Malan	MSc Biochemistry, PhD Nutrition, fatty acids and inflammatory-related morbidity	HPCSA: Medical Biological Scientist	Student supervisor and co-principal investigator of main study
North-West University	Dr Lizelle Zandberg	PhD Biochemistry, Molecular nutrition and laboratory specialist Fatty acid genetics and cardiovascular outcomes in a black South African population	NA	Investigator and student co-supervisor
North-West University	Dr Arista Nienaber	PhD Dietetics Fatty acids and inflammatory-related morbidity Therapeutic and clinical dietetics	HPCSA Dietitian	Investigator and student co-supervisor

Partner name	Team member	Qualification, knowledge, experience, skills	Professional registration	Role and responsibility
North-West University	Katlego Kekana	BSc Biochemistry and Honours Nutrition. Honours research study in n-3 fatty acids and inflammation Laboratory techniques: Fatty acid and lipid mediator blood sample extraction for chromatography mass spectrometry analysis	NA	MSc student: Maternal anthropometric, general history, socio-demographic, delivery assessment, morbidity, biological samples sheet and blood pressure data capturing, statistical analysis and writing of dissertation

1.6 Ethical approval

Ethical approval for this study has been obtained from the Health Research Ethics Committee (HREC) of the Faculty of Health Sciences of the North-West University (NWU), Potchefstroom (NWU-00186-15-A1-07), as well as from the University of Witwatersrand Human Research Ethics Committee (Medical), M150968. Approval was obtained from the RMMCH research review committee, the Gauteng Department of Health (which includes the Johannesburg Health District's District Research Committee), RMMCH ANC clinic, Zandspruit clinic, Sophia Town clinic, Florida clinic and Bosmont clinic.

1.7 Structure of the dissertation

This dissertation is for the fulfilment of the MSc degree in Nutrition and is presented in chapter format. Chapter 1 gives an introduction to the study, and presents the rationale, aim and objectives. Chapter 2 provides the literature relevant to this research topic, with a focus on gestational hypertension globally and in SA. The role of PUFA in gestational hypertension is also discussed with reference to previous studies. The relationship of PUFA with inflammation is also explored. Lastly, routine iron supplementation during pregnancy in the South African context will be covered. Chapter 3 describes the research methodology used throughout the longitudinal and

cross-sectional analysis together with the ethical considerations. The main findings of the study will be provided in Chapter 4, followed by a detailed discussion of the results in Chapter 5. Chapter 6 consists of the conclusion, limitations and recommendations for future research.

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CHAPTER 2 LITERATURE REVIEW

2.1 Introduction

Hypertension is a major public health problem associated with a reduced life expectancy (Baradaran *et al.*, 2014). Globally, hypertensive disorders are the most common risk factors for maternal and perinatal morbidity and mortality, and affect 10% of pregnancies (Barton *et al.*, 2001). In South Africa, hypertensive disorders of pregnancy account for 18% of maternal mortalities (Moodley *et al.*, 2019).

The adequate consumption and status of omega-3 polyunsaturated fatty acids (n-3 PUFA), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been associated with positive outcomes in hypertension, especially in Caucasian populations (Innes & Calder, 2020; Wang, L. *et al.*, 2016). Apart from structural modulation of vascular cell membranes, the anti-inflammatory properties of n-3 PUFA have shown protective effects on the cardiovascular system and thereby improve endothelial function, thus reducing blood pressure (Cabo *et al.*, 2012). To our knowledge, there are no studies on the association of fatty acid status and blood pressure in African women living in urban South Africa.

2.2 Gestational hypertension

Hypertension is a condition characterised by the prolonged rise in two forces that push blood against the arterial wall. These forces are defined as a systolic blood pressure (BP) of ≥ 140 mmHg and diastolic BP of ≥ 90 mmHg, confirmed by two measurements four hours apart while resting (Vest & Cho, 2014). The systolic pressure is created when the heart pumps blood to the arteries, while diastolic pressure is the resting force between heartbeats (Moini, 2015).

Hypertension may be a pre-existing condition diagnosed before (chronic hypertension) or after (gestational hypertension) the 20th week of pregnancy, and both conditions may coexist (Shah, 2001). Compared to normotensive women, women with hypertensive disorders during pregnancy have a marked increased risk of severe adverse outcomes, such as preterm birth, intrauterine growth restriction, perinatal death, acute renal or hepatic failure, antepartum haemorrhage and postpartum haemorrhage (Roberts *et al.*, 2005).

Table 2-1 presents the classification of blood pressure by the Southern African Hypertension Society (SAHS) (Seedat *et al.*, 2014).

Table 2-1: Classification of blood pressure

Blood pressure Classification	Systolic BP mmHg	Diastolic BP mmHg
Normal	<120	and<80
Prehypertension	120-139	or 80-89
Stage 1 hypertension	140-159	or 90-99
Stage 2 hypertension	≥160	Or ≥100

(Adapted from Seedat *et al.*, 2014)

Hypertensive disorders of pregnancy are classified into four groups, as recommended by the International Society for the Study of Hypertension in Pregnancy and by the National High Blood Pressure Education Program (NHBPEP), namely chronic hypertension, gestational hypertension, pre-eclampsia (gestational proteinuric hypertension) and chronic hypertension with superimposed pre-eclampsia (Brown *et al.*, 2001). Gestational hypertension is defined as elevated maternal blood pressure of ≥140 mm Hg systolic or ≥90 mm Hg diastolic that occurs after 20 gestational weeks without the presence of proteinuria (Barton *et al.*, 2001; Brown *et al.*, 2001). Prehypertension is defined as a persistent systolic blood pressure of range 120-139 mm Hg and diastolic blood pressure of 80-90 mm Hg almost approaching hypertensive levels (Chobanian *et al.*, 2003).

2.3 Polyunsaturated fatty acids

2.3.1 Structure and function

Fatty acids are carboxylic acids with a long aliphatic saturated or unsaturated carbon chain, comprised of carbon atoms and range from a length of 6-24 atoms, with a methyl group (CH3) at the omega end of the carboxyl group (COOH) (Arab, 2003). Fatty acids are further classified according to the number of double bonds in their backbone: saturated fatty acids (SFAs) have no double bonds, monounsaturated fatty acids (MUFAs) have one double bond and PUFA have two or more double bonds in their backbone (Saini & Keum, 2018). The chain length of the fatty acids determines their biological function, with the SFAs classified as short- (3-7 carbon atoms), medium- (8-13 carbon atoms), long- (14- 20 carbon atoms) and very long-chain (> 21 carbon atoms) fatty acids. The chain length categories of the unsaturated fatty acids differ from that of the saturated fatty acids with only three categories, namely short- (< 19 carbon atoms), long- (20-24 carbon atoms) and very long-chain (> 25 carbon atoms) (Kalish *et al.*, 2012).

The fatty acids are named according to the number of carbon atoms in the carbon chain, the degree of saturation and to the position of the first double bond opposite the carboxyl group (Arab,

2003; Fattore & Massa, 2018), as shown in Figure 2-1. The carbon atom (third or sixth) at the omega end opposite the carboxyl group with the first double bond is the determining factor of whether the fatty acid will be an n-3 or n-6 PUFA (Colussi *et al.*, 2016; Lunn & Theobald, 2006).

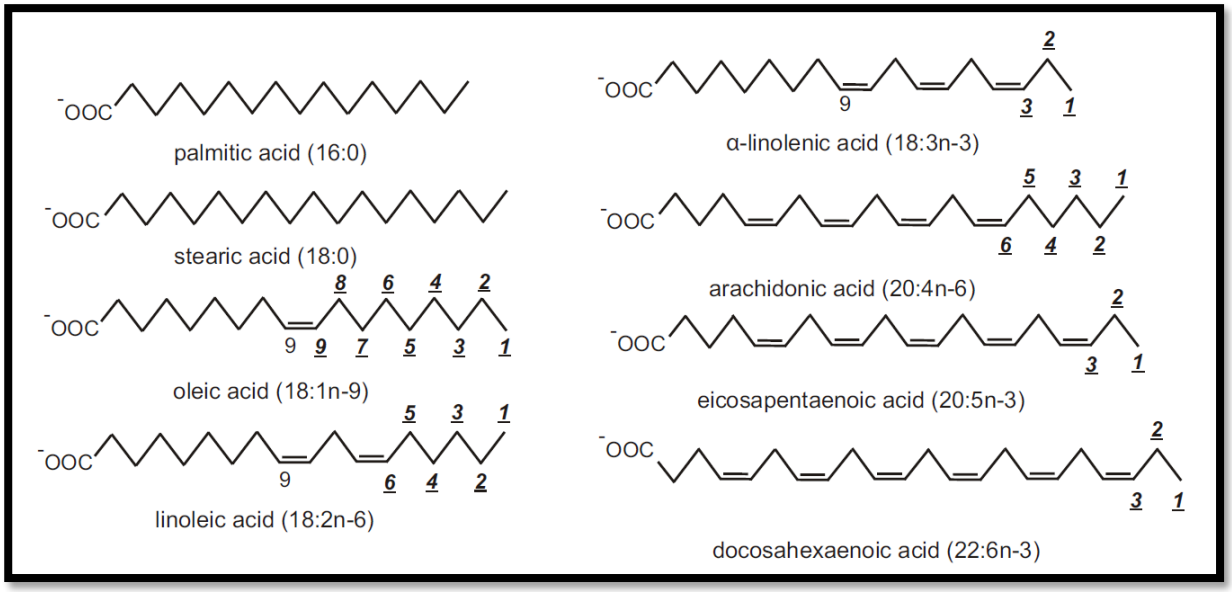

Figure 2-1: Structural formulas and shorthand notations of fatty acids (Tvrzicka *et al.*, 2011) The numbers in the picture represent carbon atoms

Fatty acids are the building blocks of triglycerides, phospholipids and other lipid classes. Apart from being a source of energy, fatty acids have important roles as components of the phospholipid bilayer, which constitute cell membranes, where they influence structure and membrane fluidity. They also act as precursors of signalling molecules named lipid mediators (i.e. prostaglandins, thromboxanes and leukotrienes) (Calder, 2015).

The two major groups of essential polyunsaturated fatty acids are n-3 and n-6 fatty acids, whereas n-7 and n-9 make up the nonessential groups (Colussi *et al.*, 2016). Alpha-linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6) are the simplest form of the n-3 and n-6 PUFAs respectively (Shek *et al.*, 2012). These essential PUFAs can be obtained only from the diet as the body cannot synthesise them (Coletta *et al.*, 2010); this is because humans lack the desaturase enzyme that inserts a double bond at the C-12 and C-15 position of the fatty acid carbon chain (Al *et al.*, 2000). Therefore, humans obtain these fatty acids from plants (Calder, 2004) and they perform important physiological functions in the body (Lunn & Theobald, 2006).

Although human beings cannot synthesise the essential/parent fatty acids, LA and ALA can be metabolised to their long-chain PUFA (LCPUFA) derivatives through a series of elongation and desaturation reactions in the liver (Calder, 2004; Kalish *et al.*, 2012).

The n-3 LCPUFAs, such as docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3), play a vital role in the structure of all human cells, the central nervous system, as well as the immune response (Greenberg *et al.*, 2008; Malan *et al.*, 2015). Within endothelial and immune cells, fatty acids form part of structural components of the cell membranes and act as precursors in the synthesis of bioactive lipid mediators. In addition, fatty acids provide fuel for energy, regulate gene expression and modify the structure of proteins (Calder, 2008). Furthermore, during pregnancy, the role of the n-3 PUFAs goes beyond the growth and development of the foetus. The longer chain products from these essential PUFAs play an essential part as precursors of prostaglandins and as organisational components of cell membranes during pregnancy (Al *et al.*, 2000). Adequate PUFA intake during pregnancy, is therefore critical (Mennitti *et al.*, 2015). The n-3 and n-6 metabolites play a crucial role in cognitive development, DHA is involved in the growth and development of the foetus and is important for healthy brain and eyes (retina development) (Akerele & Cheema, 2016), whereas AA generates pro-inflammatory mediators, which are involved in the initiation of delivery (Christian *et al.*, 2016).

The integration of fatty acids occurs rapidly in phospholipids of plasma, platelets, neutrophils, and red blood cells, however, slowly in other tissues. Omega-3 PUFAs predominantly accumulate in the cerebral cortex, retina, testes, muscle and liver, whereas n-6 is found in almost all tissues (Colussi *et al.*, 2016).

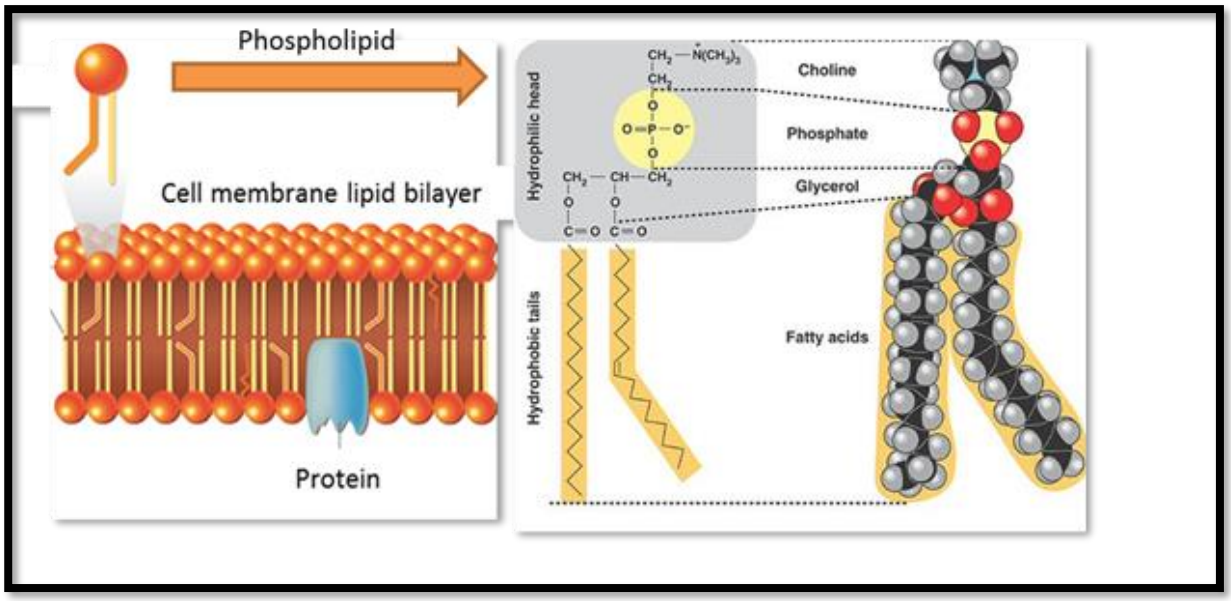

Figure 2-2: Structure of cell membranes (adapted from http://bioapwikispaces.com/Ch+5+Collaboration+2010)

All cells in the human body are surrounded by a cell membrane, which is made up of phospholipids that form the lipid bilayer (Alberts et al., 2002; Zalba & ten Hagen, 2017), as shown in Figure 2-2. A phospholipid is composed of a glycerol-3-phosphate backbone with three carbon atoms, a polar head group and two fatty acid tails adjoining the backbone. The three carbons are denoted sn-1, sn-2 and sn-3 using the stereospecific numbering system (Zalba & ten Hagen, 2017). A phosphate group sustains the connection between sn-3 and the polar head group. The phospholipid bilayer is amphipathic, as it contains both the hydrophilic (head group) and hydrophobic (fatty acid tails) elements. The hydrophobic section faces away from the aqueous solution while the polar head groups cluster together to face the outward aqueous regions on either side of the extracellular fluid. The major classes in membranes include phospholipids, sphingolipids, glycolipids and cholesterol (Alberts et al., 2002; Zalba & ten Hagen, 2017). Phosphatidylethanolamine (PE) and phosphatidylserine (PS) incorporate mostly DHA, while phosphatidylinositol (PI), phosphatidylcholine (PC) and PE incorporate mostly arachidonic acid (AA) (Rey *et al.*, 2018). The n-3 and n-6 LCPUFAs are vital constituents of phospholipids of all cell membranes (Leghi & Muhlhausler, 2016).

2.3.2 Dietary sources

Major dietary sources of n-3 fatty acids are fish oils and fatty fish (tuna, salmon, mackerel, herring, sardine) (EPA, DHA), green leafy plants as well as some plant oils such as canola oil (ALA) (Abedi & Sahari, 2014; Calder, 2011). Docosahexaenoic acid is also found in animal-derived products,

such as meat and eggs (Calder, 2016). Eicosapentanoic acid and DHA are mostly consumed through fish but also synthesised in the human body from ALA at low conversion rate, 6% for EPA and 3.8% for DHA (Gerster, 1998) The sources of n-6 fatty acids (LA) include vegetable oils, chia and pumpkin seeds, soybean oil, eggs and sunflower (Vesa *et al.*, 2008; Whelan & Fritsche, 2013); nuts, including pignolia (pine) nuts and pistachios, are also rich n-6 PUFA sources (Vesa *et al.*, 2008). Furthermore, AA is found in significant quantities in meats (Calder, 2011). The ratio of n-6 to n-3 PUFA intake has increased vastly in the diet due to westernisation, with the present ratio lying between 5 and 20 in most western populations (Blasbalg *et al.*, 2011; Shek *et al.*, 2012). The introduction of and increased intake of cooking oils and margarine in the 20th century led to the increased consumption of LA, while that of ALA remained stagnant (Shek *et al.*, 2012).

The Food and Agriculture Organisation (FAO) and the World Health Organization (WHO) have advised the intake of fat to be minimal in proportion to an individual's total energy intake. An individual should have a total fat intake of 20-35%, which should ideally consist of 10% SFAs, 15-20% of MUFAs and 6-11% of PUFAs (FAO/WHO, 2010). The recommendations for n-3 PUFA intake in healthy males and females are 0.7% of total daily energy intake for ALA, and 250 mg to 500 mg DHA plus EPA or two to three servings of oily fish per week (Elagizi et al., 2018; FAO, 2010; Flock et al., 2013a; ISSFAL, 2004; Nestel et al., 2015).

2.3.3 Polyunsaturated fatty acid elongation and desaturation metabolism

Figure 2-3 illustrates the n-3 and n-6 fatty acid metabolisms. The n-3 fatty acid ALA can be firstly biosynthesised then converted in the liver to longer chain PUFA and metabolised to form EPA and DHA through a series of enzymatic chain elongation and desaturation reactions. There exists a competition in the metabolism of both the n-3 and n-6 PUFAs as they go through the same desaturation and elongation pathway, resulting in signalling molecules that have opposing biological functions (Shek *et al.*, 2012; Wadhwani *et al.*, 2018); the rate of desaturation is very low due to the competition for the desaturase enzyme. Moreover, EPA is converted at a rate of 8% and DHA at 1% (Punia *et al.*, 2019).

The precursors of n-3 and n-6 PUFA compete for the same enzymatic pathway during the elongation and desaturation steps in the metabolic pathway. Moreover, ALA is the preferred substrate in the conversion of ALA into n-3 LCPUFA EPA by the desaturases and is favoured compared to LA conversion into n-6 LCPUFA AA. This affinity of the desaturases for ALA hinders the production of AA. The balance between n-6 and n-3 PUFA composition in cell membranes can be optimised through dietary intake modification. Research has shown populations with a higher seafood consumption to have a lower n-6 to n-3 PUFA ratio than populations with high

vegetable intake in farming prevalent regions with low n-3 PUFA consumption. The former populations have the lowest risk for cardiovascular mortality (Akerele & Cheema, 2016).

Following the consumption of the n-6 PUFA LA, the enzyme Δ-6-desaturase converts LA to gamma-linolenic acid (GLA, 18:3, n-6), this then undergoes elongation by elongase enzyme to form dihomo-GLA (DGLA, 20:3, n-6), which is further converted to AA by Δ-5-desaturase. The dietary sources of GLA generally include supplemental oils such as borage oil (starflower oil), blackcurrant seed oil and evening primrose oil (Innes & Calder, 2018). The conversion of n-3 PUFA, ALA to stearidonic acid is through the action of Δ-6-desaturase, and further elongated to eicosatetraenoic acid (20:4, n-3) by the elongase enzyme. Furthermore, Δ-5- desaturase acts on eicosatetraenoic acid to yield EPA (Akerele & Cheema, 2016). The last biosynthetic step occurs in the peroxisome, yielding DHA and DPA of the n-3 and n-6 PUFA respectively.

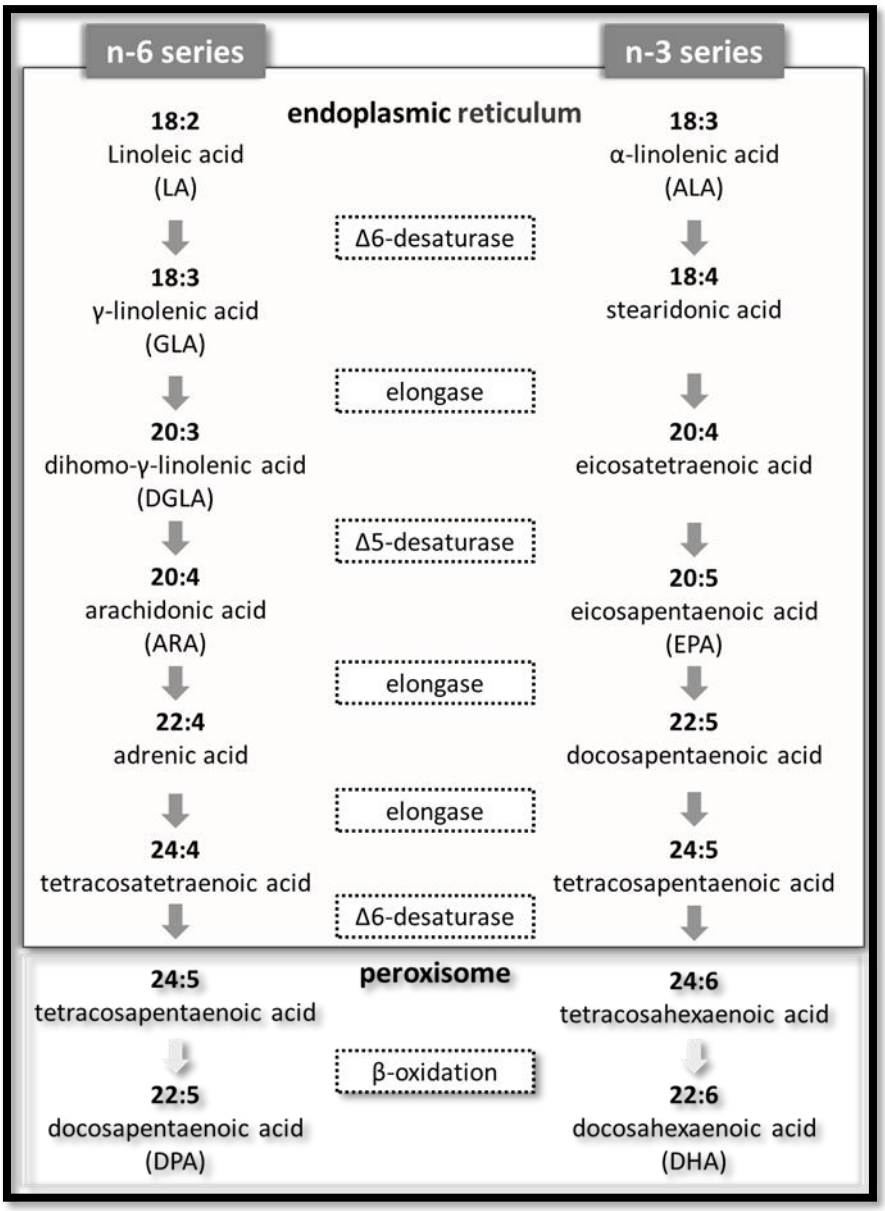

Figure 2-3: Overview of n-3 and n-6 PUFA metabolism (Edwards & O'Flaherty, 2008).

2.3.4 Polyunsaturated fatty acids and inflammation

Inflammation is the human body's defence mechanism in response to injury and foreign pathogens, characterised by swelling, redness, heat and pain of the affected area (Calder, 2009). Predominantly EPA, DHA and AA serve as precursors for the synthesis of signal molecules known as lipid mediators. They are produced in response to extracellular stimuli and the ratio of the n-3 and n-6 PUFA obtained from the diet will influence the capacity of cells to produce anti-inflammatory or pro-resolving (n-3) or pro-inflammatory (n-6) lipid mediators (Serhan & Chiang, 2008). Therefore, the diet plays an integral role in an individual's inflammatory state (Lunn & Theobald, 2006).

Lipid mediators are the most studied series of 20 carbon bioactive signalling lipid mediators. They are acquired from AA, a member of the n-6 PUFA family that produces the highly pro-inflammatory mediators and members of the n-3 PUFA family (EPA and DHA), which produce the anti-inflammatory and pro-resolving mediators (Tsikas & Zoerner, 2014). The most prominent members of the lipid mediator subfamily include prostaglandins, thromboxane, leukotrienes and lipoxins (Tsikas & Zoerner, 2014). Emerging evidence has identified the existence of a class of n-3 PUFA derived mediators, known as specialised pro-resolving lipid mediators (SPM), which are comprised of resolvins (RV), protectins (PD) and maresins (Leghi & Muhlhausler, 2016).

The inflammatory response initiates the biochemical and physical changes to resolve inflammation and its presence is characterised by the production of inflammatory cytokines, pro-inflammatory eicosanoids (prostaglandins, leukotrienes and thromboxanes) and reactive oxygen species (Mburu *et al.*, 2010; Sansbury & Spite, 2016). However, the production of these inflammatory molecules is inhibited when the ratio of n-3: n-6 intake is optimum. Since the two fatty acid classes compete for the same enzyme, metabolism favours the n-3 pathway thus decreasing the production of the AA eicosanoid precursor (Mburu *et al.*, 2010).

Resolution of inflammation is a tightly regulated on-going process controlled by the newly discovered specialised pro-resolving mediators (SPM) derived from the n-3 LCPUFAs (EPA and DHA). The process of inflammation resolution is divided into three phases, namely 1) Initiation phase, 2) Resolution phase, and 3) Termination phase, which aim to repair damaged tissue and restore homeostasis at the site of inflammation (Bosma-den Boer *et al.*, 2012; Calder, 2009). Failure in this regulation leads to chronic inflammation (Calder, 2006).

During the late stages of inflammation, more EPA and DHA (n-3 LCPUFA) derived lipid mediators are produced to maintain homeostasis at the site of injury. The n-3 LCPUFAs are excised from the phospholipids in a time- and dose-dependent manner and metabolised to inflammation resolving lipid mediators (Calder, 2009; Serhan, 2017).

A high n-3 PUFA intake leads to increases in the n-3 PUFA composition of phospholipids of cells involved in inflammation at the expense of n-6 PUFA. This mitigates the n-6 PUFA membrane composition as well as the production of pro-inflammatory lipid mediators (Colussi *et al.*, 2016). In addition, EPA and AA compete for the same cyclooxygenase and lipoxygenase enzymes, thus leading to the production of anti-inflammatory lipid mediators (Akerele & Cheema, 2016; Cabo *et al.*, 2012; Endo & Arita, 2016). A novel class of lipid mediators during which COX-2 and 5-LOX use EPA and DHA as substrates was identified more recently. EPA gives rise to the inflammation resolving E series resolvins (RV), and DHA to the D series resolvins (RV) and protectin (PD) D1 (Cottin *et al.*, 2011).

Moreover, the enzymatic competition between the n-3 and n-6 PUFAs leads to the suppression of the n-6 PUFA derived pro-inflammatory lipid mediator production downstream (Akerele & Cheema, 2016; Cabo *et al.*, 2012; Endo & Arita, 2016). This inhibitory effect is strengthened by reducing gene expression of pro-inflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α) and interleukin- (IL-6, and IL-1β) and inhibiting the production of the transcription factor, nuclear factor-kappaB (NFkB)(Akerele & Cheema, 2016). During enzyme competition, the n-3 PUFAs inhibit the production of the potent vasoconstrictor thromboxane A2 (TxA2) and elevate that of the common vasodilator prostaglandin I3 (PGI3) (Borghi & Cicero, 2007). This mechanism has been shown to reduce blood pressure levels (Tsukamoto & Sugawara, 2018)

2.4 Gestational hypertension and polyunsaturated fatty acids

It has been hypothesised that the supplementation of n-3 LCPUFAs during pregnancy may reduce the risk of pre-eclampsia and gestational hypertension (Horvath *et al.*, 2007). This could be due to the improvement of endothelial function as well as the reduction of inflammation. Several meta-analyses have shown EPA and DHA to lower both SBP and DBP by enhancing endothelial function and thus improve flow-mediated dilation and arterial compliance (Innes & Calder, 2020). Mechanisms by which n-3 PUFAs can modulate blood pressure levels and protect against hypertension-related organ damage are listed below, as described by (Colussi *et al.*, 2016):

- Reduction of stroke volume and heart rate;
- Improvement of left ventricle diastolic filling;
- Reduction of peripheral vascular resistances;
- Improvement of endothelial-dependent and -independent vasodilation;
- Anti-inflammatory, antioxidant, and antithrombotic effects;
- Reduction of arterial stiffness;
- Experimental effects on left ventricular hypertrophy and abnormal gene expression;
- Has an effect on atherosclerotic plaque progression and stability.

Table 2-2: Studies on the effect or relationship of n-6 and n-3 PUFA with gestational hypertension

Authors	Year	Study design	Setting	Ethnicity	n	Gestational age (weeks)	Exposure	Outcome	Results	Conclusion
Randomised controlled trials										
Bulstra-Ramakers et al.	1995	RCT, double-blind, multicentre	Netherlands	Caucasian	63	2 to 14 weeks of gestation onwards	3 g EPA daily to the diet and placebo	gestational hypertension	One-third of the women developed pregnancy induced hypertension.	Addition of 3 g EPA daily does not prevent recurrence of pregnancy-induced hypertension in a high risk population.
Onwude et al.	1995	RCT, double-blind	Leeds, UK	Caucasian	223	from 24 weeks of gestation	1.62 g EPA and 1.08 g DHA	gestational hypertension	No reduction in pregnancy induced hypertension. (relative risk (RR) = 0.88;95% CI 0.47–1.66).	No evidence of reduced risk of gestational hypertension from the supplementation.
Salvig et al.	1996	RCT	Aarhus, Denmark,	Caucasian	533	30th week of gestation	randomly assigned in a ratio 2:1:1 to receive fish oil (2–7 g/day n-3 fatty acids (Pikasol)), or a control regimen of either olive oil or no oil supplementation	blood pressure	Proportion of women with diastolic above 90 mmHg tended to be lower in the fish oil group compared with the olive oil group. The corresponding relative risk was RR = 0.48 (95 % CI 0.22–1.06; P = 007).	2.7 g/day of marine n-3 fatty acids provided in the third trimester of normal pregnancy showed no effect on either SBP or DBP.
Barden et al.	2006	RCT, double-blind	Australia	Caucasian	83	from 20th week until delivery at 36 weeks	4 g/day olive oil (n=43) or 4 g/day fish oil (1.1 g/day EPA and 2.2 g/day DHA; 3.3 g/day total) (n=40)	blood pressure	No significant difference in SBP or DBP between the control and fish oil groups during pregnancy at 20, 30 or 36 weeks	BP and heart rate recorded during and after pregnancy were not significantly affected by fish oil supplementation.

Authors	Year	Study design	Setting	Ethnicity	n	Gestational age (weeks)	Exposure	Outcome	Results	Conclusion
Observational studies										
Popeski et al.	1991	Retrospective survey of gestational hypertension; prospective diet survey	Keewatin, Canada	Inuit	27		self-reported consumption of fish, sea mammals and terrestrial mammals by a subgroup of the subjects	gestational hypertension	Significantly lower mean diastolic blood pressure values during the last 6 hours of pregnancy were noted for the women from the three communities with a higher consumption of fish and sea mammals (78.2 [95% confidence limits (CL) 76.6 and 79.9] mm Hg) than for those from the four communities with a lower consumption of such food (81.5 [95% CL 80.1 and 82.91 mm Hg) ($p < 0.005$).	Increased consumption of fish may be beneficial for women at risk for hypertension during pregnancy.
Olafsdottir et al.	2005	Observational prospective study	Reykjavik, Iceland	Caucasian	488	11 & 15 34 & 37	cod-liver oil	gestational hypertension	the odds ratio for developing hypertensive disorders in pregnancy for women consuming liquid cod-liver oil was 4.7 (95% CI 1.8–12.6, P = 0.002).	Consumption of high doses of n-3 LCPUFAs in early pregnancy, or other nutrients found in liquid cod-liver oil, may increase the risk of developing hypertensive disorders in pregnancy.
Lim et al.	2014	Observational	Singapore	Chinese, Malay, and Indian	751	26th –28th	plasma polyunsaturated fatty acid composition	gestational hypertension	Peripheral SBP was inversely associated with total n-3 PUFAs [0.51 (95% CI, 0.89 to 0.13) mm Hg] and long-chain n-3 PUFAs	28 pregnancy-associated hypertension cases, and 1% increase in total n-3 PUFAs was associated with a 24% lower odds of pregnancy-associated hypertension

Authors	Year	Study design	Setting	Ethnicity	n	Gestational age (weeks)	Exposure	Outcome	Results	Conclusion
									[0.52 (CI 0.92 to 0.13) mmHg]. Similar but weaker associations were observed for central SBP and PP.	(odds ratio 0.76; 95% CI 0.60 to 0.97).
Lepsch et al.	2018	Prospective cohort	Rio de Janeiro, Brazil	Hispanic	223	5th–13th, 20th–26th, 30th–36th	serum fatty acids	blood pressure	Total n-6 PUFAs positively associated with SBP throughout pregnancy.	Maternal serum concentrations of total SFAs, MUFAs and n-6 PUFAs were positively associated with BP levels in normotensive pregnant women.

BP: blood pressure; DBP: diastolic blood pressure; MUFAs: mono-unsaturated fatty acids; n-3: omega-3; PP: pulse pressure; PUFA: polyunsaturated fatty acids; RCT: randomized controlled trial; SBP: systolic blood pressure; SFAs: saturated fatty acids

There is mixed evidence regarding the relationship of LCPUFA intake and status with blood pressure in pregnancy (Table 2-2). Evidence from observational studies conveyed that n-3 PUFA may be protective against gestational hypertension, however four randomised controlled trials reported that n-3 PUFA supplementation had no significant effect on blood pressure (Barden *et al.*, 2006; Bulstra-Ramakers *et al.*, 1995; Onwude *et al.*, 1995; Salvig *et al.*, 1996).

A study conducted by Barden *et al.* (2006) aimed at examining the effect of fish oil supplementation during pregnancy on blood pressure, and maternal lipids in 83 Caucasian women with allergic disease (Barden *et al.,* 2006). They found 4 g fish oil administered from the 20 – 36th week of gestation until delivery to significantly increase red blood cell composition of EPA, DHA and AA $P < 0.001$ compared with the control group receiving 4 g olive oil. However, in that study fish oil did not affect either SBP and DBP or heart rate. A potential explanation for the results obtained by Barden *et al*. (2006) could be that the fish oil (EPA & DHA) consumed improved the synthesises of anti-inflammatory lipid mediators to resolve inflammation as the women were in an allergic state, and the intake of fish oil was relatively low.

Conversely, some cohort and cross-sectional studies showed an adequate PUFA dietary intake or supplementation, thus better n-3 LCPUFA status, was associated with significantly lower blood pressure (Lim *et al.*, 2015; Popeski *et al.*, 1991). Popeski *et al.* (1991) reported a beneficial effect of increased self-reported fish consumption in decreasing the risk of developing gestational hypertension in the Canadian Inuit. Although Popeski *et al.* (1991) found a reduction in DBP from blood pressure readings during the last six hours of pregnancy, the study did not hold statistical power due to small sample size. An observational study by Lim *et al.* (2015) identified the potential benefit of n-3 PUFA in lowering the odds (odds ratio 0.76; 95% CI 0.60 to 0.97) of developing gestational hypertension in Chinese, Malay, and Indian women. However, Olafsdottir *et al.* (2006) showed conflicting results, as they found an increased risk of developing hypertensive disorders in a community with traditionally high fish and cod-liver oil consumption; this may have been due to the other constituents of the cod liver oil.

Supporting the notion that inflammation plays a role, maternal serum concentrations of the pro-inflammatory precursor group, n-6 PUFA, were shown in a prospective cohort study to be associated positively with blood pressure in normotensive pregnant women (Lepsch *et al.*, 2018). The plasma total phospholipid n-6 PUFA and n-3 PUFA composition have shown to be associated with hypertension in the Prospective Urban Rural Epidemiology (PURE) study in Potchefstroom and Ganyesa, South Africa, in Black adults. In contrast with most other research, a positive association between EPA status and blood pressure, and an inverse association between n-6 PUFA and blood pressure, were found throughout the 10 year period (Zec *et al.*, 2019).

2.5 Routine iron supplementation during pregnancy in the South African context

Anaemia is a major public health problem, with a worldwide prevalence of 32.9% reported in 2010 and the WHO analysis reporting a prevalence of 42% in pregnant women in 2008 (Pasricha, 2014; Thejpal, 2015). The South African National Health and Nutrition Examination Survey (SANHANES-1) conducted in 2012, reported a total prevalence of 17.5% of anaemia in the adult population. Women of childbearing age had a prevalence of 22%, with 15.4% of these cases attributed to iron deficiency (ID). Furthermore, the South African Demographic and Health Survey estimated that women of childbearing age (15- 49 years) had a prevalence of 33% of anaemia in 2016. However, recent studies conducted in South Africa on iron deficiency anaemia (IDA) concluded that IDA is a moderate public health problem compared with other African countries (Mwangi *et al.*, 2017; Nojilana *et al.*, 2007). It affects both low- and middle-income countries and has dire consequences on the health of pregnant women and on birth outcomes (WHO, 2008).

Iron deficiency is the major cause of anaemia in South Africa, accounting for a minimum of 50% of all reported cases (Nojilana *et al.*, 2007; Visser & Herselman, 2013). Iron deficiency anaemia (IDA) is a special case where ID (ferritin levels < 15 gμ/l) reaches the phase of breaking down red blood cells leading to haemoglobin levels of less than 11 g/dl (12 g/dl in pregnancy) (Nojilana *et al.*, 2007; Yip, 1996). Iron deficiency is most prevalent in women of reproductive age from low- and middle-income countries like South Africa (Control, 1989; Remacha, 2000). Studies have shown the Black ethnic group and those from poverty-stricken homes are more susceptible to anaemia, and that Black women had an average haemoglobin concentration of 8 g/L lower than that of White women (Control, 1989; Remacha, 2000). The South African Demographic and Health Survey estimated that the prevalence of anaemia in Black African women was 32% opposed to 11% in White women, which was almost three times lower. The causes of ID, such as inadequate dietary intake, Pica and poor availability of nonheme iron often coexist (Bopape *et al.*, 2008). Women with short inter-pregnancy intervals are also at increased risk of ID owing to the pregnant mother's iron being transferred with every pregnancy, thus depleting her iron reserves (Felkner *et al.*, 2005).

Anaemia during pregnancy is perceived to stem from ID and many other micronutrient deficiencies (e.g. folate, riboflavin, vitamins A, vitamin C and B12), acute and chronic infections (e.g. malaria, tuberculosis (TB) and human immunodeficiency virus (HIV), and hereditary disorders that hinder haemoglobin synthesis, red blood cell production or red blood cell survival (e.g. haemoglobinopathies) (WHO, 2012). Pregnant women of all ages require 27 mg/day to meet iron demands throughout pregnancy (Adu-Afarwuah *et al.*, 2017a). The utilisation and need for absorbed iron increases by up to 4 mg/d during pregnancy to accommodate the growing foetus

and when these requirements are not met, the risk of pre-term or low birth weight delivery as well as maternal and perinatal deaths increases (Nojilana *et al.*, 2007; Yip, 1996).

It is critical for the body to have adequate iron stores to perform fundamental functions during pregnancy and to avoid adverse effects of ID (Nojilana *et al.*, 2007). Moreover, the production of red blood cells requires iron, a component of the oxygen carrier haemoglobin, which has to be sufficient to meet the foetal needs and maternal erythropoiesis (Friedman *et al.*, 2012). Infants born to mothers who are anaemic are more likely to have poor intellectual development due to them being prone to IDA (van den Broek & Letsky, 2000). This occurs as a consequence of the increased oxygen demand of the brain during development which cannot be met (Alemayehu *et al.*, 2019).

Major natural food sources of mineral iron include cooked green leafy vegetables, meat and organ meat, cooked lentils and fortified cereals (Nojilana *et al.*, 2007). In South Africa, due to a high prevalence of ID, compulsory iron fortification of staple foods, such as maize and wheat flour (35 mg electrolytic iron per kg), as well as the endorsement of the food-based dietary guidelines to help prevent ID were introduced in 2003 (Mchiza *et al.*, 2018; Thejpal, 2015; van Jaarsveld *et al.*, 2015).

Iron absorption is tightly regulated as the body has no excretory mechanism for iron. In addition, homeostasis is an effective process in that when the body has adequate iron stores, absorption is inhibited. The uptake, transport, storage and utilisation of iron is controlled by feedback regulation. Moreover, the protein hepcidin plays an intricate role in iron homeostasis through its effect on the iron exporter ferroportin s concentrations in the enterocyte, macrophage and hepatocyte. Furthermore, hepcidin production has been shown to be increased by inflammation especially in pregnancies associated with chronic inflammation, thereby impair iron absorption and leads to the development of anemia of chronic diseases (Gozzelino and Arosio., 2016).

In pregnancy, the WHO recommends a daily oral iron supplementation of 30 to 60 mg of elemental iron and 0.4 mg folic acid to prevent anaemia during foetal development (WHO, 2012). Following this recommendation, the Integrated Nutrition Programme of the South African Department of Health, supplements 200 mg ferrous sulphate tablets daily (equivalent to ~60 mg elemental iron) to all pregnant and lactating women through healthcare facilities from the second trimester, without first determining anaemia or iron status. In addition, they receive 1000 mg calcium and 5 mg folic acid tablets daily to prevent complications such as pre-eclampsia and neural tube defects (DoH, 2015). Several markers assess iron status during pregnancy: haemoglobin (Hb), serum ferritin (storage form of iron in the blood) and soluble transferrin

receptor (sTfR). Unlike ferritin, sTfR is less altered by inflammation and is therefore deemed the best indicator of iron status (Nair *et al.*, 2004).

Iron supplementation during pregnancy can have detrimental effects, such as nausea, vomiting, diarrhoea and constipation (WHO, 2012), which may be worse and more prevalent in those who have adequate iron status (Goodchild, 2018). More seriously, there have been cases of iron sufficient pregnant women receiving elemental iron having increased risk of haemoconcentration at delivery and small, for gestational age (SGA), infants (Shastri *et al*., 2015). Excessive iron can be detrimental to health and lead to increased oxidative stress, causing lipid peroxidation through the generation of free radicals and also damage the DNA and protein structures (McArdle *et al.*, 2014). Oxidative stress is characterised by the disturbance in homeostasis between the production of oxygen radicals and the inability of an individual to neutralise free radicals during periods of high metabolic demand (Mavangira & Sordillo, 2018). It is therefore of utmost importance that iron levels remain at equilibrium in the body to avoid a high accumulation in the tissues. Accumulation can be toxic, and bring about the production of reactive oxygen species and trigger unfavourable outcomes correlated with oxidative stress, such as cellular damage and cell death (Ma *et al.*, 2010; Tussing-Humphreys *et al.*, 2012; Wessling-Resnick, 2010).

A small randomised clinical trial conducted by Ziaei and colleagues on pregnant women with haemoglobin ≥13.2 g/dL receiving 50 mg/d of iron from the second trimester, found an association between iron excess and the risk of developing hypertension compared with the placebo group (Chen *et al.*, 2019; Ziaei *et al.*, 2007). However, a randomised double-blind controlled trial in China found conflicting results. They enrolled pregnant women before 20 weeks of gestation with haemoglobin concentrations of <13.2 g/dL and found that multiple micronutrient supplementation containing 30 mg iron reduced the risk of pregnancy-induced hypertension (Chen *et al.*, 2019). Oxidative stress and inflammation are both involved in hypertension, but whether inflammation is a cause or effect of hypertension is not well understood (Dinh *et al.*, 2014).

In addition, plasma iron levels can be altered by inflammation (Thurnham *et al.*, 2010). The rate at which iron is absorbed during inflammatory activity decreases due to ferroportin losing its protein component required in transporting iron out of cells. This down-regulation is caused by the up-regulation of hepcidin in the liver as pro-inflammatory cytokines such as IL-6 are released (Cherayil, 2011). Even though it has been documented that serum ferritin is the best indicator of iron levels in the blood and can be used to diagnose ID, inflammation also increases ferritin levels, as it is an acute phase protein (Cherayil, 2011). Ferritin levels, therefore, increase regardless of the iron status if inflammation is present (Brindle *et al.*, 2017). The WHO suggested that ferritin be measured together with C-reactive protein (CRP) and α1-acid glycoprotein (AGP) to be able

to adjust for acute phase response during inflammation (Knowles *et al.*, 2013; Namaste *et al.*, 2017).

2.6 Markers of inflammation

C- reactive protein and AGP are the most prominent acute phase proteins (APPs) used as markers of inflammation (Thurnham *et al.*, 2010). C- reactive protein and AGP are classified as positive APPs since their concentrations increase in plasma in response to inflammation (Gabay & Kushner, 1999), whilst albumin, transferrin, and retinol-binding protein are some of the examples of negative APPs (Grover *et al.*, 2016).

During acute inflammation, CRP is released from the liver by the hepatocytes, and its activity is promoted by the pro-inflammatory cytokine IL-6, where its concentration increases drastically to show the presence of inflammation (Sacks *et al.*, 2004; Wang, Y. *et al.*, 2015). C- reactive protein is both pro-inflammatory and anti-inflammatory and is reported to be synthesised in neurons, atherosclerotic plaques, monocytes, and lymphocytes, in small quantities (Jialal *et al.*, 2004). C-reactive protein typically responds to inflammation in 24-48 hours, therefore it will not be a useful marker in measuring inflammation during convalescence in seemingly healthy individuals due to its short half-life of 19 hours (Shinoda *et al.*, 2013; Thurnham *et al.*, 2010; Thurnham *et al.*, 2015). A better alternative marker would be AGP, which reaches its maximum concentration in 3-5 days and remains elevated after the infection (Thurnham *et al.*, 2010). α1-acid glycoprotein is secreted by hepatocytes during inflammation and its activity is promoted by IL-1, IL-6, and glucocorticoids (Fournier *et al.*, 2000). The physiological function of AGP, especially during pregnancy is still unclear, however, it has been reported to have immunomodulatory effects (Sauder *et al.*, 2019; Fournier *et al.*, 2000). Four categories of inflammation exist as determined from measures of APPs (CRP and AGP), namely no inflammation, incubation, early and late convalescence (Knowles *et al.*, 2013; Shinoda *et al.*, 2013)

C- reactive protein is a component of the innate immune system and can activate the classical complement pathway, stimulate phagocytosis and bind to immunoglobulin receptors (Black *et al.*, 2004). It is associated with the signalling of pro-inflammatory cytokines (IL-6, IL-1β, and TNF) released by the visceral adipose tissue, thus showing its association with obesity (Salazar *et al.*, 2014). Pregnant obese women are, therefore, at increased risk of being in a pro-inflammatory state, which may give rise to the various risk factors, such as foetal growth restriction. Furthermore, neonatal complications such as preterm birth, low birth weight, and small size for gestational age are associated with low-grade inflammation, as shown by high concentrations of CRP (Ernst *et al.*, 2011; Gaillard *et al.*, 2016). High CRP levels during low-grade inflammation are

also associated with preeclampsia and gestational diabetes (Adu-Afarwuah *et al.*, 2017b; Wang, Y. *et al.*, 2015).

2.7 Conclusion

Hypertension during pregnancy is a global and local public health problem and can lead to adverse effects for both the mother and neonate. LCPUFA intake and status, through its role in inflammation and other mechanisms, may modulate blood pressure, but research on the association is inconclusive. Routine iron supplementation may contribute to the development of oxidative stress and inflammation, which may influence blood pressure and the development of hypertension. Further research is required to ascertain a link between n-3 PUFA status and BP, and if iron status and inflammation have an influence on this relationship.

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CHAPTER 3 RESEARCH METHODOLOGY

3.1 Study design

This is a prospective cohort study that followed 250 women from < 18 to 36 weeks of gestation. This sub-study is nested within a larger study, titled Nutrition during Pregnancy and Early Development (NuPED) and consists of both longitudinal and cross-sectional analyses using secondary data.

3.2 Study setting and population

3.2.1 Study site and infrastructure

The setting of the research, and specifically recruitment, was in Primary Health care clinics and the ante-natal clinic (ANC) at Rahima Moosa Mother and Child Hospital (RMMCH) within municipal Regions B and C of the COJ (City of Johannesburg, 2015a, 2015b) in the Gauteng Province. The COJ is divided into seven regions (See Figure 3.1) (City of Johannesburg, 2015c). RMMCH (formerly known as Coronation Hospital) is a provincial hospital focusing on maternal and paediatric healthcare and is situated in Coronationville, which falls in Region B. This is the only government hospital in South Africa (SA) focusing on maternal and paediatric care and therefore serves as an ideal point of focus to address the research aim. Sampling participants from this region would also supply results representative of most urban areas in SA. After recruitment participants were followed-up at RMMCH.

Region A	Diepsloot, Kya Sands, Dainfern, Midrand, Lanseria, Fourways
Region B	Randburg, Rosebank, Emmarentia, Greenside, Melville, Mayfair, Northcliff, Rosebank, Parktown, Parktown North
Region C	Roodepoort, Constantia Kloof, Northgate, Florida, Bram Fischerville
Region D	Doornkop, Soweto, Dobsonville, Protea Glen
Region E	Alexandra, Wynberg, Sandton, Orange Grove, Houghton
Region F	Inner City, Johannesburg South
Region G	Orange Farm, Weilers Farm, Ennerdale, Lenasia, Eldorado Park, Protea South

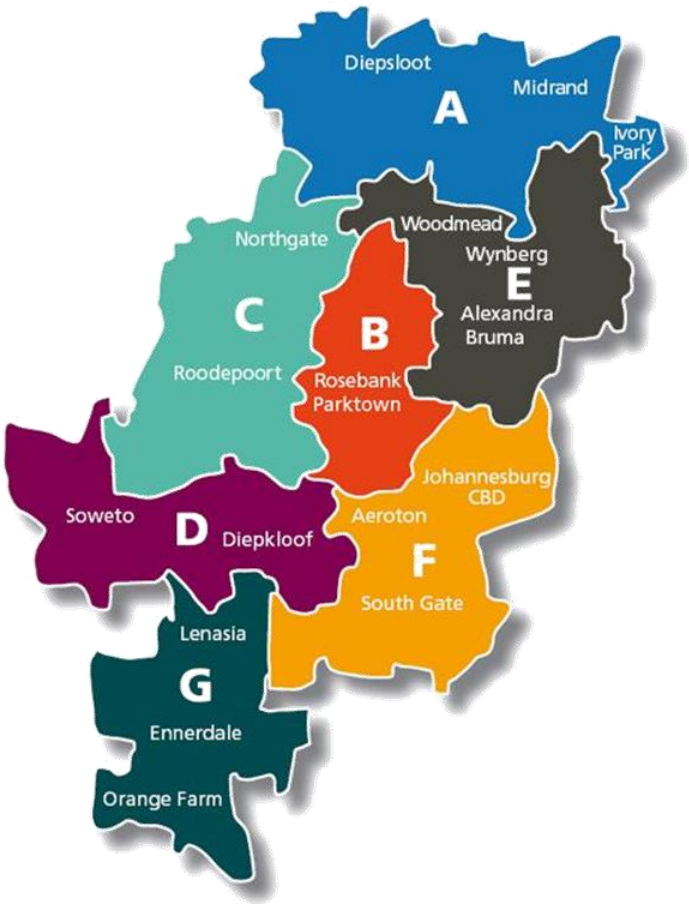

Figure 3-1: The seven regions of the City of Johannesburg (obtained from: http://www.htxt.co.za/wp-content/uploads/2014/06/jra-map.jpg)

3.2.2 Study population

3.2.2.1 Inclusion and exclusion criteria

The study population are urban pregnant women attending ANC facilities in selected clinics within regions B and C of the COJ, complying with the inclusion and exclusion criteria listed in Table 3-1.

Table 3-1: Summary of inclusion and exclusion criteria at sampling

Inclusion criteria	Exclusion criteria
Confirmed pregnancy and planning to deliver the baby at RMMCH <18 weeks gestational age	>18 weeks gestational age
Born in South Africa, Lesotho, Swaziland, Zimbabwe, Botswana or Namibia	<18 years and >39 years
Able to communicate effectively in one of the following languages: English, Afrikaans, Sotho, Zulu or Xhosa	Multiple foetus pregnancy
	Women using illicit drugs (self-confessed)
	Smoking (current and/or in past year)
	Known non-communicable diseases (NCDs) such as diabetes, renal disease, history of high blood cholesterol, and hypertension
	Known infectious disease such as tuberculosis and hepatitis*
	Known serious illness such as cancer, lupus or psychosis

*HIV positive participants were included in the study

The inclusion and exclusion criteria are dependent on the larger NuPED study. For this sub-study, all women enrolled in the NuPED study were included.

3.2.3 Sample size calculation

For this sub-study, all participants of the larger study were included (n = 250). In the absence of reliable data regarding possible effect size in the sub-study, a measure of standardised effect size was considered. Power calculations were performed using G*power vers.3.1.9.2. The power

calculation was performed considering the regression f^2 as standardised effect size ($f^2 = R^2/(1-R^2)$). According to our calculations, a sample size of 250 participants was sufficient to achieve a type-II error rate below 20% (power ≥ 80%) to detect a medium to large effect size in a multivariate model with up to four covariates. A second power calculation considering a logistic regression confirmed a sample size of 250 subjects for large to very large effect sizes (odds ratio ≥ 1.7).

3.2.4 Recruitment, enrolment and informed consent

The first step of the recruitment process was facilitated by the nursing operational managers of the identified clinics by acting as mediators during the process on the basis that the necessary authorities approved. The nursing staff was thoroughly trained on the aspects of the recruitment process. The patients visiting the clinics were informed about the study and given a detailed information brochure about the project, written in simple language (English, Afrikaans, Sotho, Xhosa and Zulu). Those interested were screened for eligibility in a private room by a trained fieldworker in a one-on-one setup. Further details of the study were explained including the inclusion and exclusion criteria (see Table 3-1). Patients who did not meet the inclusion criteria were advised on healthy habits during pregnancy and received a pamphlet. Those interested and eligible received a further detailed explanation with the informed consent form. They were given two options if they were interested to voluntarily participate: 1) they were given time alone or with any person of their choice to consider signing the written informed consent form; 2) they returned home with the informed consent form to discuss with those they wished to, and would return on the next appointment. Nursing staff, the fieldworker(s) and a research team member were available for any questions. A few short questions were asked before signing the informed consent form to ensure the respondent comprehended the information supplied. Participants were informed they could withdraw from the study at any time without consequences.

3.3 Research procedures and data collection

The sub-study sample was assessed at <18, 22 and 36 weeks' gestation, categorised as phase 1, 2 and 3 (see Table 3-2 for details). Participants were recruited from the clinics and from there they were referred for ANC at RMMCH for the duration of the larger study. Participants were reimbursed for the additional travelling at a standard amount. In the first phase, participants were assessed in early pregnancy (<18 weeks) at RMMCH.

Table 3-2: Schedule of study activities

Phase	1[a]	2	3
Approximate gestational age (weeks)	18 weeks or less	22 weeks	36 weeks
Window		±1 week	±1 week
Informed consent	X		
Medical history	X	X	X[e]
Demographics	X		
Inclusion/Exclusion	X		
Anthropometric measurements[c, d]	X[b]	X	X
Quantitative Food Frequency Questionnaire (QFFQ)	X	X	
24-hour recall	X	X	X
Blood sample	X	X	X
Blood pressure	X	X	X

a: Recruitment was done at four different primary healthcare clinics: Florida clinic; Zandspruit clinic; Bosmont clinic; Sophiatown, clinic as well as at the antenatal care clinic of RMMCH.

b: Height was measured only at phase 1.

c: Anthropometric measurements included: height; weight; MUAC

d: Weight and MUAC measurements were taken at Ward 6 (where admission was done).

e: Medical history from Maternity Case Record determined adverse outcomes or change in health status

All female fieldworkers were fully trained on all aspects of extensive data collection. The two study co-ordinators were responsible for overseeing the safe storage and transport of samples.

3.3.1 Medical history

Medical history included the development of hypertension, diabetes, tuberculosis and human immunodeficiency virus infection during pregnancy; bacterial vaginosis; smoking; the number of births; etc. This was done as part of routine medical assessment.

3.3.2 Anthropometric data

Anthropometric data, including weight, height and mid-upper arm circumference (MUAC), were collected at all three phases. The procedures were performed according to the National Health

and Nutrition Examination Survey (NHANES) Anthropometry Procedures Manual 2007, using a calibrated digital scale for weight, a mobile stadiometer for height and non-stretchable metal measuring tape for MUAC.

3.3.3 Diet history

Dietary intake data were obtained by means of single 24-hour recall (24-HR) questionnaires administered at phases 1, 2 and 3 by trained fieldworkers. The purpose of the 24-HR was to collect diet history as well as supplement use information. An additional section on the compliance of supplement use was included in the questionnaire. The second dietary assessment method, a validated quantified food frequency questionnaire (QFFQ) (MacIntyre *et al.*, 2001), obtained dietary intake data and assessed individual and total omega-3 and omega-6 fatty acid intake at <18 week's gestation.

3.3.4 Brachial blood pressure

Brachial blood pressure was measured and recorded by trained fieldworkers with Dinamap® ProCare at all three phases. It was correctly calibrated if Pressure Transducer Verification was out of tolerance. The second of the two measurements was used. Hypertensive status was defined as DBP ≥ 90 or SBP ≥ 140, and pre-hypertensive as DBP ≥ 80 or SBP ≥ 120 (Weber *et al.*, 2014). Pulse pressure (PP), defined as the difference between SBP and DPB, was calculated and PP > 60 mm Hg was considered as high.

3.3.5 Biological sampling and invasive procedures

Maternal venous blood was collected by a qualified professional at the laboratory of the hospital. Venous blood samples were drawn into EDTA-coated and trace element free evacuated tubes (Becton Dickinson) via antecubital venepuncture. Blood samples were processed within 1 hour after the blood was drawn by a trained research team member at an allocated space in the medical facility. Plasma was separated and aliquots were stored at -20°C on-site and transported to the UNISA laboratories of the Sciences campus in Florida, where it was stored in locked facilities at -80°C. There was restricted access to all on-site freezer facilities. Red blood cells (RBCs) were washed with normal saline and aliquots were stored at -20°C on-site and transported on dry ice (or portable freezer) to the NWU laboratories for storage at -80°C for up to seven years. Storage temperature was monitored and logged for the entire duration of the study. All biochemical analyses were performed at the Centre of Excellence for Nutrition (CEN) by qualified laboratory personnel. There is laboratory facilities and expertise for gas chromatography-mass

spectrometry at CEN.

3.4 Biochemical analyses

3.4.1 Inflammation biomarkers

C-reactive protein (CRP) and α1-acid glycoprotein (AGP) were analysed in serum samples with a multiplex enzyme-linked immunosorbent assay (ELISA) on the Quansys Q-View Imager Pro (Logan, Utah, USA). Elevated inflammation, was defined as CRP >5 mg/L and AGP >1 g/L (Thurnham *et al.*, 2010).

3.4.2 Iron status

Haemoglobin was analysed with HemoCue® Hb 201+ System (Angelholm, Sweden), performed by the NuPED laboratory technician (Mathee *et al.*, 2014). Ferritin and soluble transferrin receptor (sTfR) concentrations were determined using the Q-Plex Human Micronutrient Array (7-plex, Quansys Bioscience, Logan, UT, USA) (Brindle *et al.*, 2017). This fully quantitative chemiluminescent multiplex assay also included the acute phase proteins CRP and AGP. Ferritin concentrations were adjusted for inflammation using the correction factors recommended by Thurnham *et al*. (2015). Iron deficiency was defined as adjusted ferritin <15 µg/L (WHO, 2011), iron deficient erythropoiesis (IDE) was defined as sTfR >8.3 mg/L (Erhardt *et al*., 2004) and iron deficiency anaemia (IDA) as ferritin <15 µg/L plus Hb<11 g/dL (WHO, 2017)**.**

3.4.3 Red blood cell total phospholipid fatty acid status

Total phospholipids were extracted from RBCs with chloroform: methanol (2:1 vol: vol; containing 0.01% butylated hydroxytoluene) using a modification of the method of Folch *et al*. Lipid extracts were concentrated, and the neutral lipids separated from the phospholipids by 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 was removed from the thin-layer chromatography plate and trans-methylated with methanol: sulphuric acid (95:5 vol: vol) at 70ºC for 2HR to yield fatty acid methyl esters (FAMEs). The resulting FAMEs were extracted with water and hexane. The organic layer was evaporated, redissolved in hexane and analysed using quadrupole gas chromatography–electron impact–mass spectrometry on an Agilent Technologies 7890A Gas Chromatograph system, equipped with an Agilent Technologies 7000GC/MS 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), using helium as the carrier gas at a flow rate of 2.2 mL/min. The gas chromatography

injector was held at a temperature of 270ºC, and the mass spectrometry source at 250ºC. The injection volume of the sample solution was 1 µL by using a split ratio of 1:20. The oven temperature was programmed from 50 to 170ºC at 30 ºC/min, then from 170 to 215ºC at 2ºC/min, and rose at 4ºC/min to 230ºC. After that, the temperature was held isothermally at 230ºC for 7 min. The total analysis time was 38.25 min. Mass spectrometry with 70 eV electron ionisation was carried out in a multiple reaction monitoring mode, with at least two transitions per compound. Quantification of FAME was done 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 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.

Groups were defined based on RBC phospholipid EPA plus DHA compositions as follows: ≤ 3.8% = very low, > 3.8–5.7 %= low, > 5.7–7.6% = moderate, > 7.6% = high (Stark *et al.*, 2016).

3.5 Sample storage quality assurance

Maternal venous blood samples were collected from the study site, RMCH, every week and transported to the UNISA laboratories of the Sciences Campus in Florida, where it was stored in locked facilities at -80°C. There was restricted access to all on-site freezer facilities. The samples were then transported to CEN on dry ice for storage at -80°C for up to 7 years. Storage temperature was monitored and logged for the entire duration of the study.

3.6 Data collection, management and quality control, storage and archiving

All collected data (original data and hard copies stored in locked cabinets in the researcher's office, while electronic data were password protected) are stored at CEN (NWU) for 7 years. Extreme values were identified according to a three standard deviation rule, and data were checked for unrealistic values, as well as typing and quantisation errors. Random checking of 10% of the data was performed by double-checking data entries for correctness. Data will be deleted from the student's computer after completion of her degree.

3.7 Statistical analysis

Statistical analyses were performed using SPSS software (SPSS Inc, Chicago, IL, USA). IBM SPSS Statistics (version 26; IBM Corporation). Model assumption of linearity and normality of

residuals was evaluated using graphical approaches such as scatterplot and histogram visual inspection. In case of clear model violation, outcome and covariate transformation or non-parametric tests were used. Normally distributed data were described using means and standard deviations; and non-normally distributed data as medians and 25th and 75th percentiles. Frequencies and percentages were used to describe categorical variables. Continuous data were analysed using a generalised linear model approach, both unadjusted and corrected for covariates in two different models. Cross-sectional analysis was conducted at each time-point with Pearson's correlation and linear regression (ANOVA and ANCOVA). Longitudinal analysis of normal data was performed with the repeated measures general linear regression test (ANOVA or ANCOVA) and Bonferroni corrections for between group analyses. Sphericity was assumed when $P > 0.05$ with Mauchly's test. Greenhouse-Geisser was used when sphericity was violated. Longitudinal analysis for skewed data was performed with Friedman's test and differences between phases were tested with Wilcoxon signed rank tests. Categorical associations were analysed with the Chi-square test for independent data and McNemar's test for dependant data. Analysis with ANCOVA for the relationship of fatty acid status and blood pressure was adjusted for age, gestational age, parity, aspirin intake, and MUAC in the partially adjusted model and additionally for the intake of the respective individual fatty acid analysed in the fully adjusted model. Pearson's (normal data) or Spearman's (skewed data) correlations were used to test for collinearity among potential covariates and collinear covariates were not included in analyses. The influence of inflammation and iron status on associations between fatty acid status and blood pressure were verified by testing for loss of significance with the addition of inflammation and iron status markers at <18 week's gestation as covariates to the fully adjusted models. The level of significance was set at $P < 0.05$.

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CHAPTER 4 RESULTS

4.1 Introduction

The following chapter provides the main findings of the research project of the 250 pregnant women, from different antenatal clinics in the city of Johannesburg, who participated in the study. The participants' characteristics and fatty acid intake of the total sample and in relation to blood pressure status at enrolment (<18 week's gestation) are provided in section 4.2. Section 4.3 presents the status of the women throughout pregnancy, at <18, 22 and 36 week's gestation. This includes the 1) participant anthropometric status, 2) inflammatory status, 3) blood pressure, hypertensive status and aspirin intake, 4) iron status and 5) red blood cell (RBC) total phospholipid fatty acid status. Section 4.4 focuses on the relationship between fatty acid status and blood pressure, taking into account possible confounders. Lastly, section 4.5 provides data on the influence of inflammation and iron status on the relationship between fatty acid status and blood pressure.

4.2 Participant characteristics

Table 4-1 provides the demographic and anthropometric characteristics of the total study sample grouped by blood pressure status at <18 week's gestation. The study participants had a mean age of 27.7 ± 5.0, ranging from 18 to 38 years. Most of the participants were from African descent and in terms of ethnicity, 87.6% (n = 219) were Black, followed by 11.2% (n = 28) Coloured, and a small portion were Indian and White, both 0.4% (both n = 1). Participants had a median weight of 67.4 (58.0, 77.8) kg, a mean height of 159.2 ± 6.2 cm and a mean mid-upper arm circumference (MUAC) of 30.3 ± 4.7 cm at <18 week's gestation. Most of the participants had attained Grade 11-12 (58%) and almost a quarter had attained a tertiary qualification (23.2%). Twenty-five percent (25.6%) of the participants were human immunodeficiency virus (HIV) positive.

There was no significant difference in the ethnicity, country of birth, education level, HIV status, inflammatory marker concentrations and iron status markers between the normotensive and prehypertensive group. Gestational age on the day of data collection (<18 week's gestation visit) was lower (p=0.047) and parity tended to be higher (p=0.061) in the prehypertensive group. Weight was higher in the prehypertensive group (all p<0.001), whereas height tended to be higher in the prehypertensive group (p=0.065). Anthropometric status according to body mass index (BMI) classification and MUAC was different between the groups, with a higher prevalence of obesity and lower prevalence of normal weight and underweight (p<0.001) and a higher mean MUAC (p<0.001) in the prehypertensive group.

Table 4-2 provides the results of the associations of the fatty acid intake with the blood pressure status at <18 week's gestation. There were no significant differences in dietary fatty acid intake between the normotensive and prehypertensive groups, both in the unadjusted model and when corrected for total fat intake.

Table 4-1: Characteristics of the normotensive and prehypertensive participants at <18 week's gestation

	Total sample n=250	Normotensive n=208[4]	Prehypertensive n=40	p-value
Demographics				
Age (years)	27.7 ± 5.0[1]	27.6 ± 5.1	28.5 ± 4.8	0.279
Gestational age (days)	96.2 ± 20.2	97.5 ± 20.0	90.6 ± 20.1	0.047
Parity	1 (0, 2)	1 (0, 2)	2 (0, 2)	0.061
Ethnicity • Black • Coloured • Indian • White	 219 (87.6)[2] 28 (11.2) 1 (0.4) 1 (0.4)	 181 (87.4) 24 (11.6) 1 (0.5) 1 (0.5)	 36 (90.0) 4 (10.0) 0 (0.0) 0 (0.0)	0.922
Country of birth • South Africa • Zimbabwe • Lesotho • Swaziland	 172 (72.0) 60 (25.0) 4 (2.0) 3 (1.0)	 146 (72.6) 49 (24.4) 4 (2.0) 1 (1.0)	 25 (69.4) 10 (27.8) 0 (0.0) 1 (2.8)	0.644
Education • None/primary • Gr 8-10 • Gr 11-12 • Tertiary	 9 (3.6) 37 (14.8) 145 (58.0) 58 (23.2)	 8 (3.9) 31 (15.0) 117 (56.5) 51 (24.6)	 1 (2.5) 5 (12.5) 28 (70) 6 (15)	0.723
HIV Positive	64 (25.6)	53 (25.5)	11 (27.5)	0.789
Anthropometry				
Weight (kg)	67.4 (58.0, 77.8)[3]	65.9 (56.6, 74.8)	81.3 (68.7, 92.7)	< 0.001
Height (cm)	159.2 ± 6.2	158.9 ± 6.2	160.9 ± 6.1	0.065
BMI (kg/m^2)	26.3 (23.0, 30.6)	25.9 (22.5, 29.9)	31.4 (25.9, 37.8)	< 0.001
Mid-upper arm circumference (cm)	29.9 (27.1, 33.1)	29.5 (26.7, 32.2)	32.8 (29.6, 37.4)	< 0.001
Weight status • Underweight (<18.5 kg/m^2) • Normal weight (18.5–24.9 kg/m^2) • Overweight/ Pre-obese (25–29.9 kg/m^2) • Obese (>30 kg/m^2)	 8 (3.0)[2] 89 (36.0) 81 (33.0) 71 (28.0)	 7 (3.4) 83 (40.1) 68 (32.9) 22 (23.7)	 0 (0.0) 5 (12.5) 13 (32.5) 49 (55.0)	< 0.001
Inflammation				
AGP	0.75 (0.63, 0.86)	0.74 (0.62, 0.86)	0.80 (0.71, 0.89)	0.207
CRP	6.48 (3.1, 14.4)	6.47 (3.10, 14.12)	8.32 (3.72, 18.38)	0.708
Iron status				
Ferritin	47.6 (21.3, 98.7)	44.2 (21.0, 92.0)	56.2 (21.3, 107,7)	0.947
TfR	4.8 (3.8, 6.6)	4.7 (3.7, 6.4)	5.6 (4.3, 8.4)	0.647

Differences between normotensive and prehypertensive groups were analysed with independent t-tests. [1]Values are means ± SD, [2]values are n (%), [3]values are medians (25th, 75th percentiles), [4]two data points missing. $p<0.05$ was considered significant. Abbreviations: AGP, α1-acid glycoprotein; BMI, Body mass index (cut-off categories are for non-pregnant individuals); CRP, C-reactive protein; TfR, transferrin receptor.

Table 4-2: Fatty acid intake in relation to blood pressure status at <18 week's gestation

Fatty acid intake (g)	Total sample n=250	Normotensive n=208	Prehypertensive n=40	p-value[1]	p-value[2]
Linoleic acid	22.2 ± 11.4	22.0 ± 11.5	23.6 ± 11.1	0.404	0.649
α-Linolenic acid	0.57 ± 0.31	0.60 ± 0.30	0.6 ± 0.34	0.641	0.747
Arachidonic acid	0.15 ± 0.11	0.14 ± 0.11	0.17 ± 0.11	0.120	0.174
Eicosapentaenoic	0.10 ± 0.11	0.09 ± 0.10	0.12 ± 0.12	0.092	0.124
Docosahexaenoic acid	0.20 ± 0.21	0.19 ± 0.21	0.24 ± 0.22	0.178	0.232
Total n-3 PUFA	0.91 ± 0.55	0.90 ± 0.54	1.02 ± 0.61	0.206	0.310
Total n-6 PUFA	22.4 ± 11.5	22.2 ± 11.6	23.9 ± 11.2	0.396	0.636
n-6/n3 EFA ratio	43.7 ± 24.4	43.7 ± 25.0	45.0 ± 21.7	0.747	0.585
n-3 LCPUFA	0.33 ± 0.34	0.31 ± 0.34	0.40 ± 0.37	0.125	0.167
n-6 LCPUFA	0.21 ± 0.13	0.21 ± 0.13	0.25 ± 0.14	0.084	0.117
n6/n3 LCPUFA ratio	1.12 ± 1.12	1.17 ± 1.18	0.90 ± 0.67	0.161	0.146

[1]Differences between normotensive and prehypertensive groups were analysed with independent t-tests. [2]Differences between normotensive and prehypertensives groups were analysed with ANCOVA, adjusted for total fat intake. p<0.05 was considered significant. Abbreviations: LCPUFA: long-chain polyunsaturated fatty acids; PUFA, polyunsaturated fatty acids.

4.3 Participant status during pregnancy

The women were followed during pregnancy at <18, 22 and 36 week's gestation and anthropometric, blood pressure, inflammatory and iron status markers, as well as phospholipid fatty acid composition in RBC, were measured. Table 4-3 shows the weight, BMI and MUAC increase during pregnancy (all p>0.001).

Table 4-3: Participant anthropometric status during pregnancy

	Phase 1 (<18 weeks) n=250	Phase 2 (22 weeks) n=234	Phase 3 (36 weeks) n=199	Change over pregnancy p-value
Anthropometry				
Weight (kg)	67.4 (58.0, 77.8)[c1]	70.9 (61.9, 80.8)[b2]	77.3 (67.4, 86.2)[a2]	<0.001
BMI (kg/m^2)	26.3 (23.0, 30.6)[c]	27.8 (24.6, 32.1)[b]	30.2 (26.8, 34.1)[a]	<0.001
Mid-upper arm circumference (cm)	29.9 (27.1, 33.1)[c]	30.25 (27.6, 33.4)[b5]	30.85 (28.3, 34.3)[a5]	<0.001

Change in weight and mid-upper arm circumference over pregnancy was tested with Friedman's test. Differences between phases were tested with Wilcoxon signed-rank tests. Values in the same row with different letters differ significantly. [1]Values are medians (25th, 75th percentiles), P<0.05 was considered significant. Abbreviation: BMI, Body mass index.

Table 4-4 summarises the results of inflammatory markers. The prevalence of elevated α-1-Acid glycoprotein (AGP) (> 1 g/L) was 11.2% and that of elevated c-reactive protein (CRP) (> 5g/L) was 59.6% at <18 weeks and decreased to 1.6% and 38.0% 22 and 36 week's gestation (both p<0.001), respectively (Table 4-4).

AGP and CRP concentrations were not different between HIV positive and HIV negative women during the three phases of pregnancy (<18weeks: p=0.162 and 0.397; 22 weeks: 0.699 and p=0.266; 36 weeks: p=0.512 and 0.195, respectively, results not shown). The AGP and CRP concentrations were higher or tended to be higher in overweight and obese women combined, compared with normal and underweight combined in the first two phases of pregnancy (<18 weeks: p=0.005 and p=0.068; 22 weeks: p=0.006 and p=0.102), but not at 36 weeks (both p=0.224, results not shown).

Table 4-4: Inflammatory status during pregnancy

Inflammatory status	**Phase 1 (<18 weeks) n=250**	**Phase 2 (22 weeks) n=234**	**Phase 3 (36 weeks) n=199**	**Change during? pregnancy p-value**	
α-1-Acid glycoprotein (g/L)	0.75 (0.63, 0.86)[a1]	0.61 (0.53, 0.74)[b2]	0.55 (0.47, 0.65)[c3]	<0.001	
C-Reactive protein (mg/L)	6.48 (3.1, 14.4)[a]	6.55 (3.1, 12.8)[a3]	4.77 (2.8, 9.8)[b]	<0.001	
Inflammation prevalence				**p-value[4]**	**p-value[5]**
Chronic inflammation α-1-Acid glycoprotein (>1 g/L)	28 (11.2)[6]	9 (3.6)[3]	4 (1.6)[3]	0.002	<0.001
Acute inflammation C-Reactive Protein (>5 mg/L)	149 (59.6)	136 (54.4)[3]	95 (38.0)[3]	0.784	<0.001

Change in inflammatory status during the pregnancy was tested with Friedman's test. Differences between phases were tested with Wilcoxon signed-rank tests. Values in the same row with different letters differ significantly. [1]Values are medians (25th, 75th percentiles), [2]two data points missing, [3]three data points missing, [4]difference between <18 and 22 weeks was tested with Mcnemar's test, [5]difference between <18 and 36 weeks was tested with Mcnemar's test, [6]values are n (%). P<0.05 was considered significant.

Table 4-5 provides the blood pressure, hypertensive status and aspirin intake of the participants throughout pregnancy. The median systolic and diastolic (both p<0.001) blood pressures were higher <18 and 36 weeks and lower at 22 weeks. There was no change in pulse pressure (p=0.638) or prehypertension status over the three time points of pregnancy (all p>0.05). The highest percentage of participants received aspirin at 22 weeks (8.8%).

Table 4-5: Blood pressure, hypertensive status and aspirin intake during pregnancy

Blood Pressure	**Phase 1 (<18 weeks) n=250**	**Phase 2 (22 weeks) n=234**	**Phase 3 (36 weeks) n=199**	**Change over pregnancy p-value**	
Systolic blood pressure (mmHg)	108 (102, 115)[a1,2]	107 (100, 114)[b]	108 (102, 114)[a3]	<0.001	
Diastolic blood pressure (mmHg)	69 (63, 75)[a2]	67 (62, 72)[b]	68 (63, 73)[a3]	<0.001	
Pulse pressure (mmHg)	39 (34, 43)[2]	40 (34, 45)	40 (36, 43)[3]	0.638	
Hypertension status				**p-value[5]**	**p-value[6]**
Hypertensive (Systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg) n (%)	2 (1.6)[4]	5 (2.1)	3 (1.5)[3]	1.000	<0.001
Pre-hypertensive plus hypertensive (Systolic blood pressure ≥120 mmHg or diastolic blood pressure ≥80 mmHg) n (%)	40 (16.1)	31 (13.2)	28 (14.3)[3]	0.229	0.137
Aspirin intake					
75 mg n (%)	2 (0.8)	18 (7.2)	10 (4.0)		
100 mg n (%)	2 (0.8)	3 (1.2)	0 (0.0)		
150 mg n (%)	0 (0.0)	1 (0.4)	1 (0.4)		
Total intake n (%)	4 (1.6)	22 (8.8)	11 (4.4)	0.040	0.837

Change in blood pressure over pregnancy was tested with Friedman's test. Differences between phases were tested with Wilcoxon signed-rank tests. Values in the same row with different letters differ significantly. [1]Values are medians (25th, 75th percentiles), [2]two data points missing, [3]three data points missing, [4]values are n (%), [5]difference between <18 and 22 weeks gestation were tested with Mcnemar's test, [6]difference between <18 and 36 weeks gestation was tested with Mcnemar's test. p<0.05 is considered significant.

Table 4-6 presents the iron status and anaemia markers. There was a decline in ferritin concentrations throughout pregnancy (p<0.001), and an increase of sTfR (p<0.001). At <18 weeks, iron deficiency (ID) and iron deficiency erythropoiesis (IDE) prevalence among the women was 14.8% and 15.2% respectively. Both ID and IDE increased over pregnancy (both p<0.001), except for ID from <18 to 22 weeks (p=0.058). Haemoglobin concentrations decreased from <18 to 22 weeks (p<0.001) and the prevalence of anaemia increased from <18 to 22 weeks (p<0.001) and from <18 to 36 weeks (p=0.003). The median percentage compliance to iron supplementation during pregnancy was estimated at 100% (0-227), and the median iron intake of 55 (0-125) mg/day was indicative of routine iron supplementation (Symington *et al.,* 2018).

Table 4-7 reveals that arachidonic acid (AA), eicosapentaenoic acid (EPA), the n-3 index, n-6 long-chain polyunsaturated fatty acid (LCPUFA) and n-3 LCPUFA decreased during pregnancy (all p<0.001, except n-3 index p=0.011), with the main decrease taking place between at <18 weeks and 22 weeks (all p<0.001 except n-3 index p=0.004).

Table 4-6: Iron status during pregnancy

	Phase 1 (<18 weeks) n=250	Phase 2 (22 weeks) n=234	Phase 3 (36 weeks) n=199	Change over pregnancy p-value	
Iron status and anaemia markers					
Ferritin (µg/L)	47.6 (21.3, 98.7)[1]	31.7 (17. 8, 58.6)[2]	20.8 (13.9, 40.1)	<0.001	
Serum transferrin receptor (sTfR) (mg/dL)	4.8 (3.8, 6.6)	6.8 (5.4, 8.3)[2]	8.1 (6.5, 10.5)	<0.001	
Haemoglobin (Hb) (g/dL)	11.7 (10.8, 12.7)	11.2 (10.1, 12.1)	11.2 (10.0, 12.1)	<0.001	
Iron deficiency and anaemia prevalence				**p-value[3]**	**p-value[4]**
ID (ferritin <15 µg/L)	37 (14.8)[5]	44 (17.6)[2]	65 (26.0)	0.058	<0.001
IDE (sTfR >8.3 mg/dL)	38 (15.2)	59 (25.4)[2]	93 (46.7)	<0.001	<0.001
Anaemia (Hb <11.0 g/dL)	70 (28.8)	103 (44.0)	87 (45.1)	<0.001	0.003

Change in iron status and anaemia markers over pregnancy was tested with Friedman's test. Differences between phases were tested with Wilcoxon signed-rank tests. Values in the same row with different letters differ significantly. [1]Values are medians (25th, 75th percentiles), [2]two data points missing [3]difference between at <18 and 22 weeks was tested with Mcnemar's test, [4]difference between at <18 and 36 weeks was tested with Mcnemar's test, [5]values are n (%). P<0.05 was considered significant. Abbreviations: ID, iron deficiency; IDE, iron deficiency erythropoiesis.

Table 4-7: Red blood cell total phospholipid fatty acid status during pregnancy

Fatty acid (% of total fatty acids)	**Phase 1 (<18 weeks) n=243**	**Phase 2 (22 weeks) n=230**	**Phase 3 (36 weeks) n=190**	**Change over pregnancy p-value**
Arachidonic acid (AA)	14.40 ± 1.75[1a]	12.41 ± 1.17[b]	12.12 ± 1.60[b]	<0.001
Eicosapentaenoic acid (EPA)	0.29 ± 0.15[a]	0.24 ± 0.12[b]	0.21 ± 0.11[b]	<0.001
Docosahexaenoic acid (DHA)	5.28 ±1.16	5.12 ±1.03	5.16 ± 1.02	0.062
n-3 index	5.57 ± 1.23[a]	5.36 ± 1.10[b]	5.37 ±1.07[b]	0.011
n-6 LCPUFA	21.45 ± 2.26[a]	19.43 ± 1.83[2b]	19.35 ± 2.63[b]	<0.001
n-3 LCPUFA	7.44 ±1.50[a]	7.05 ±1.34[b]	6.70 ± 1.36[b]	<0.001
n6/n3 LCPUFA ratio	2.99 ± 0.62	2.86 ± 0.61[2]	2.87 ± 0.60	0.070

Change in fatty acids composition over pregnancy and between groups were tested with repeated ANCOVA measures using the Greenhouse-Geisser test and post hoc Bonferroni correction. Values in the same row with different letters differ significantly. [1]Values are means ± SD, [2]One data point missing, P<0.05 was considered significant. Abbreviation: LCPUFA, long-chain polyunsaturated fatty acid.

4.4 The relationship of fatty acid status with blood pressure during pregnancy

Table 4-8 gives the correlation coefficients for the associations between RBC fatty acid composition with systolic and diastolic blood pressure, as well as pulse pressure. The compositions of AA, EPA, DHA and n-3 LCPUFA correlated positively with systolic blood pressure (r=0.133, p=0.044; r=0.208, p=0.002; r=0.217, p=0.001 and r=0.213, p=0.001) at 22 weeks. Eicosapentaenoic acid, DHA and total n-3 LCPUFA correlated positively with diastolic blood

pressure (r=0.149, p=0.024; r=0.226, p=0.001 and r=0.210, p=0.001) and EPA with pulse pressure (r=0.133, p=0.045) at 22 weeks. The n6/n3 LCPUFA ratio correlated negatively with both systolic blood pressure and diastolic blood pressure at 22 weeks (r=-0.170, p=0.010 and r= -0.175, p=0.008 respectively) and with pulse pressure at <18 weeks (r= -0.125, p=0.053). Results were unchanged when participants who received low dose aspirin any time during pregnancy were excluded.

Table 4-9 shows an unadjusted, partially adjusted and fully adjusted model of the associations between fatty acid status and blood pressure status during pregnancy. At <18 weeks, DHA showed a tendency (p=0.059) to be higher in the prehypertensive group in the unadjusted model. Compositions of EPA, DHA, n-3 LCPUFA and the n6/n3 LCPUFA ratio (all p<0.05) were higher in the prehypertensive than in the normotensive group in the unadjusted and adjusted models of 22 weeks. Results were unchanged when participants who received low dose aspirin any time during pregnancy were excluded.

4.5 Influence of inflammation and iron status on the association between fatty acid status and blood pressure

Table 4-10 illustrates there was no loss of significance when additionally adjusting for AGP, CRP, Ferritin or TfR at <18 week's gestation, after adjustment for age, gestational age, parity, aspirin intake, MUAC and the respective individual or grouped fatty acid intake of the status marker which was assessed. However, both ferritin and TfR showed a trend to be significant in the model (p=0.087 and p=0.100).

Table 4-8: Pearson's correlations of red blood cell fatty acid composition with blood pressure during pregnancy

	Phase 1 (18 weeks) n=243			Phase 2 (22 weeks) n=229			Phase 3 (36 weeks) n=188		
Fatty acid (% of total fatty acids)	**SBP**	**DBP**	**PP**	**SBP**	**DBP**	**PP**	**SBP**	**DBP**	**PP**
Arachidonic acid (AA)	r=0.063[1]	r=0.009	r=0.085	**r=0.133**	r=0.081	r=0.102	r=-0.046	r=-0.042	r=-0.021
	p=0.332	p=0.894	p=0.189	**p=0.044**	p=0.220	p=0.124	p=0.532	p=0.564	p=0.772
Eicosapentaenoic acid (EPA)	r=0.057	r=0.104	r=- 0.043	**r=0.208**	**r=0.149**	**r=0.133**	r=0.077	r=0.019	r=0.102
	p=0.380	p=0.106	p=0.505	**p=0.002**	**p=0.024**	p=**0.045**	p=0.295	p=0.795	p=0.165
Docosahexaenoic acid (DHA)	r=0.058	r=0.101	r= -0.036	**r=0.217**	**r=0.226**	r=0.058	r=-0.008	r=-0.011	r=0.001
	p=0.369	p=0.119	p=0.573	**p=0.001**	**p=0.001**	p=0.379	p=0.915	p=0.880	p=0.986
n-6 LCPUFA	r=0.009	r=-0.066	r=0.096	r=0.066	r=0.036	r=0.055	r=-0.057	r=-0.022	r=-0.065
	p=0.885	p=0.306	p=0.135	p=0.322	p=0.586	p=0.409	p=0.44	p=0.766	p=0.374
n-3 LCPUFA	r=0.027	r=0.093	r=-0.075	**r=0.213**	**r=0.210**	r=0.071	r=-0.012	r=-0.009	r=-0.009
	p=0.682	p=0.150	p=0.246	**p=0.001**	**p=0.001**	p=0.288	p=0.867	p=0.903	p=0.905
n6/n3 LCPUFA ratio	r=- 0.035	**r=- 0.125**	r=0.102	**r=- 0.170**	**r=- 0.175**	r=- 0.048	r=0.012	r=0.029	r=- 0.017
	p=0.592	**p=0.053**	p=0.114	**p=0.010**	**p=0.008**	p=0.471	p=0.867	p=0.692	p=0.817

[1]r = Pearson correlation coefficient. p<0.05 was considered significant. Abbreviations: DBP, diastolic blood pressure; LCPUFA, long-chain polyunsaturated fatty acid; SBP, systolic blood pressure; PP, pulse pressure.

Table 4-9: Fatty acid status in relation to blood pressure status during pregnancy in adjusted models

	Blood pressure status		Unadjusted model		Partially adjusted model		Fully adjusted model	
Fatty acids	**Normotensive**	**Prehypertensive**	**B-value**	**p-value**	**B-value**	**P value**	**B-value**	**p-value**
<18 weeks gestation (n=250)								
Arachidonic acid (AA)	14.37 ± 1.61[1]	14.63 ± 2.36	0.264 (-0.339, 0.868)[2]	0.389	-0.103 (-0.716, 0.511)	0.742	-0.103 (-0.716, 0.511)	0.742
Eicosapentaenoic acid (EPA)	0.29 ± 0.16	0.31 ± 0.15	0.022 (-0.032,0.076)	0.418	0.011 (-0.046, 0.068)	0.700	0.011 (-0.046, 0.068)	0.700
Docosahexaenoic acid (DHA)	5.23 ±1.14	5.61 ± 1.20	0.380 (-0.015,0.775)	**0.059**	0.267 (-0.145, 0.679)	0.203	0.267 (-0.145, 0.679)	0.203
n-6 LCPUFA	21.48 ± 2.08	21.39 ± 3.04	-0.095 (-0.873,0.683)	0.811	-0.408 (-1.213, 0.396)	0.318	-0.408 (-1.213, 0.396)	0.318
n-3 LCPUFA	7.40 ± 1.49	7.72 ± 1.53	0.321 (-0.195, 0.837)	0.221	0.158 (-0.385, 0.701)	0.567	0.158 (-0.385, 0.701)	0.567
n6/n3 LCPUFA ratio	3.01 ± 0.63	2.85 ± 0.55	-0.165 (-0.378, 0.049)	0.130	-0.146 (-0.373, 0.080)	0.203	-0.146 (-0.373, 0.080)	0.203
22 weeks gestation (n=234)								
Arachidonic acid (AA)	12.39 ± 1.22	12.58 ± 0.78	0.190 (-0.269, 0.649)	0.416	0.097 (-0.375, 0.568)	0.687	0.097 (-0.375, 0.568)	0.687
Eicosapentaenoic acid (EPA)	0.23 ± 0.11	0.30 ± 0.16	0.075 (0.029, 0.121)	**0.002**	0.076 (0.028, 0.124)	**0.002**	0.076 (0.028, 0.124)	**0.002**
Docosahexaenoic acid (DHA)	5.02 ± 1.00	5.86 ± 0.93	0.834 (0.446, 1.223)	**0.000**	0.709 (0.307, 1.112)	**0.001**	0.709 (0.307, 1.112)	**0.001**
n-6 LCPUFA	19.44 ± 1.90	19.46 ± 1.22	0.023 (-0.694, 0.741)	0.949	-0.024 (-0.768, 0.720)	0.950	-0.024 (-0.768, 0.720)	0.950
n-3 LCPUFA	6.92 ± 1.31	7.97 ± 1.24	1.058 (0.549, 1.568)	**0.000**	0.949 (0.417, 1.480)	**0.001**	0.949 (0.417, 1.480)	**0.001**
n6/n3 LCPUFA ratio	2.91 ± 0.61	2.51 ± 0.51	-0.399 (-0.632, -0.166)	**0.001**	-0.350 (-0.593, -0.107)	**0.005**	-0.350 (-0.593, -0.107)	**0.005**
36 weeks gestation (n=199)								
Arachidonic acid (AA)	12.14 ± 1.63	12.13 ± 1.35	-0.010 (-0.663, 0.642)	0.975	-0.066 (-0.759, 0.626)	0.850	-0.066 (-0.759, 0.626)	0.850
Eicosapentaenoic acid (EPA)	0.20 ± 0.09	0.24 ± 0.19	0.038 (-0.006, 0.081)	**0.091**	0.033 (-0.013, 0.079)	0.159	0.033 (-0.013, 0.079)	0.159
Docosahexaenoic acid (DHA)	5.15 ± 0.98	5.26 ± 1.27	0.112 (-0.311, 0.534)	0.603	0.053 (-0.384, 0.489)	0.812	0.053 (-0.384, 0.489)	0.812
n-6 LCPUFA	19.44 ± 2.68	19.18 ± 1.89	-0.253 (-1.314, 0.807)	0.638	-0.126 (-1.244, 0.991)	0.824	-0.126 (-1.244, 0.991)	0.824
n-3 LCPUFA	6.94 ± 1.31	7.13 ± 1.67	0.194 (-0.366, 0.754)	0.496	0.123 (-0.458, 0.704)	0.677	0.123 (-0.458, 0.704)	0.677
n6/n3 LCPUFA ratio	2.88 ± 0.58	2.83 ± 0.73	-0.048 (-0.294, 0.198)	0.702	0.001 (-0.257, 0.258)	0.995	0.001 (-0.257, 0.258)	0.995

Unadjusted associations between the respective fatty acid compositions and blood pressure status were tested with ANOVA. The partially adjusted model was adjusted for age, gestational age, parity, aspirin intake and mid-upper arm circumference (MUAC). The fully adjusted model was additionally adjusted for the respective individual or grouped fatty

acid intake of the status marker which was assessed. The normotensive and prehypertensive groups were classified on the basis of their blood pressure with each phase. Nornotensive ≤ (SBP 120-139, DBP 80-89), prehypertensive ≥ (SBP 120-139, DBP 80-89). [1]Values are means ± SD, [2] B unstandardised regression coefficient (95% CI). p<0.05 was considered significant. Abbreviations: LCPUFA, long-chain polyunsaturated fatty acid.

Table 4-10: Mediation analysis of iron status and inflammation at 22 weeks gestation

Fatty acids	**Normotensive**	**Prehypertensive**	**Fully adjusted p-value[1]**	**p-value after additional adjustments[2]**			
				AGP	CRP	Ferritin	TfR
Eicosapentaenoic acid (EPA)	0.23 ± 0.11	0.30 ± 0.16	**0.002**	**0.001**	**0.002**	**0.002**	**0.002**
Docosahexaenoic acid (DHA)	5.02 ± 1.00	5.86 ± 0.93	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**
n-3 LCPUFA	6.92 ± 1.31	7.97 ± 1.24	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**
n6/n3 LCPUFA ratio	2.91 ± 0.61	2.51 ± 0.51	**0.005**	**0.005**	**0.005**	**0.005**	**0.005**

Associations between the respective fatty acid compositions and blood pressure status were tested with ANCOVA. The fully adjusted model was adjusted for age, gestational age, parity, aspirin intake and mid-upper arm circumference (MUAC) and the respective individual or grouped fatty acid intake of the status marker which was assessed. [2]Additional adjustments to test for loss of significance was made individually for AGP, CRP, ferritin or TfR at <18 week's gestation [3]Values are means ± SD. Abbreviations: AGP, α1-acid glycoprotein; CRP C-reactive protein, TfR, transferrin receptor; LCPUFA, long-chain polyunsaturated fatty acid.

CHAPTER 5 DISCUSSION

5.1 Introduction

To the best of our knowledge, this is the first study to assess the relationship of long-chain polyunsaturated fatty acid (LCPUFA) status with blood pressure in pregnant predominantly black African women residing in urban South Africa. This chapter will describe the relationship of n-6 and n-3 LCPUFA with blood pressure in pregnant women receiving routine iron supplementation and whether inflammation or iron status mediated the association. Section 5.2 describes the participant characteristics at enrolment at <18 week's gestation. Section 5.3 provides the status of participants from <18 until 36 week's gestation, including the anthropometry, inflammatory status, blood pressure and hypertensive status as well as aspirin intake, iron status and also red blood cell (RBC) total phospholipid n-3 and n-6 LCPUFA status. The relationship of fatty acid composition with blood pressure is explored in section 5.4. Lastly, the influence of inflammation and iron status on the relationship between RBC phospholipid fatty acid cell composition and blood pressure is further explored in section 5.5, while section 5.6 provides concluding remarks.

5.2 Participant characteristics at enrolment (<18 week's gestation)

Several studies have investigated the relationship between n-3 and n-6 LCPUFA status and blood pressure in Caucasian, Inuit, Chinese, Malay, Indian and Hispanic people, but not in Africans (Barden *et al.*, 2006; Bulstra-Ramakers *et al.*, 1995; Lepsch *et al.*, 2018; Lim *et al.*, 2015; Olafsdottir *et al.*, 2006; Onwude *et al.*, 1995; Popeski *et al.*, 1991; Salvig *et al.*, 1996). This prospective study followed 250 pregnant women in the city of Johannesburg. A strength of the study was that the sample did not only consist of South African nationality (72%) but also participants from Zimbabwe (25%), as well as some from Lesotho (2%) and Swaziland (1%), were included. Our sample was also unique as a quarter was human immunodeficiency virus (HIV) positive, which is well representative of the black South African female population (Mabaso *et al.*, 2019). However, in this study, both HIV status and country of birth were not different between normotensive and hypertensive groups.

As was expected and established from the literature, the prehypertensive participants had a higher weight status compared to the normotensive group (Golinska-Grzybala *et al.*, 2020). Nevertheless, there was no significant difference in fatty acid intake in the normotensive and prehypertensive groups, before or after adjusting for total fat intake. In congruence with current knowledge, our prehypertensive sample tended to be taller than the normotensive women (Cruickshank *et al.*, 2005). On the other hand, women who had higher parity in our study also had higher blood pressure, which disagrees with a large Australian study (Lupton *et al.*, 2013).

The fact that our results differed may have been due to ethnicity differences as well as higher age and body weight in women with more children in our study. No significant differences were found in terms of fatty acid intake between normotensive and hypertensive groups, however the mean docosahexanoic acid (DHA) intake in our study population met the recommendation as set out by the European Commission with the International Society for the study of Fatty Acids and Lipids (ISSFAL) that pregnant and lactating women should consume at least 200 mg DHA per day (Koletzko *et al.*, 2008).

5.3 Participant status during pregnancy

5.3.1 Anthropometric status

As expected, the anthropometric status of the participants changed throughout pregnancy, as evident in body mass index (BMI) and mid-upper arm circumference (MUAC) increases. MUAC has been shown to be directly related to maternal weight gain and can be used to indicate the maternal nutritional status instead of pre-pregnancy weight (Ricalde *et al.*, 1998). However, the weight recorded in the first trimester of pregnancy is representative of pre-pregnancy weight (Amorim *et al*.,2008). It is normal for fat mass to increase by up to 3 kg from the 10th to the 30th week of gestation during pregnancy, thus resulting in weight gain (Lauritzen & Carlson, 2011). In addition, the participants in our study had a high prevalence of overweight and obesity with the prehypertensive group being more obese. This high overweight and obesity prevalence may relate to the high inflammatory status observed in our study participants (Christian & Porter, 2014). Significant increases in BMI were also observed during pregnancy in other studies and a higher BMI during this period was also associated with high inflammation in these studies (Perichart-Perera *et al.*, 2017).

5.3.2 Inflammatory status

In this study sample, the level of C-reactive protein (CRP) decreased significantly from 22 weeks to 36 week's gestation. This finding is consistent with previous studies and current knowledge which supports the notion that CRP levels become elevated from as little as four weeks of gestation (Sacks *et al.*, 2004) and then decline throughout pregnancy (Christian & Porter, 2014).

Pregnancy is a period characterised by elevated CRP and AGP levels, especially during the earliest phases of implantation of the embryo (Mor *et al.*, 2011; Sacks *et al.*, 2004). In our study chronic inflammation also declined significantly from 11.2% to 1.6% over pregnancy. The decline in AGP from <18 to 36 weeks of gestation seen in our study is in concordance with other studies (Sauder *et al.*, 2019). A study conducted in rural Nepal in pregnant women receiving micronutrients showed a 39 % mean decrease in AGP from the first trimester to the third trimester

(Sauder *et al.*, 2019). A study conducted in Boston by Ferguson *et al.* (2014) in pregnant white, African American as well as women of other races also found elevated CRP levels during early pregnancy. Ferguson *et al.* (2014) further saw a decline in CRP concentrations as pregnancy progressed in their study. Alpha-1 acid glycoprotein (AGP) is known to modulate immune function and has mostly pro-inflammatory effects (Sauder *et al.*, 2019). However, a few studies disagree that AGP and CRP decline during pregnancy. One such study is a randomized control trial conducted in Chinese pregnant women receiving folic acid, iron-folic acid and multiple micronutrients (Mei *et al.*, 2016). They found CRP levels to increase with gestational age. In women from Nepal, it was also found that CRP concentrations almost doubled from the first to the third trimester. However, in this study, participants had a very low inflammatory state [median CRP of 0.56 (0.25, 1.54) mg/l] at the start of pregnancy (Sauder *et al.*, 2019). Others have also found CRP to increase as pregnancy progresses in African American pregnant women of age <30 years compared to non-pregnant women of Pittsburgh, PA (von Versen-Hoeynck *et al.*, 2009).

In our study a large number of women had elevated CRP (60%) and AGP (11.2%) concentrations at enrolment compared to other studies (Ferguson *et al.*, 2014; Sauder *et al.*, 2019; von Versen-Hoeynck *et al.*, 2009). There can be various reasons for the high inflammatory status in our study.

In our sample, a quarter (26%) of the women were HIV positive. HIV infection is associated with chronic systemic inflammation with or without the use of antiretroviral drugs (Wilkinson *et al.*, 2017). A study conducted in Tanzania in HIV positive and negative pregnant women found that HIV positive women had higher levels of inflammation compared to HIV negative women and that disruption of the maternal cytokine profiles leads to unfavourable birth outcomes such as small birth weight (Wilkinson *et al* 2017). However, in our study HIV status did not associate with inflammation during pregnancy.

Our study sample had a high prevalence of overweight (33%) and obesity (28%). Obesity has been shown to be associated with a high inflammatory status as adipocytes facilitate the production of proinflammatory cytokines (Christian & Porter, 2014). This context may explain the high inflammation prevalence in our sample and is supported by findings reporting that obese pregnant women show high proinflammatory markers in contrast with normal-weight pregnant women (Christian & Porter, 2014). This finding in our sample further add to the current evidence that African ethnicity may be prone to have CRP levels higher than other ethnic groups (Kelley-Hedgepeth *et al.*, 2008).

5.3.3 Blood pressure, hypertensive status and aspirin intake

It is common for blood pressure to decline from the first trimester <18 weeks to 22 weeks then increase until delivery (Macdonald-Wallis *et al.*, 2011; Macdonald-Wallis *et al.*, 2012). Our finding that both systolic and diastolic BP declined from <18 to 22 weeks gestation and increased again at 36 weeks gestation agrees with other studies (Macdonald-Wallis *et al.*, 2012; Stone *et al.*, 1995). Such as the Avon Longitudinal Study which also demonstrated a decrease in systolic and diastolic blood pressure from early pregnancy and an increase from mid-pregnancy until birth (Macdonald-Wallis *et al.*, 2011). The drop in blood pressure could be attributed to the great expansion of the plasma volume throughout pregnancy (Strevens *et al.*, 2001). However, factors like age, parity and weight could be risk factors of developing hypertensive disorder of pregnancy (Macdonald-Wallis *et al.*, 2011). Despite the general decrease in blood pressure during pregnancy, some studies have found blood pressure to increase from as soon as 8 weeks gestation (Nama *et al.*, 2011; Thompson *et al.*, 2009). In South Africa, pregnant women with hypertensive disorders without proteinuria during pregnancy are administered low dose aspirin with the intension to reduce the risk of developing preeclampsia (Moodley *et al.*, 2019). Aspirin prevents platelet aggregation and vasospasm in preeclampsia (ACOG, 2018). Hypertension was well managed in our cohort, as blood pressure were largely stable from at <18 to 36 weeks gestation. This was probably due to the provision of low dose aspirin to women exhibiting a risk for preeclampsia. It is possible that aspirin intake could have confounded our findings with regards to the relationship of LCPUFA with blood pressure as hypertension was well managed. Nevertheless, the association between fatty acid status and blood pressure at 36 weeks did not change (no association) when analysing the sample without participants receiving aspirin any time during pregnancy. Therefore, aspirin use was not the main confounder.

5.3.4 Iron status and prevalence of anaemia during pregnancy

The change in iron status and anaemia prevalence during pregnancy of the NuPED study participants has been described previously (Symington *et al.,* 2018). The prevalence of iron deficiency (ID) was high throughout pregnancy while that of anaemia increased significantly between <18 and 22 weeks gestation and then stabilised. This increase at 22 weeks could be attributed to the increase in RBC mass and plasma volume expansion as pregnancy progresses (Nair *et al.*, 2004), thus resulting in the decline in haemoglobin (Hb) levels which is an indicator of anaemia prevalence. Ferritin levels have been shown to have a direct association with inflammation as it is an acute-phase protein (Nair *et al.*, 2004), therefore we corrected ferritin values using the Thurnham method (Thurnham *et al.*, 2010). In our cohort, we found ferritin levels to decline over pregnancy. The median transferrin receptor (TfR) level stayed below the cut off of 8.3 mg/dl for iron deficiency erythropoiesis (IDE) throughout pregnancy. However, TfR increased

significantly over pregnancy and between time points, despite iron supplementation from <18 weeks. This finding is similar to that observed in a prospective study of Indian pregnant women receiving iron supplementation from 19 weeks and whose TfR levels increased significantly throughout pregnancy (Nair *et al.*, 2004). The increase in TfR from the second trimester may be attributed to increased erythropoiesis as pregnancy progresses (Nair *et al.*, 2004). The prevalence of ID and anaemia increased or did not improve throughout pregnancy despite the participants receiving routine iron supplementation from < 18 weeks of gestation. This finding may be attributed to the high inflammatory status of the women (Symington *et al.,* 2018).

5.3.5 Red blood cell total phospholipid n-3 and n-6 LCPUFA status during pregnancy

Both n-3 and n-6 LCPUFA RBC total phospholipid status, but not the n6/n3 LCPUFA ratio, declined or showed a tendency to decline during pregnancy in our study. The decline could be attributed to increased demand and transfer of LCPUFA to the foetus, most preferably DHA and arachidonic acid (AA) that needs to be incorporated in the membranes of the growing tissues of the foetus (Christian *et al.*, 2016; Lauritzen & Carlson, 2011). Our finding agrees with those of Otto *et al.* (1997) where they compared the fatty acid status of the Dutch, Hungarian, Finnish, English and Ecuadorian pregnant women where they found a decline in LCPUFA composition during pregnancy.

Our study sample had a low n-3 PUFA index 5.57 ± 1.23 (EPA+ DHA) at <18 weeks according to the erythrocyte classifications of ≤4 (very low), >4–6 (low), >6–8 (moderate), >8 (high) of Stark *et al*. (2016) and remained in this class (low) during pregnancy.

5.4 Fatty acid status in relation to blood pressure status during pregnancy

The results of this study demonstrated significant positive associations of EPA, DHA and total n-3 LCPUFA status with blood pressure in pregnant women at 22 week's gestation. These relationships were not influenced by the participant's age, gestational age, parity, aspirin intake or MUAC. Moreover, the associations were independent from the dietary intake of the respective n-3 LCPUFA, indicating that it was likely driven by an endogenous metabolic response.

These results from our study that blood pressure associates positively with n-3 LCPUFA status, seem contradictory to current knowledge. Adequate consumption of the n-3 LCPUFA EPA and DHA have been shown to modulate blood pressure, but research are especially in Caucasian populations (Innes & Calder, 2020; Wang *et al.*, 2016). In addition to the anti-inflammatory properties of n-3 PUFA in improving endothelial function, n-3 PUFA also modulates the structure of vascular cell membranes and these effects offer protective benefits to the cardiovascular system, resulting in reduced blood pressure (Cabo *et al.*, 2012).

Our results are also different from other studies in pregnant women where they found n-3 PUFA supplementation to not have any effect on blood pressure (Barden *et al.*, 2006; Bulstra-Ramakers *et al.*, 1995; Onwude *et al.*, 1995; Salvig *et al.*, 1996). Observational studies in pregnant women also showed that intake of fish or plasma n-3 PUFA status associated inversely with blood pressure (Lim *et al.*, 2015; Popeski *et al.*, 1991). However, in the study showing an inverse association with n-3 PUFA status, 39% of the study sample supplemented with n-3 LCPUFA during pregnancy (Lim *et al*., 2015).

Seemingly in congruence with our results, only one study found that n-3 PUFA intake in the form of cod liver oil (Olafsdottir *et al* 2005) was positively associated with hypertension in pregnancy. However, cod liver oil also contains other components such as vitamins A, D and E, which could have confounded the association and this study was designed to evaluate intake and not status. It is important to note that none of the mentioned studies was in women from black African ethnicity.

Our results agree with those seen in a cohort of apparently healthy black South African men and women from Potchefstroom and Ganyesa, South Africa (Zec *et al*., 2019). In this study they found a positive association between EPA status and blood pressure throughout a ten year follow-up period (Zec *et al.*, 2019).

We hypothesise that our results may represent a metabolic response in women of black African ethnicity to the combination of pregnancy, higher blood pressure and possibly also the routine iron supplementation. It is known that a high dose of non-heme iron supplementation (equal to 60 mg elemental iron), as was routinely taken by these pregnant women, increases oxidative stress and inflammation (Casanueva & Viteri, 2003; Symington *et al.*, 2018). In turn, evidence that oxidative stress increases blood pressure is emerging (Van Zyl *et al.*, 2016). There is evidence that individuals from black African ancestry are more efficient in producing LCPUFA from their essential precursors (Ford *et al.*, 2016; Mathias *et al.*, 2011; Sergeant *et al.*, 2012). Furthermore, desaturase enzymes have a higher affinity for n-3 PUFA than for n-6 PUFA (Hofacer *et al.*, 2011; Thanuthong *et al.*, 2011; Tocher, 2003), which in turn lead to the unexpected good n-3 LCPUFA status in black Africans with low n-3 LCPUFA intake (Ford *et al.*, 2016). Also, pregnancy leads to mobilisation of fat, particularly from 10 to 30 week's gestation (Lauritzen & Carlson, 2011). The second trimester of pregnancy is characterised by high metabolic demands to accommodate the growing foetus, possibly explaining the strong associations at this time point (Haggarty, 2004). A pregnant woman's fat mass increases by about 3.5 kg from 10 to 30 weeks of gestation where it reaches a plateau (Lauritzen & Carlson, 2011). Mobilisation from the mother's adipose tissue takes place and non-esterified fatty acids provide the major source of fuel for the foetus during the first two trimesters (Haggarty, 2004). The possibility that aspirin intake may have been the

reason that there was an association at 22 weeks but not at 36 week's gestation. Women at risk for preeclampsia received low dose aspirin treatment that starting largely at 22 week's gestation. However, this was tested by doing a sensitivity analysis by excluding individuals taking aspirin and proved to make no difference to the result. Moreover, it is known that in the third trimester, glucose is mainly transported over the placenta and serve as the major energy source for the foetus and not fatty acids as in the first two trimesters (Haggarty, 2004). We, therefore, propose that the positive association of blood pressure with n-3 LCPUFA at 22 week's gestation may be a metabolic response of this population, which is known to be good converters of essential PUFA to LCPUFA, to the pregnancy combined with the increase in blood pressure in the setting where they receive routine iron supplementation.

The results of this study showed a small but significant positive association of AA status with blood pressure in pregnant women. In agreement with our results, Lepsch *et al*. (2018) found total serum n-6 PUFA to be associated with blood pressure, particularly systolic blood pressure throughout pregnancy (Lepsch *et al.*, 2018). We found AA to be associated with systolic blood pressure at 22 weeks of gestation. One of the mechanisms by which n-3 LCPUFA can affect blood pressure is through lowering of inflammation (O'sullivan *et al.*, 2012). The positive association found of blood pressure with AA status in our study agree with researchers proposing that n-6 PUFA may lead to increased blood pressure and that a lower n-6/n-3 PUFA ratio to avoid the production of proinflammatory lipid mediators by AA may be beneficial (O'sullivan *et al.*, 2012). The long-chain n-3 PUFA DHA and EPA are thought to have cardioprotective effects due to their anti-inflammatory metabolites in lipid mediator metabolism. Eicosapentaenoic acid and DHA compete with AA for the same enzyme in the PUFA metabolism pathway. This competition favours the increased synthesis of the vasodilatory prostacyclin I2 than thromboxane A2, which acts as a vasoconstrictor (Tsukamoto & Sugawara, 2018).

5.5 Influence of inflammation and iron status on the relationship between red blood cell fatty acid composition and blood pressure

Routine iron supplementation may contribute to the development of oxidative stress and inflammation which may influence blood pressure and the development of gestational hypertension, as well as possibly the upregulation of the production of endogenous n-3 LCPUFA in response to counteract the inflammatory state. However, we found no influence of markers of inflammation (AGP and CRP) or iron status (ferritin and TfR) on the association between the LCPUFA status and blood pressure. Folic acid supplementation could be a possible confounder in our study as it has been shown in several studies to be associated with a lower risk of gestational hypertension (Hernández-Díaz *et al*., 2002). Oxidative stress markers like reactive

oxygen species (ROS) could have been a more sensitive biomarker and has been shown to have an interdependent relationship with inflammation (Tan *et al.*, 2018).

5.6 Conclusion

The findings from this study demonstrate a positive relationship between n-3 LCPUFA status with blood pressure at 22 week's gestation and, even more so, neither dietary LCPUFA intake, nor inflammation or iron status influenced this relationship. Our sample's inflammatory status was high during early pregnancy indicating metabolic stress. However, further research is warranted to explore the mechanism of the involvement of n-3 LCPUFA status and inflammation/iron status with blood pressure in the context of routine iron supplementation during pregnancy in women of African descent.

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CHAPTER 6 CONCLUSION AND RECOMMENDATIONS

6.1 Introduction

The purpose of this chapter is to summarise the main findings of this study and also discuss the limitations of the current study and recommendations for future studies. The aim of this dissertation was to assess the relationship of n-6 and n-3 LCPUFA with blood pressure in pregnant African women receiving routine iron supplementation in urban SA. Our objectives were to:

- characterise the RBC total phospholipid AA, total n-6 LCPUFA, EPA, DHA and total n-3 LCPUFA status at <18, 22 and 36 week's gestation;
- characterise the blood pressure at <18, 22 and 36 week's gestation;
- assess the association between AA, EPA, DHA and total n-6 and n-3 and LCPUFA compositions with blood pressure over pregnancy

and, if associations are found, to

- assess whether n-3, n-6, essential PUFA and LCPUFA intake influence the association; • assess whether iron status and/or inflammation influence the association.

6.2 Attainment of objectives

Objective 1: Characterization of red blood cell total phospholipid AA, total n-6 LCPUFA, EPA, DHA and total n-3 LCPUFA status at <18, 22 and 36 week's gestation

The results of this study showed the fatty acid status of our cohort to decline between <18, 22 and 36 weeks' gestation. The n-3 index was low, despite adequate DHA intake. The results support the notion that fatty acid status declines during pregnancy due to placental transfer (Haggarty, 2004). Adequate n-3 PUFA during pregnancy has been shown to influence inflammation with their inflammation-resolving capacity as well as blood pressure modulation attributed to their cardioprotective effects (Cabo *et al.,* 2012; Innes & Calder, 2020). Adequate fatty acid intake during pregnancy is therefore critical (Mennitti *et al*., 2015). Our study is the first to report on red blood cell total phospholipid AA, total n-6 LCPUFA, EPA, DHA and total n-3 LCPUFA status longitudinally throughout gestation and contributes to current knowledge.

Objective 2: Characterization of the blood pressure at <18, 22 and 36 week's gestation

The findings revealed that blood pressure declined during early pregnancy and increase from the second trimester until the third trimester. The same trend was observed in the Avon longitudinal study by Macdonald-Wallis *et al*.,2011 where they also observed both systolic and diastolic blood pressure to decline first and then increase to reach a plateau by birth.

Objective 3: Assessment of the association of AA, EPA, DHA and total n-6 and n-3 and LCPUFA compositions with blood pressure over pregnancy

The study investigated the association between n-3 and n-6 LCPUFA of interest with blood pressure. A persistent positive association between n-3 LCPUFA with blood pressure were evident at 22 week's gestation. These results from our study that blood pressure associates positively with n-3 LCPUFA status, appear inconsistent to existing knowledge. Adequate intake of the n-3 LCPUFA EPA and DHA have been proven to modulate blood pressure, but research data is more prominent in Caucasian populations. We hypothesise that our results may represent a metabolic response in women of black African ethnicity to the combination of pregnancy, higher blood pressure and possibly also the routine iron supplementation. These results also add to the growing body of evidence that shows that the second trimester of pregnancy is characterised by high metabolic demand to accommodate the growing foetus, thus the association of n-3 LCPUFA with blood pressure.

Objective 3: Assessment whether n-3 and n-6 LCPUFA intake influence the fatty acid status–blood pressure association

Our results show that dietary n-3 and n-6 LCPUFA were independent of the relationship between LCPUFA status and blood pressure, and that it was likely controlled by an endogenous metabolic response. The participants' intake was below that recommended during pregnancy of EPA plus DHA of 500mg, however DHA intake alone (200mg) was adequate. The results indicate our population to have a high n-6 PUFA intake as expected due to urbanisation.

Objective 3: Assessment whether iron status and/or inflammation influence the fatty acid status–blood pressure association

Our study participants were routinely administered 60 mg elemental iron during antenatal care during pregnancy and reported a high adherence rate (Symington *et al*., 2018). The prevalence of anaemia and iron deficiency anaemia was high regardless of routine iron supplementation. In this study it was evident that iron status and markers of inflammation had no influence on the relationship between blood pressure and LCPUFA status. However, we found the markers of

inflammation (AGP and CRP) or iron status (ferritin and TfR) to not have an influence on the association between the LCPUFA status and blood pressure. In addition, they have an interdependent relationship with inflammation.

6.3 Strengths and limitations

Our study had several strengths. The study design was longitudinal and focussed on the same participants at three time points. The sample did not only represent the South African nationality (72%) but also participants from Zimbabwe (25%), as well as some from Lesotho (2%) and Swaziland (1%). Some unique feature of our sample was their HIV status, a quarter was HIV positive and the sample was therefore well representative of the Black South African female population (Mabaso *et al*., 2019).

There were some limitations. The assessment of maternal fatty acid intake by means of a dietary questionnaire subjects the participants to reporting bias. The study experienced great loss to follow up to 25% due to participants moving out of the province. Further, interpretation of our study results on whether inflammatory/iron status influence the relationship between fatty acid status and blood pressure may have been limited by the small sample size. Causal relationships could not be assessed due to the study design being observational.

6.4 Recommendations

More research on LCPUFA status association with blood pressure in pregnant women of African descent is needed. There is evidence that individuals from black African ancestry are more efficient in producing LCPUFA from their essential precursors, and that they may be genetically advantaged to produce n-3 LCPUFA more efficiently than n-6 LCPUFA. These genetic variations should be further investigated. Additionally, the interaction between iron and fatty acid metabolism during pregnancy should be further investigated to find their influence on inflammation and oxidative stress in women being supplemented with iron during pregnancy. Prior to routine iron supplementation, pregnant women's ferritin in addition to Hb could be measured as Hb alone is not a sensitive marker of iron status. CRP levels should also be measured to correct for inflammatory status. Furthermore, the association of n-3 LCPUFA with blood pressure in the second trimester of pregnancy as observed in our study is of special interest and should be validated with replication studies. Pregnant women should be provided with nutrition education on the intake of adequate n-3 and n-6 LCPUFA and essential PUFA.

6.5 References

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ANNEXURES

ETHICAL APPROVAL FROM NORTH-WEST UNIVERSITY ETHICS COMMITTEE

Private Bag X1290, Potchefstroom
South Africa 2520

Tel: 086 016 9698
Web: http://www.nwu.ac.za/

North-West University Health Research Ethics Committee (NWU-HREC)

Tel: 018 299-1206
Email: Ethics-HRECApply@nwu.ac.za (for human studies)

7 August 2020

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Health Research Ethics Committee (NWU-HREC) on 07/08/2020, the NWU-HREC hereby approves your study as indicated below. This implies that the NWU-HREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: The relationship of long-chain polyunsaturated fatty acid status with blood pressure in pregnant African women: The NuPED study
Principal Investigator/Study Supervisor/Researcher: Dr L Malan
Student: KK Kekana - 29955416

Ethics number:

N	W	U	-	0	0	1	8	6	-	1	5	-	A	1	-	0	7
Institution				Study Number						Year			Status		Sub-study		

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Sub-study
Commencement date: **07/08/2020**
Expiry date: 31/08/2021

Risk: **Minimal**

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is due at the end of August annually until completion.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-HREC:*
  - *annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-HREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *Annually a number of studies may be randomly selected for active monitoring.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-HREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*

- *withdraw or postpone approval if:*
  - *any unethical principles or practices of the study are revealed or suspected;*
  - *it becomes apparent that any relevant information was withheld from the NWU-HREC or that information has been false or misrepresented;*
  - *submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
  - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-HREC can be contacted for further information via Ethics-HRECApply@nwu.ac.za or 018 299 1206*

Special conditions of the research approval due to the COVID-19 pandemic:

Please note: Due to the nature of the study i.e. statistical analysis of previously collected data from the NUPED study, this study will be able to proceed during the current alert level, following receipt of the approval letter. No additional COVID-19 restrictions have been placed on the study except that the researcher must ensure that before proceeding with the study that all research team members have reviewed the North-West University COVID-19 Occupational Health and Safety Standard Operating Procedure.

The NWU-HREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-HREC for any further enquiries or requests for assistance.

Yours sincerely,

Digitally signed by Prof Petra Bester
Date: 2020.08.07 14:21:08 +02'00'

Chairperson NWU-HREC

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20 August 2019
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