

Effects of pre- and postnatal iron and n-3 fatty acid depletion, alone and in combination, on bone development in rats

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PREFACE

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ABSTRACT

A third of women and a fifth of men over the age of 50 worldwide are estimated to endure an osteoporotic fracture (Svedbom *et al.*, 2013), and 75% of hip fractures are predicted to occur in developing countries by 2050 (Genant *et al.*, 1999). It is also believed that the prevalence is increasing as a result of an aging population in developed and developing countries (Woolf and Pflieger, 2005, Handa *et al.*, 2008, Mushtaq *et al.*, 2014). Iron and omega-3 (n-3) polyunsaturated fatty acids (PUFAs) are vital nutrients during early development and may also play an important role in bone development (Palacios, 2006, Claassen *et al.*, 1995, Haag *et al.*, 2003, Kruger and Schollum, 2005, Lau *et al.*, 2013). The aim of this study was therefore to investigate the effects of pre- and postnatal iron and n-3 PUFA depletion, alone and in combination, on bone development in rats, and to determine whether effects are sex-specific. Female Wistar rats were randomly allocated to one of four diets: 1) Control, 2) iron deficiency (ID), 3) n-3 fatty acid deficiency (FAD) or 4) ID and n-3 FAD, and were maintained on the respective diets throughout pregnancy and lactation. Offspring continued on the respective diets after weaning until post-natal day 42-45, when bone mineral density (BMD) and bone strength were determined using dual X-ray absorptiometry and three-point bending tests, respectively. Results from this study showed that a pre- and post-natal ID has negative effects on the BMD and bone strength of offspring in early adolescence. A pre- and post-natal n-3 FAD might have an additive effect by further decreasing BMD and bone strength. Further research is needed to determine whether the effects of a pre- and post-natal ID on bone development in the offspring can be reversed if offspring is switched to a control diet after weaning, or if dams receive iron supplementation during pregnancy and lactation.

Key terms:

Iron (Fe), omega-3 polyunsaturated fatty acids (n-3 PUFAs), bone mineral density, bone strength, bone development, osteoporosis

ABBREVIATIONS

°C	Degrees Celsius
AA	Arachidonic acid
AAS	Atomic absorption spectrometry
AIN	American Institute for Nutrition
ALA	Alpha-linolenic acid
ANCOVA	Analysis of covariance
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
Ca-ATPase	Calcium adenosine triphosphatase
CEN	Centre of Excellence for Nutrition
cm	centimetre
DHA	Docosahexaenoic acid
dl	decilitre
DXA	Dual X-ray Absorptiometry
EPA	Eicosapentaenoic acid
ESCEO	European Society for Clinical and Economic Evaluation of Osteoporosis and Osteoarthritis
EU	European Union
FA	Fatty acid
FAD	Fatty acid deficiency

FAME	Fatty acid methyl esters
Fe	Iron
g	gram
GD	Gestational day
GS-MS-MS	Gas chromatography tandem mass spectrometry
Hb	Haemoglobin
ID	Iron deficiency
ISCD	International Society for Clinical Densitometry
kg	kilogram
LA	Linoleic acid
mg	milligram
ml	millilitre
mm	millimetre
MUFAs	Mono-unsaturated fatty acids
N	newton
n	number
n-3	omega-3
n-6	omega-6
NBHA	National Bone Health Alliance
NHANES	National Health and Nutrition Examination Survey
NOFSA	National Osteoporosis Foundation of South Africa
NWU	North-West University

Pa	Pascal
PBM	Peak bone mass
PBS	Phosphate buffered saline
PCDDP	Preclinical Drug Development Platform
PND	Postnatal day
PPE	Personal protective equipment
PUFAs	Poly-unsaturated fatty acids
QP	Quadrupole
RBC	Red blood cell
RDA	Recommended Dietary Allowance
SA	South Africa
SD	Standard deviation
SEM	Standard error of the mean
SPSS	Statistical Program for Social Sciences
TLC	Thin layer chromatography
USA	United States of America
WHO	World Health Organization

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CHAPTER 1: INTRODUCTION

1.1 Background and motivation

In 2007 the World Health Organization (WHO) stated that osteoporosis affected more than 75 million people in the United States of America (USA), Europe and Japan alone (World Health Organization, 2004). A study conducted across nine industrialised countries (USA, Canada, Australia, Japan and five European countries) in 2014 found that approximately 24 to 49 million people over the age of 50 years had osteoporosis (Wade *et al.*, 2014). In the European Union it was found that osteoporosis affected approximately 6% of men and 21% of women between the ages of 50 and 84 years (Hernlund *et al.*, 2013). The National Osteoporosis Foundation of the USA estimated that 10.5% of the USA adult population 50 years and older had osteoporosis at the femoral neck or lumbar spine and 43.9% had low bone mass at one of these skeletal sites (Wright *et al.*, 2014). Furthermore, it is estimated that a third of women and a fifth of men over the age of 50 worldwide will endure an osteoporotic fracture (Svedbom *et al.*, 2013).

The impact of osteoporosis in developing countries is difficult to determine owing to lack of information. There is, however, a general belief that the prevalence is increasing as a result of an aging population (Woolf and Pflieger, 2005, Handa *et al.*, 2008, Mushtaq *et al.*, 2014). It is suspected that osteoporosis will become more prevalent in developing countries, with an estimated 75% of hip fractures occurring in developing countries by 2050 (Genant *et al.*, 1999). The incidence of osteoporosis in South Africa (SA) appears to be similar to that found in developed countries in Caucasian, Asian and mixed race populations (Van Schoor, 2011). Generally, it is believed that Caucasians are at highest risk of hip and vertebral fractures, followed by Asians, while blacks have the lowest risk (Genant *et al.*, 1999). It is also suggested that the bone mineral density (BMD) of sub-Saharan black women differ from that of US black and white women (Mukwasi *et al.*, 2015). Inconsistent results have been found when the BMD was compared between black and white South African women (Daniels *et al.*, 1995, Conradie *et al.*, 2014, Conradie *et al.*, 2015). Nevertheless, an absence of standardized country-specific prevalence estimates makes it challenging to predict the future potential global impact of osteoporosis (Wade *et al.*, 2014).

Many pregnant women, unfortunately, are at increased risk of iron deficiency (ID), considering that ID is the most prevalent nutrient deficiency globally, particularly in developing countries (Zimmermann and Hurrell, 2007). Premenopausal women are at particularly high risk of ID owing to blood loss during menstruation. Pregnant women have an added risk because of iron

stores often being insufficient for the increased demands during pregnancy (World Health Organization, 1998a, Scholl, 2005). The South African National Food Consumption Survey, conducted in 2005, showed that approximately 20% of South African women of reproductive age had a poor iron status, and about 30% suffered from anaemia (Labadarios *et al.*, 2008). ID during pregnancy can have many detrimental effects on the mother and infant, which are further described later.

Another nutrient essential for healthy pregnancies and optimal growth and development of the foetus is omega-3 (n-3) polyunsaturated fatty acids (PUFAs) (Cetin and Koletzko, 2008). Unfortunately, no biochemical markers or acceptable dietary intake levels are available to indicate an n-3 fatty acid (FA) deficiency (Innis and Friesen, 2008). It has been reported, however, that populations with a low consumption of fish, and/or a high intake of fat and oils rich in n-6 but low in n-3 FAs, are at risk of inadequate n-3 FA intake (Briend *et al.*, 2011). Intakes of alpha-linolenic acid (ALA, 18:3n-3) and docosahexaenoic acid (DHA, 22:6n-3), both n-3 PUFAs, are often insufficient in pregnant and lactating women in developing countries (Huffman *et al.*, 2011). Although suboptimal n-3 FA status is observed not only in low-income countries, an increase in the intake of omega-6 (n-6) FAs in developed countries has resulted in an increased n-6:n-3 ratio of 15-25:1 in Western diets (Simopoulos, 2011). Currently no data are available on the n-3 FA status of South African women of childbearing age. However, in a previous study conducted by the Centre of Excellence for Nutrition, the dietary assessment revealed a high n-6:n-3 FA intake ratio of approximately 60:1 in rural school children in the South African province of KwaZulu-Natal (Baumgartner *et al.*, 2012b). It is likely then that pregnant women, especially in lower socioeconomic classes, have an ID as well as an inadequate n-3 FA status due to poor quality diets (Briend *et al.*, 2011, Stoltzfus, 2011).

Iron and n-3 PUFAs are both essential nutrients for optimal foetal and infant development (Georgieff, 2008, Gambling *et al.*, 2011, Swanson *et al.*, 2012). Besides the importance of iron and n-3 PUFAs for growth, brain and immune development, adequate iron and n-3 PUFA status during early development may play an important role in the development of bones. It has been suggested that iron acts as a cofactor of enzymes involved in collagen bone matrix synthesis and the conversion of vitamin D to its active form (Palacios, 2006). n-3 PUFAs can influence bone development by increasing calcium absorption and by influencing the differentiation of mesenchymal cells into osteoblasts (Claassen *et al.*, 1995, Haag *et al.*, 2003, Kruger and Schollum, 2005, Lau *et al.*, 2013). The foetal and neonatal period is the most vulnerable period of development. Ensuring optimal bone development before adolescence is likely to reduce the risk of osteoporosis later in life. Thus, it is important to investigate the biochemical and

functional consequences of maternal ID and n-3 FA deficiency (FAD) on bone health, and potential interactive effects of these two common deficiencies when occurring in combination.

1.2 Aim, objectives and hypothesis

Using the study design as presented in the following section, the aim of this study was to investigate the effects of pre- and postnatal iron and n-3 PUFA depletion, alone and in combination, on bone development in rats, and to determine whether effects are sex-specific.

The specific objectives are:

- To investigate the effects of pre- and postnatal iron and n-3 FA deficiency, alone and in combination, on BMD and bone strength at postnatal day 42-45 (adolescence).
- To investigate whether outcomes are sex-specific.

Hypotheses stated:

- Pre- and postnatal iron and n-3 FA deficiency, alone and in combination, would decrease BMD and bone strength at postnatal day 42-45 (adolescence), and may have an interactional or additive effect.
- There will be no sex differences.

1.3 Study design

This MSc project is a sub-study of a larger project with the aim to investigate the effects of maternal iron and n-3 FA depletion and repletion, alone and in combination, on the development and health of offspring. The animal trial was conducted at the vivarium of the Preclinical Drug Development Platform (PCDDP) of the North-West University (NWU), Potchefstroom, SA.

Fifty-six female Wistar rats at 21 ± 3 days of age (postnatal day [PND] 21) were placed on the control diet for a two week period of preconditioning. At the age of five weeks (the end of the preconditioning phase) the rats were randomly allocated to one of four diet groups, as shown in Figure 1-1. The diet groups were: 1) Control; 2) ID; 3) n-3 FAD; or 4) ID+n-3 FAD. The rats that were randomly allocated to one of the n-3 FAD diet groups (n-3 FAD and ID+n-3 FAD) were switched to an n-3 FAD diet for seven weeks before mating. At nine weeks of age (three weeks before mating) the female rats that had been allocated to one of the ID diet groups (ID and ID+n-3 FAD), were placed on an ID or ID+n-3 FAD diet, respectively.

At 12 weeks of age, the female rats were mated with 12-week-old male breeders of the same strain. After conception, the females maintained their pre-pregnancy diets throughout pregnancy

and lactation. Within three to five days of birth, the litters were culled to eight pups (to maintain nutritional adequacy) with ideally four males and four females per litter [eight pups/litter; minimum of three litters/group]. The remaining pups were weaned from the dams and randomly allocated to receive either the control diet (not for the purposes of this MSc) or were maintained on their respective experimental diets for three weeks until PND 42-45, when 24 (12 male and 12 female) offspring from each group (n = 96) were euthanized and samples were collected.

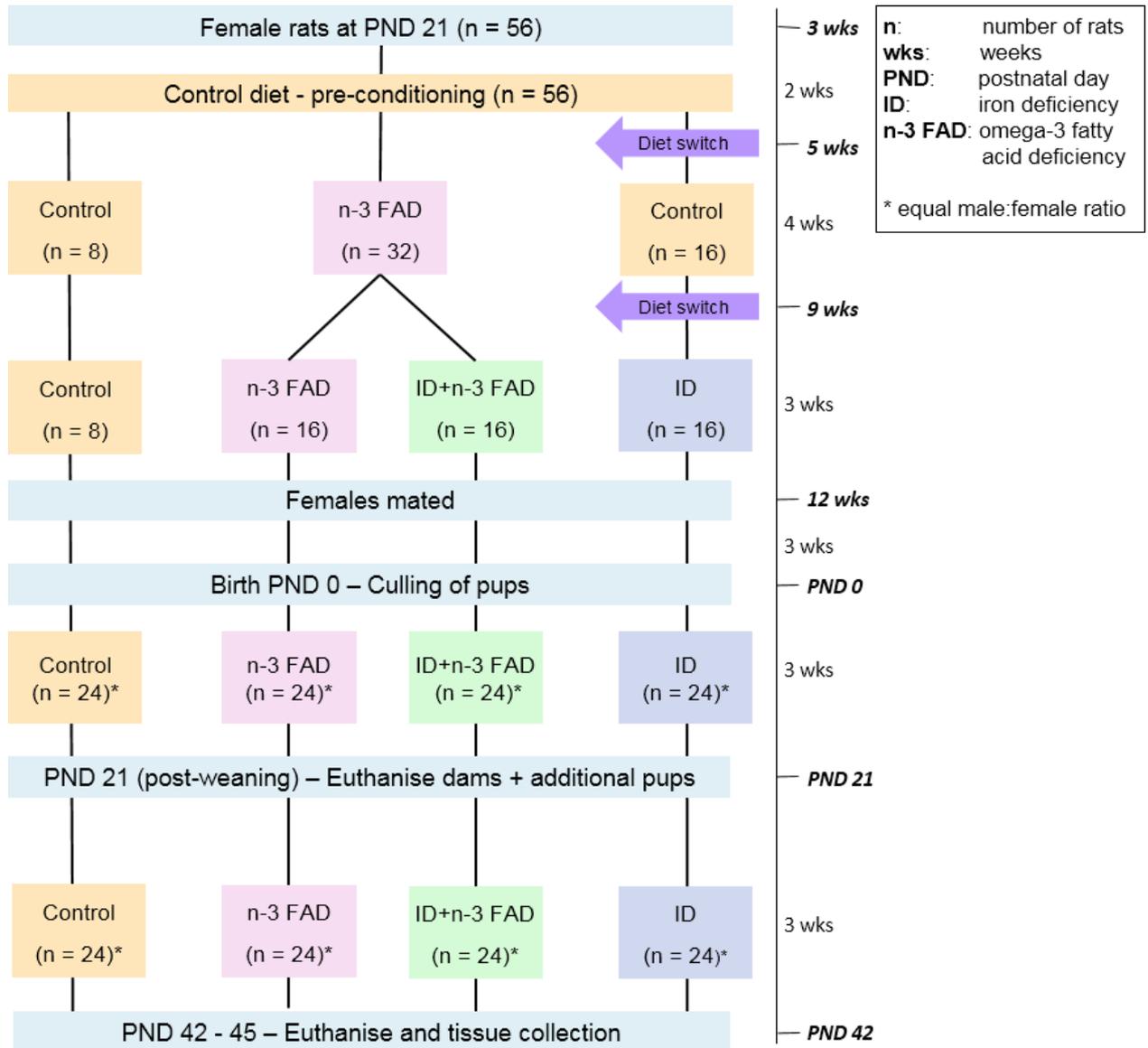


Figure 1-1: Flow diagram of animal trial

1.4 Research team and authors' contribution

Project head (PI) large study:

Prof Marius Smuts, Centre of Excellence for Nutrition (CEN), NWU Potchefstroom

Project Supervisor (Co-PI) large study and supervisor of MSc student:

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Investigator and co-supervisor of MSc student:

Prof Salome Kruger, CEN, NWU Potchefstroom

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Ms Erna Kemp, CEN, NWU Potchefstroom

Collaborator:

Mr Philip Venter, School of Mechanical and Nuclear Engineering, NWU Potchefstroom

MSc student:

Ms Estelle Strydom

Involved in organization and execution of larger study (i.e. feeding and weighing of rats; data capturing); responsible for planning, organization and execution of MSc sub-study: data collection, implementation and optimization of tree-point bending test, analyses of samples (three-point bending tests and preparing samples for DXA scans); data capturing and statistical analyses; reporting of findings.

1.5 Other study contributors

Technical assistance:

Prof Marlana Kruger, Massey Institute of Food Science and Technology, Massey University, New Zealand (expert in studying the role of nutrition in bone health using rodent models).

Prof Johann Markgraaff, School of Mechanical and Nuclear Engineering, NWU Potchefstroom (assistance with biomechanical testing of rat femurs).

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1.6 Structure of this mini-dissertation

This mini-dissertation is presented in article format according to the NWU's guidelines for postgraduate students, where the main outcomes are presented in chapter 3 as an article prepared for publication in an accredited journal. Four chapters are included in this mini-dissertation. All relevant references are provided at the end of the mini-dissertation.

Chapter 1 serves as a brief introduction and explains the rationale for conducting this study. The study design derived from the larger study is provided in brief, along with the consequent aim and objectives. The research team and all contributors are acknowledged.

Chapter 2 includes a literature review on the importance of bone health and the role of iron and n-3 PUFAs in bone development, providing background information and further explaining the rationale of this study. The methods used to determine the BMD and bone strength are also explained.

Chapter 3 will provide the key data findings as an article to be submitted for publication in the nutrition research journal. This article, titled "Effects of pre- and postnatal iron and omega-3 fatty acid depletion, alone and in combination, on bone development in rats", is presented according to the journal's formatting guidelines. Tables and references used in the article are thus provided separately at the end of the chapter.

Chapter 4 consists of a summary and conclusion based on the specific objectives provided in chapter 1. Limitations and recommendations for future research are also included.

Annexures attached include the standard operating procedures developed by the student for the measurement of BMD and bone strength, which were adjusted from the methodology used by Massey University in New Zealand, to be compatible with equipment available at the North-West University. The study design is also elaborated on and the animal housing conditions, ethical considerations, experimental diets, monitoring sheets and certificates of analyses of bedding and diets are provided. Author guidelines for the Nutrition Research Journal is also provided.

CHAPTER 2: LITERATURE REVIEW

2.1 Osteoporosis and bone development

2.1.1 Osteoporosis

The World Health Organization (WHO) defines osteoporosis as “a disease characterised by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk” (World Health Organization, 1994, Genant *et al.*, 1999). Osteoporosis is a major cause of fractures, as well as one of the main diseases that cause people to become bedridden with serious complications (World Health Organization, 2004). Osteoporosis can lead to disability, decreased quality of life and mortality. Inexpensive, safe and effective interventions with high compliance rates are therefore needed to prevent fractures and decrease morbidity (Genant *et al.*, 1999).

Bone mineral density (BMD) is a known indicator of fracture risk later in life (World Health Organization, 2004, Prentice, 2004) and it is possible that bone mass during early life is also associated with childhood fractures (Clark *et al.*, 2006). Early detection of low BMD is recommended to ensure intervention to prevent future fractures (Genant *et al.*, 1999).

2.1.2 Bone development

Bone tissue is constantly remodelled through resorption via osteoclasts and bone formation via osteoblasts. Bone mass can change greatly in different life stages (Ilich and Kerstetter, 2000). During infancy, childhood and young adulthood, bone increases in size, thickness and density until the peak bone mass (PBM) is reached. Peak bone mass can be explained as the amount of bone that is gained by the time that a stable skeletal state has been reached during young adulthood and may refer to a person’s maximal potential for bone strength (Weaver *et al.*, 2016). The exact age at which PBM is reached varies with skeletal region and with method of measuring bone mass (Heaney *et al.*, 2000). For example, most of the skeletal mass of multiple skeletal sites has been found to be accumulated by late adolescence in Caucasian females (Matkovic *et al.*, 1994). After PBM is reached, bone loss will slowly start occurring, where bone resorption predominates over formation, and will continue until the end of life. In contrast, rats were found to have reached PBM at 12 weeks of age and PBM levels are suggested to be maintained until 36 months of age (Sengupta *et al.*, 2005).

Interventions for preventing osteoporosis can be classified into two categories: 1) maximising PBM before adulthood; or 2) preventing bone loss after PBM is reached. It is widely believed

that achieving a higher PBM during childhood or adolescence will have a prolonged effect on BMD, and will therefore decrease the risk of osteoporosis later in life (Heaney *et al.*, 2000, Office of the Surgeon, 2004, Warden *et al.*, 2007). Using computer simulations, it has been predicted that a 10% increase in PBM would delay osteoporosis for up to 13 years (Hernandez *et al.*, 2003). Many public policies and guidelines are thus aimed at factors maximizing PBM before adulthood, such as increasing calcium intake or physical activity during childhood and adolescence. It is important to note that there is no general agreement that higher PBM will be associated with higher BMD later in life (Gafni *et al.*, 2002, Gafni and Baron, 2007). Quality research investigating this question is very difficult to conduct because of the large timeframe in humans.

The tracking of bone strength indicators from childhood to adolescence further supports the notion that prevention of osteoporosis should begin in the early stages of bone development. The tracking of a certain trait means that individuals are likely to maintain their ranked position in a distribution curve over a time period (Foley *et al.*, 2009). Several studies have shown that bone mass, BMD and bone mineral content (BMC) are tracked from childhood to adolescence or skeletal maturity, even after correcting for confounders such as body size, pubertal stage, energy intake and sex (Foley *et al.*, 2009, Budek *et al.*, 2010, Kalkwarf *et al.*, 2010, Fujita *et al.*, 2011, Wren *et al.*, 2014). This means that children with low bone strength indicators are unlikely to catch-up by adolescence. Variations in tracking correlations between studies indicate that lifestyle factors can influence bone strength positively and negatively (Weaver *et al.*, 2016).

Studies have also suggested that birth weight and body weight at one year of age, reflecting growth during conception and infancy, are associated with BMC at the age when PBM is reached, as well as later in life (Cooper *et al.*, 1995, Cooper *et al.*, 1997, Baird *et al.*, 2011). Both lean mass and fat mass have been associated with BMD in a meta-analysis of 44 studies, but lean mass appears to be a more important determinant of BMD than fat mass (Ho-Pham *et al.*, 2014).

Apart from lowering the risk of osteoporosis later in life, increasing bone mass, density or strength before reaching PBM may have additional current benefits for children and adolescents (Heaney *et al.*, 2000). Caucasian girls with forearm fractures had lower BMD at the ultradistal radius, radius, lumbar spine, hip and total body than case-matched girls with no history of fractures (Goulding *et al.*, 1998). A meta-analysis, mentioned previously, included eight case-control studies and showed an association between BMD and fractures in children (Clark *et al.*, 2006). Results of prospective studies are, however, needed to clarify the role of BMD in current fracture risk in children further.

2.1.3 Sex differences

No sex differences were found in the BMD and BMC of infants (Unal *et al.*, 2000, Kurl *et al.*, 2002). Reports of sex differences of BMD in children and adolescents are inconsistent (Nelson *et al.*, 1997, Willing *et al.*, 2005, Macdonald *et al.*, 2006). These gender differences might also be site-specific (Bell *et al.*, 1991, Kröger *et al.*, 1993, Willing *et al.*, 2005). After the attainment of PBM men usually have higher BMD, and thus lower fracture risks than women. This may be due to a larger body frame and therefore larger bone mass, or a heavier body mass leading to increased mechanical loading on the bones (Havill *et al.*, 2007). The most prominent sex differences in BMD become evident after menopause and are believed to be due to a decrease in oestrogen levels (Järvinen *et al.*, 2003).

2.2 The rat as an experimental model in studies of nutrition and bone health

As it is not ethical to purposely make or leave women of childbearing age deficient in vital nutrients, rodent models are more suitable for research on the causal effects of nutrient deficiencies on bone health. Similarities in the pathophysiologic responses between the human and rat skeleton make the rat a valuable model for research on bone health (Frost and Jee, 1992). Skeletally immature rats are seen as an appropriate model for researching the nutritional factors that can influence bone development, as a lower PBM is seen as a risk factor for fracture in humans (Lelovas *et al.*, 2008). Furthermore, using a rat model, more invasive methods of determining bone strength can be used, such as the three-point bending test, which is explained in section 2.3.2.

2.3 Measuring bone development or bone strength in rats

2.3.1 Bone mineral density

Bone mineral density is the amount of bone mass per unit volume (volumetric density measured three-dimensionally in g/cm^3) or per unit area (areal density measured two-dimensionally in g/cm^2), depending on measurement technique (Kanis *et al.*, 2008a, Kanis *et al.*, 2008b). There are several techniques available to measure BMD, including dual X-ray absorptiometry (DXA), quantitative ultrasound, quantitative computed tomography (often referred to as micro-computed tomography), digital X-ray radiogrammetry and radiographic absorptiometry (Kanis *et al.*, 2008a), and interpretation differs depending on technique.

The National Osteoporosis Foundation of South Africa (NOFSA) recommends that BMD measured by DXA scans be used to diagnose and monitor the development of osteoporosis (Hough *et al.*, 2010). Several national and international organisations have developed different

diagnostic and treatment guidelines for osteoporosis in humans as summarized by Wright *et al* (2014) (Wright *et al.*, 2014).

DXA is a non-invasive technique to determine body composition and can quantify lean tissue mass, fat mass, bone mineral mass and BMD (Stone and Turner, 2012). DXA can be used to determine the body composition of mammals *in vivo* or *ex vivo* with high precision and accuracy, and has been used extensively on humans and rodents in the past (World Health Organization, 1998b, Lelovas *et al.*, 2008, Stone and Turner, 2012). DXA is based on the principle that the intensity of X-rays passed through tissue are decreased in proportion to tissue mass (Stone and Turner, 2012). See annexure C for the methodology of DXA used in this study.

The WHO published diagnostic criteria for osteoporosis in postmenopausal women based on the T-scores for the BMD of the average value for healthy young women (Hernlund *et al.*, 2013). The WHO classifies osteoporosis as a BMD of less than 2.5 standard deviations (SD) below the reference mean (T-score <-2.5 SD). A standard deviation less than 2.5 but more than 1 under the reference BMD (-1 SD > T-score > -2.5 SD) is regarded as osteopenia (low bone mass). These criteria are currently widely used as diagnostic and treatment thresholds (World Health Organization, 1998a). The recommended reference range currently used internationally is the Third National Health and Nutrition Examination Survey (NHANES III) reference database for the BMD of the femoral neck in white women between 20 and 29 years (Kanis *et al.*, 2008b). Different skeletal areas in humans have, however, been recommended by various organisations, as shown in Table 2-1.

Table 2-1: Skeletal areas recommended to measure bone mineral density

Organisation	Skeletal area recommended	References
World Health Organization (WHO)	Femoral neck	(World Health Organization, 2004)
European Society for Clinical and Economic Evaluation of Osteoporosis and Osteoarthritis (ESCEO)	Femoral neck	(Kanis <i>et al.</i> , 2008a, Kanis <i>et al.</i> , 2008b, Kanis <i>et al.</i> , 2013)
The National Osteoporosis Foundation of the USA and the National Bone Health Alliance (NBHA) Working Group	Hip and lumbar spine	(Cosman <i>et al.</i> , 2014)
International Society for Clinical Densitometry (ISCD)	Femoral neck, lumbar spine or total hip	(Kanis <i>et al.</i> , 2005, Lewiecki <i>et al.</i> , 2008)

Most guidelines also mention that a measure of BMD alone might not be sufficient to diagnose osteoporosis, and that clinical aspects such as fracture risk must be taken into account (Lewiecki *et al.*, 2008, Hernlund *et al.*, 2013, Kanis *et al.*, 2013, Siris *et al.*, 2014).

2.3.2 Bone strength

BMD is a good predictor of bone strength. However, it is only a surrogate determinant of bone strength and other parameters could also be used to evaluate bone strength (Ammann and Rizzoli, 2003). Bone strength or the risk of fractures are dependent not only on the quality (composition) of the bone, but also the quantity and structure (distribution) of the bone (Cooper *et al.*, 1997, Warden *et al.*, 2007). Increases in BMD in humans are not always related to fracture reduction in humans (Divittorio *et al.*, 2006). While bone strength cannot be measured directly in living humans (it can only be estimated), biomechanical bone strength can be measured in rodents *ex vivo* using three-point bending tests of the long bones such as the tibia, femur or humerus (Ammann and Rizzoli, 2003, Lelovas *et al.*, 2008).

Three-point bending tests, also called flexure tests, are performed on a servohydraulic machine that measures the elongation (measured in millimetres [mm]) of the bone with the corresponding force applied (measured in newton [N]). The bone is supported at the end-points on two bottom rods, while a third rod (bending rod) is used to apply a force at the midpoint between the bottom rods (Crenshaw *et al.*, 1981, Jepsen *et al.*, 2015).

The apparatus used for the three-point bending tests in this study has been designed by the mechanical engineering department of the NWU to accommodate the size of the rat bones (see Figure 2-1). The design has been adapted from the guidelines provided by Massey University.

The bending rod and the bottom rods are 3 mm wide with rounded points, and 4 mm deep. The length between the two bottom rods can be adjusted to accommodate different size bones from rats of different ages: for the femurs of rats aged 42-45 days, the length was set at 8 mm.

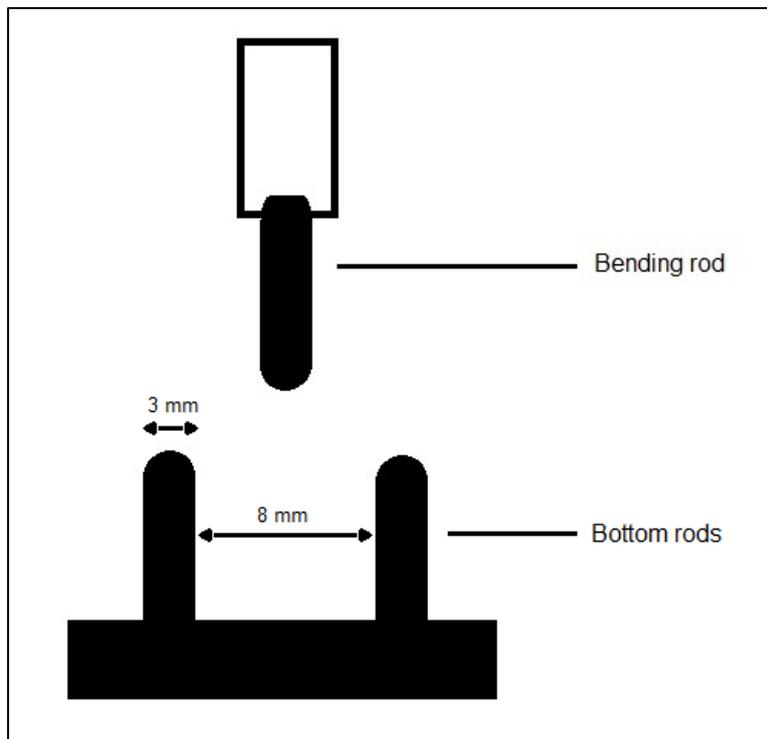


Figure 2-1: Three-point bending apparatus adjusted for rat femurs measured at postnatal day 42-45

During a bending test where force is applied from the top, the top fibres of the bone (concave surface) are compressed, while the bottom fibres (convex surface) are pulled apart through tensile forces (Crenshaw *et al.*, 1981, Raab *et al.*, 1990). Rat femurs used in this study were tested in the anterior-posterior direction for practical and anatomical reasons. The load is the force applied to the bone and the elongation is the perpendicular displacement that the bone endures in response to the force. These two parameters are measured simultaneously over time, allowing for a load-displacement curve to be plotted. The load-displacement curve can then be used to determine the following parameters for each bone (see Figure 2-2 for a visual representation) (Crenshaw *et al.*, 1981, Raab *et al.*, 1990, Jepson *et al.*, 2015, Silva, 2016) :

- Ultimate load: the force required to break the bone (point B on Figure 2-2), measured in newton (N).

- Ultimate displacement: the amount of displacement (elongation) at the point of ultimate load (point B on Figure 2-2), measured in millimetres (mm).
- Ultimate stress: determined by the ultimate load (point B on Figure 2-2) over the cross-sectional bone area where the force was applied, measured in Pascal (Pa).
- Load (N) and stress (Pa) measured at the yield point (point A on Figure 2-2): transition from the elastic to the plastic deformation region. The elastic region of the load-deformation curve is where the bone is elongated only to a point where it will still return to its original position without any permanent damage. Once the bone reaches the yield point, and is in the plastic region, permanent damage has been done (without the bone necessarily breaking completely), and this can be regarded as a fracture.
- Stiffness: the force needed to produce a certain displacement, measured as the slope of the elastic region in newton per millimetre (N/mm).

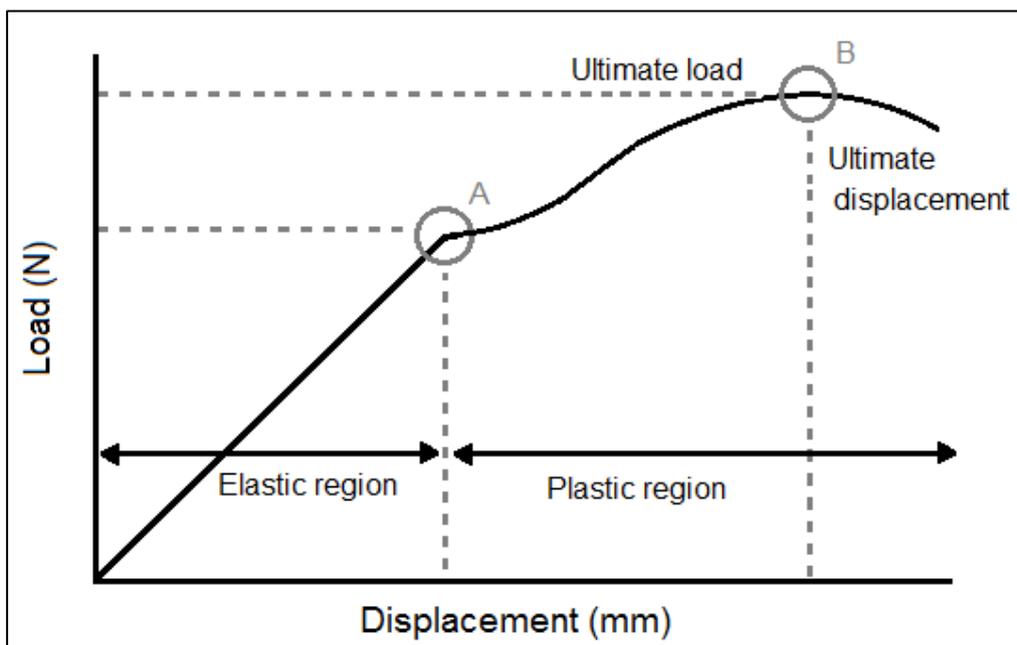


Figure 2-2: Load-displacement curve indicating the yield point (A) and the point of the ultimate load and displacement (B)

See annexure D for the methodology of three-point bending tests used in this study.

2.4 The role of nutrition in bone development

It has been shown that genetics plays a major role in the bone mass of an adult. Environmental factors, however, may also contribute to bone mass (Pocock *et al.*, 1987, Krall and Dawson-Hughes, 1993, Gafni and Baron, 2007). Studies suggest that 50 to 90% of the variance in PBM and osteoporosis risk is due to genetics, while the remaining 10 to 50% is attributed to environmental factors (Heaney *et al.*, 2000, Recker and Deng, 2002, Weaver *et al.*, 2016). Nutrition is one of these modifiable factors and can play an important role in the development and maintenance of bone mass. Between 80% and 90% of the mineral content of bone consists of calcium and phosphorous. Protein is also incorporated into the organic matrix of bone for collagen structure (Ilich and Kerstetter, 2000). It is, however, important to remember that heredity and the environment cannot be seen as completely separate, as they can influence one another (Heaney *et al.*, 2000).

Several human studies have shown that different components of maternal nutrition have been associated with bone development in the offspring. Maternal pre-pregnancy body mass index (BMI) (Macdonald-Wallis *et al.*, 2010), fat intake (Jones and Riley, 2000, Yin *et al.*, 2010) skinfold thickness (Godfrey *et al.*, 2001, Harvey *et al.*, 2010), vitamin D status (Javaid *et al.*, 2006, Curtis *et al.*, 2014), magnesium intake (Jones and Riley, 2000, Tobias *et al.*, 2005, Yin *et al.*, 2010), potassium intake (Jones and Riley, 2000, Tobias *et al.*, 2005), dietary folate intake (Tobias *et al.*, 2005, Ganpule *et al.*, 2006), phosphorous intake (Jones and Riley, 2000), calcium supplementation or intake (Raman *et al.*, 1978, Ganpule *et al.*, 2006, Curtis *et al.*, 2014) and milk intake (Yin *et al.*, 2010) may be associated with BMD in children at various ages.

2.4.1 Nutritional role of iron in the body

About 60% of the iron in the body can be found in haemoglobin of circulating erythrocytes (red blood cells), where it is responsible for the transportation of oxygen from the atmosphere to the living tissues. The rest can be found in myoglobin of muscle tissue or stored in the form of ferritin or hemosiderin in predominantly the liver, spleen and bone marrow. Adult men need to absorb approximately 1 mg of iron per day, whereas menstruating women and pregnant women, respectively, need to absorb 1.5 mg and 4 – 5 mg of iron per day to maintain iron balance. The Recommended Dietary Allowance (RDA) for iron is 8 mg/day for men, 18 mg/day for premenopausal women and 27 mg/day for pregnant women (Institute of Medicine, 2005).

Dietary iron is available in two forms, haeme and non-haeme, with the former being more bioavailable. Haeme iron can only be found in animal food sources, such as meat, poultry, fish,

eggs and milk, while non-haeme iron is available in animal and plant food sources, such as broccoli, potato and legumes (Whitney, 2013).

Iron deficiency (ID), which can cause ID anaemia, is the most common nutritional deficiency worldwide. ID can be due to blood loss, as in the case of menstruation, injury or parasitic infections, poor absorption owing to infection or inflammation, or insufficient dietary intake, for example in vegetarians (Mahan, 2012). Other health outcomes of ID during early development include increased risk of prematurity and low-birthweight infants, motor and cognitive developmental delay and increased risk of morbidity and mortality, especially from infections (World Health Organization, 2012).

Women of reproductive age are at particular risk of developing ID owing to dietary intake not able to meet increased requirements during pregnancy or losses such as menstruation or poor absorption during infection or inflammation. Therefore, iron supplementation is generally recommended to pregnant women to meet the iron needs of both the mother and foetus (Scholl, 2005). However, ID and ID anaemia in pregnant women remain a major public health concern particularly in low- and middle-income countries, owing to limited accessibility to iron supplementation, late antenatal care attendance or poor compliance with supplementation.

2.4.2 Potential role of iron in bone mineral density and bone strength

Even though 80 – 90% of the mineral content of bone consists of calcium and phosphorous (Ilich and Kerstetter, 2000), other elements such as iron have also been shown to play an important role in bone strength (Maciejewska *et al.*, 2014). However, iron can have a detrimental effect on bone as well when provided in toxic amounts; an association between iron overload and decreased BMD levels was observed in patients with hereditary haemochromatosis (Valenti *et al.*, 2009) and sickle cell anaemia (Sadat-Ali *et al.*, 2011), as well as in a healthy population with iron overload (Kim *et al.*, 2012, Kim *et al.*, 2013).

Studies using rodent models have shown that ID can lower BMD and bone strength parameters when rats are fed an iron deficient diet (5 – 8 mg Fe/kg for four to five weeks) (Medeiros *et al.*, 2004, Medeiros *et al.*, 2002, Katsumata *et al.*, 2006) or a severely iron-deficient diet (amount of iron not reported) (Katsumata *et al.*, 2009) post-weaning (from three weeks old). One study found no differences in bone strength parameters such as peak load, yield load, stiffness, resilience and absorbed energy between iron-deficient rats and a control group (Lobo *et al.*, 2009). This could, however, be due to a less severe ID than in the studies done by Katsumata and colleagues (2009). To the knowledge of the researcher, no or very little information is available on the consequences of maternal ID on bone development in offspring.

An epidemiological study in Turkey has shown that serum iron levels were significantly lower in postmenopausal women with known osteoporosis than in those without osteoporosis (Okuyay *et al.*, 2013). Serum ferritin (iron-storage protein) levels were positively associated with BMD in elderly men (Lee *et al.*, 2014), while other studies found no correlation between serum iron levels and BMD in post-menopausal women (Liu *et al.*, 2009). In Korean pre-menopausal women, serum ferritin was associated with BMD of the lumbar spine, but not in the femur, and not in post-menopausal women (Chon *et al.*, 2014). Higher serum ferritin levels have also been associated with a lower risk of osteoporosis in the femoral neck and the lumbar spine in post-menopausal women (Heidari *et al.*, 2015). An association between dietary iron intake and BMD has also been observed in post-menopausal women (Farrell *et al.*, 2009). No information on the effects of ID early in life on human bone development could be found.

Iron acts as a cofactor of enzymes involved in collagen bone matrix synthesis, as well as in 25-hydroxycholecalciferol hydroxylase, which is important for the conversion of vitamin D to its active form (Palacios, 2006). As vitamin D is necessary for calcium absorption, it can also influence bone development (Palacios, 2006). Another study established that serum 1, 25-dihydroxycholecalciferol (most active form of vitamin D) concentrations were decreased when rats were fed an iron-deficient diet for four weeks post-weaning (Katsumata *et al.*, 2009). Other proposed mechanisms include the effect of hypoxia in stimulating bone resorption or acidosis which can induce osteoclast activation and bone loss (Toxqui and Vaquero, 2015).

In view of the observations that a maternal ID can lead to prematurity or decreased birthweight of the infant (World Health Organization, 2012), and that birthweight and weight at one year of age has been associated with BMC later in life (Cooper *et al.*, 1995, Cooper *et al.*, 1997, Baird *et al.*, 2011), as mentioned previously, it is possible that the mother's iron status may have an influence on the bone health of her offspring even later in life.

2.4.3 Nutritional role of omega-3 fatty acids in the body

Fatty acids (FAs) consist of a carbon chain, a carboxyl end (hydrophilic) and a methyl end (hydrophobic), and mostly occur in nature bound to other molecules such as a glycerol backbone in the case of triglycerides. Fatty acids can be classified according to the number of carbon atoms, the number of double bonds between the carbon atoms (also called the degree of saturation), and the position of the double bonds (Mahan, 2012).

The most common dietary FA can be classified by degree of saturation into saturated FAs (no double bonds) and unsaturated FAs (one or more double bonds). Mono-unsaturated fatty acids (MUFAs) contain only one double bond and poly-unsaturated fatty acids (PUFAs) more than

one double bond. Unsaturated FAs can be further divided into groups of chain lengths: Unsaturated carbon chains with 19 or fewer carbon atoms are classified as short-chain unsaturated FAs, those with 20 to 24 carbon atoms as long-chain FAs and those with 25 or more carbon atoms as very-long-chain FAs (FAO: Fats, 2010).

Omega nomenclature of FAs indicates the length of the carbon chain and the number of double bonds in the chain, while a lower-case omega symbol (“ ω ” or “n”) is used to indicate the location of the first double bond from the methyl end of the carbon chain. Omega-3 (n-3) and omega-6 (n-6) are two of the most important PUFA families in the human context. These two families can be described as essential FAs, as they cannot be synthesized by the human body (Mahan, 2012).

Linoleic acid (LA: 18:2n-6) can be found in most vegetable oils and has been described as the parent of the n-6 PUFA family, since it can be elongated and desaturated to form longer-chain n-6 PUFAs in the human body. Arachidonic acid (AA: 20:4n-6) is the primary precursor for n-6 eicosanoids and is found in meat, eggs and fish. The parent of the n-3 PUFA family, α -linolenic acid (ALA: 18:3n-3), is found in plant oils such as flaxseed oil, canola oil and soybean oil, and can also be elongated and desaturated to form longer-chain n-3 PUFAs. Two of the most important n-3 PUFAs in human nutrition are eicosapentaenoic acid (EPA: 20:5n-3) and docosahexaenoic acid (DHA: 22:6n-3), which can both be found in oily fish such as salmon, herring, anchovies and mackerel. The elongation and desaturation pathways of n-6 and n-3 PUFAs are independent of each other, but use the same enzymes, therefore competition between the two metabolic pathways takes place. See Figure 2-3 for a visual demonstration of these pathways. Excess dietary intake of n-6 PUFAs can thus inhibit the formation of EPA and DHA from ALA (FAO: Fats, 2010). The conversion of ALA to EPA and DHA is also not very efficient, therefore dietary intake of EPA and DHA is seen as important in the human context (Arterburn *et al.*, 2006).

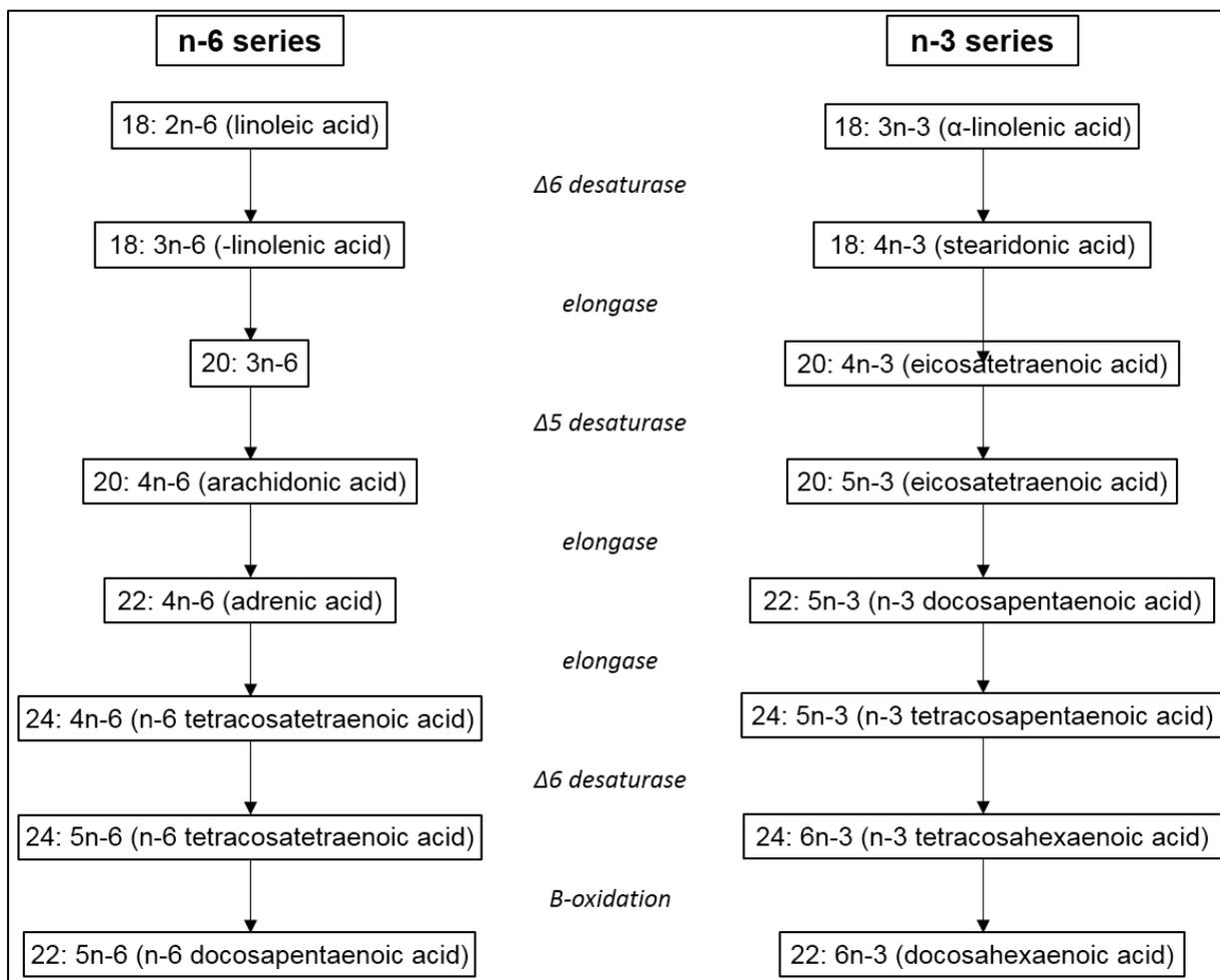


Figure 2-3: Metabolic pathways of linoleic acid and α -linolenic acid to long-chain PUFAs (FAO: Fats, 2010)

Longer chain FAs are important structural components of cellular membranes, and are precursors of eicosanoids (act as local hormones), such as prostaglandins, thromboxanes and leukotrienes, where they can influence blood vessel functioning, blood clotting and inflammation processes (Mahan, 2012).

2.4.4 Potential role of omega-3 fatty acids in bone mineral density and bone strength

There is sound evidence for the essential role of n-3 PUFAs in foetal growth and development, including the development of the brain and immune system (Rogers *et al.*, 2013). However, official recommendations on n-3 PUFA supplementation during pregnancy and lactation have not yet been issued by influential organisations such as the United States Institute of Medicine and the WHO.

N-3 PUFAs have been shown to play an important role in bone development in animal models. Fish oil has been shown to increase BMD compared to soybean oil in growing rats when provided for 35 days, starting post-weaning (Green *et al.*, 2004). An n-3 PUFA-rich diet has also been shown to increase BMD (Shen *et al.*, 2006) and biomechanical strength (Shen *et al.*, 2007) in middle-aged rats and ovariectomized (postmenopausal) rats (Sun *et al.*, 2003). Other studies have found minimal differences in BMD when different fatty acid diets were compared (Sirois *et al.*, 2003, Lukas *et al.*, 2011, Macri *et al.*, 2012, Li *et al.*, 2010). In mouse studies it has been shown that the type of n-3 FA might play a role in bone development, with DHA providing the best protection against bone loss (Fallon *et al.*, 2014) and EPA improving bone strength (Bonnet and Ferrari, 2011). A decreased n-6/n-3 ratio in bone compartments also seem to be beneficial to bone development by increasing bone modelling and strength (Reinwald *et al.*, 2004). Laying hens have also been found to have higher bone-breaking points and greater bone strength when receiving n-3 FA supplementation (Tarlton *et al.*, 2013). However, another study found that BMD and bone strength parameters increase when supplementing the diets of rats on a high-fat diet, but there were no differences in the results between different fat sources used (n-3, n-6 and saturated FAs) (Lau *et al.*, 2010).

Several rodent studies have shown that maternal fatty acid intakes can influence the bone development of the offspring. Table 2-2 contains a list of rodent studies investigating the effects of a maternal diet that restricts or supplements different fatty acids on the BMD or bone strength of the offspring. A highly saturated FA diet has been shown to increase BMD; however, it is not clear whether this outcome will have a long-lasting effect that persists until young adulthood (Miotto *et al.*, 2013). Some studies show that maternal n-3 PUFA can have a positive effect on offspring bone development (da Costa *et al.*, 2015, Korotkova *et al.*, 2005, Weiler *et al.*, 2012), while others show conflicting results (Korotkova *et al.*, 2004). Some studies have also found different results in male and female offspring (Lanham *et al.*, 2010, Yin *et al.*, 2014).

One study showed that the offspring of rats exposed to an essential fatty acid deficient diet 10 days before delivery (late gestation) and during lactation had lower femur BMD as adults, even when weaned off to a normal diet, which suggests that regulatory mechanisms can be programmed early in life (Korotkova *et al.*, 2005). In a study done on rats and guinea pigs the offspring responded positively to maternal supplementation of AA (n-6) and DHA (n-3) provided during pregnancy and lactation, with an increase in lumbar spine BMD after three weeks of life. In other bone markers (BMC and BMD) the offspring responded to AA and DHA supplementation when they were born of normal size as well as when they were small in size because of growth restriction *in utero* (Weiler *et al.*, 2012).

Table 2-2: Rodent studies investigating the effects of a maternal diet that restricts or supplements different fatty acids on bone development parameters of the offspring

Type of rodent	Control diet [number of animals per group]	Experimental diet [number of animals per group]	Duration of experimental diet	Age of endpoint in offspring	Effect of experimental diet on bone mineral density (BMD) and bone mineral content (BMC)	Effect of experimental diet on bone strength parameters	Reference
Wistar rats	Control diet with 7g/100g soybean oil [females: n=7; males: n=9]	High saturated FA diet with 20g/100g lard [females: n=9; males: n=9]	10 weeks pre-conception, pregnancy and lactation. Post-weaning, all offspring received the control diet.	19 days	No significant differences in BMC. Increased whole femur BMD in females but not significant in males	Not measured	(Miotto <i>et al.</i> , 2013)
	Control diet with 7g/100g soybean oil [females: n=9; males: n=9]	High saturated FA diet with 20g/100g lard [females: n=9; males: n=9]		3 months	No significant differences in BMC or BMD	No significant differences	
Mice	Standard chow (fat source: corn oil) [females: n=5; males: n=4]	High-fat diet with 18g/100g animal lard added [females: n=5; males: n=4]	7 weeks pre-conception, pregnancy and lactation, until 7 months of age	7 months	Not measured	No significant differences in females. Males in the high fat group had increased maximum load. No other significantly different parameters	(Lanham <i>et al.</i> , 2010)
Wistar rats	Control diet with soybean oil [males: n=12]	Flaxseed flour diet (high in ALA) [males: n=12]	During lactation until 21 days of age	21 days	Increased total body BMC. No significant differences in BMD	Increased maximum load, breaking load, resilience and stiffness. No statistical differences in maximum deformation and tenacity	(da Costa <i>et al.</i> , 2015)
Sprague-Dawley rats	Control diet (soybean oil) [males: n=7]	Essential FA-deficient diet (lard) [males: n=7]	10 days pre-conception and lactation. Post-weaning: control diet	10 months	Increased cortical femur BMC due to increased bone area. Decreased BMD in the metaphysis of the femur	Not measured	(Korotkova <i>et al.</i> , 2005)

Type of rodent	Control diet [number of animals per group]	Experimental diet [number of animals per group]	Duration of experimental diet	Age of endpoint in offspring	Effect of experimental diet on bone mineral density (BMD) and bone mineral content (BMC)	Effect of experimental diet on bone strength parameters	Reference
Sprague-Dawley rats	Sunflower seed oil diet (n=6) [females: n=10]	Linseed oil diet (n=3) [females: n=10]	10 days pre-conception and lactation. Post-weaning: control diet	7 months	No significant differences in BMD in the metaphysis of the femur	Not measured	(Korotkova <i>et al.</i> , 2004)
Sprague-Dawley rats	Control diet (AIN93G/R) [n=36]	LC-PUFA enriched diet (AA and DHA) [n=36]	During lactation	21 days	Increased lumbar spine and tibia BMC. No significant differences in femur BMC. Increased whole body and tibia BMD.	Not measured	(Weiler <i>et al.</i> , 2012)
Guinea pigs	Control diet (AIN93G/R) [n=19]	LC-PUFA enriched diet (AA and DHA) [n=19]	During lactation	21 days	No significant differences in BMC. Increased lumbar spine BMD in female offspring but not in males	Not measured	
Guinea pigs	Control diet [females: n=12; males: n=11]	DHA enriched diet [females: n=14; males: n=15]	During pregnancy and lactation	3 days and 21 days - combined	Decreased whole body BMC in males at 21 days but increased body BMC in females. Decreased body and femur BMD in males. Increased body and spine BMD in females		(Yin <i>et al.</i> , 2014)

To test the effect of specifically n-3 FA supplementation, rodent dams were fed either a control diet consisting of the typical Western diet composition – 40% saturated FA; 40% MUFA and 20% PUFA (5% total fat and 0.1% DHA) – or a diet enriched with n-3 PUFA (5% total fat and 1% DHA) from the beginning of gestation to post-weaning of the offspring (Fong *et al.*, 2011). After weaning the offspring were fed standard rodent chow. Bone outcomes in offspring were measured in different life stages: childhood (three weeks), adolescence (six weeks) and adulthood (three months). Maternal n-3 FA supplementation resulted in an increase in serum DHA levels up to six weeks of age, even when offspring were fed standard chow after weaning. At three weeks of age, male offspring showed increased bone volume and decreased osteoclast activity, but no lasting effects were observed in adulthood. No effects were observed in female offspring.

In a review, the authors stated that studies are limited to conclude whether ALA plays a definitive role in bone health, whereas DHA and EPA were shown to play a more consistently positive role in BMD and bone strength (Lau *et al.*, 2013). The authors further concluded that benefits of ALA on BMD and bone strength may be programmed as early as gestation, as an ALA-rich diet provided *in utero* showed positive results but not when only provided postnatally. Some of the knowledge gaps that the authors have identified will be addressed in this study, i.e. the optimal time period and duration of n-3 PUFA intake on bone development, as well as the identification of any possible sex differences.

Few human studies investigated the effect of dietary fats on bone. One review showed that supplementation with flaxseed oil (ALA) has a marginal effect on bone (Kim and Ilich, 2011), while another study concluded that n-3 FA supplementation might increase BMD in older populations (Mangano *et al.*, 2014). A higher n-6 to n-3 FA ratio has been associated with lower BMD of the hip in older people (Weiss *et al.*, 2005). In healthy eight-year-old boys, however, serum ALA, DHA or total n-3 FA were not associated with BMD of the total body, femur or lower spine (Eriksson *et al.*, 2009). In contrast, in young men (22 – 24 years) n-3 FA concentrations were positively associated with bone mineral accumulation and therefore peak BMD (Griel *et al.*, 2007). It has also been shown that n-3 FA may reduce the symptoms of certain bone/joint diseases in humans (Watkins *et al.*, 2001). Secondary analyses of a cross-sectional study in postmenopausal women found that PUFAs, as well as n-3 FAs, had significant inverse associations with total body BMD and lumbar spine BMD (Harris *et al.*, 2015). No studies, however, could be found determining associations of maternal n-3 FA status and bone development in offspring in humans.

One possible mechanism of the role of n-3 FA in bone health is by increasing calcium absorption, probably by regulating calcium-adenosine triphosphatase (Ca-ATPase), in the small intestine where more calcium may be available for incorporation into the bone matrix (Claassen *et al.*, 1995, Haag *et al.*, 2003, Kruger and Schollum, 2005, Lau *et al.*, 2013). It is also possible that n-3 PUFA status affects bone marrow development during early development by affecting the differentiation of mesenchymal stem cells into osteoblasts, which are responsible for bone formation (Lau *et al.*, 2013).

It is unknown whether there are sex differences in the role of n-3 FA in bone development. Previous work has shown that female rats may have an increased ability to convert shorter chain n-3 PUFA to DHA which leads to increased DHA concentrations in liver and heart tissue, plasma and erythrocytes, but not brain tissue (Kitson *et al.*, 2012).

2.4.5 Iron and omega-3 fatty acids in bone mineral density and bone strength

Iron deficiency and n-3 FAD may interact directly via iron-dependent hepatic desaturase and elongase enzymes (Cunnane and McAdoo, 1987, Nakamura and Nara, 2004), which are responsible for the conversion of essential precursor FAs into their respective long-chain PUFAs, such as DHA and EPA. Furthermore, ID and n-3 FAD may interact indirectly by affecting shared mechanisms. However, data on potential interactions between ID and low n-3 FA status, particularly in relation to functional health outcomes, are scarce. To the knowledge of the researcher, no studies have been published on the maternal effect of combined iron and n-3 FA deficiency on the bone development of the offspring in animals or humans.

2.5 Summary of literature

In summary, there are many studies indicating that both ID and n-3 FAD may influence bone development in general, and biological mechanisms are available to explain these effects. It is still unknown, however, whether maternal ID will have an effect on offspring bone development, and conflicting results have been reported on the effects of maternal n-3 FAD. Interactions between ID and n- FAD have been shown, but reports on the effects on functional health outcomes are rare. This study will be the first study, to the knowledge of the researcher, to investigate the maternal effects of ID and a combination of ID and n-3 FAD on the bone development of offspring in a rodent model. Sex differences in these possible effects will also be novel findings.

CHAPTER 3: EFFECTS OF PRE- AND POSTNATAL IRON AND OMEGA-3 FATTY ACID DEPLETION ON BONE DEVELOPMENT IN RATS

(Article to be submitted for publication - formatted as specified by the journal *Nutrition Research*)

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Abbreviations:

ID: iron deficiency, n-3 FAD: omega-3 fatty acid deficiency, BMD: bone mineral density, PBM: peak bone mass, PUFAs: poly-unsaturated fatty acids, ALA: alpha-linolenic acid, DHA: docosahexaenoic acid, FA: fatty acid, AIN: American Institute of Nutrition, PND: post-natal day, RBC: red blood cell, DXA: dual X-ray absorptiometry, Hb: haemoglobin.

Abstract

The aim of this study was to investigate the effects of pre- and postnatal iron and omega-3 (n-3) poly-unsaturated fatty acid depletion, alone and in combination, on bone development in rats, and to determine whether effects are sex-specific. Fifty-six female Wistar rats were allocated to one of four diets: 1) Control, 2) iron deficiency (ID), 3) n-3 fatty acid deficiency (FAD) or 4) ID and n-3 FAD, and were maintained on the respective diets throughout pregnancy and lactation. Offspring (n=96) continued on the respective diets after weaning until post-natal day 42-45. Bone mineral density (BMD) and bone strength were determined using dual X-ray absorptiometry and three-point bending tests, respectively. Results showed that a pre- and post-natal ID resulted in significantly lower BMD in the spine and right femur and n-3 FAD resulted in significantly lower BMD in the right femur. The ID and n-3 FAD diets alone did not significantly lower BMD compared to the control diet in the femur; however, the combination of ID with n-3 FAD resulted in significantly lower femur BMD compared to the control diet, indicating an additive effect of ID and n-3 FAD. Pre- and postnatal ID also resulted in significantly lower bone strength parameters, but almost no effects of n-3 FAD on bone strength were found. Outcomes were not sex-specific. In conclusion, these effects indicate that ID during early life may influence bone development negatively, with potential additive effects of n-3 FAD.

Keywords: Iron (Fe), omega-3 polyunsaturated fatty acids (n-3 PUFAs), bone mineral density, bone strength, bone development

3.1 Introduction

Bone tissue is constantly remodelled through resorption via osteoclasts and bone formation via osteoblasts. From infancy to young adulthood, bone increases in thickness and density until the peak bone mass (PBM) is reached [1]. After PBM is reached, bone loss will slowly start occurring, whereby bone resorption predominates over formation, and will continue until the end of life. It is widely believed that achieving a higher PBM during childhood or adolescence will decrease the risk of osteoporosis later in life [2-4]. In 2007 osteoporosis affected more than 75 million people in the United States of America, Europe and Japan alone [5]. The impact of osteoporosis in developing countries is difficult to determine owing to lack of information, but it is estimated that the incidence is increasing owing to general aging of the population [6].

It is suggested that 20 to 40% of the variance in PBM and osteoporosis risk is attributed to environmental factors, such as nutrition [2, 7]. Several human studies have indicated that different components of maternal nutrition, such as fat intake, vitamin D status and calcium intake, can influence bone development in the offspring [8-11].

Iron intake is suggested to be one of the environmental factors that may play a role in bone development [12]. Iron deficiency (ID) is the most prevalent nutrient deficiency globally [13]. Pregnant women have an added risk because iron stores are often insufficient to satisfy the increased demands during pregnancy [14, 15]. To the best of our knowledge, no information exists on the effects of ID during early life on bone development in humans; however, rodent studies have found that an ID induced post-weaning can lower bone strength parameters [16-19]. In elderly humans, serum iron,

serum ferritin (iron-storage protein) and dietary iron intake have been positively associated with bone mineral density (BMD) [20-22]. Nevertheless, to the best of our knowledge, no information is available on the effects of pre- and post-natal ID on bone development.

Omega-3 (n-3) polyunsaturated fatty acids (PUFAs) are another group of nutrients that can influence bone development [23-26]. Intakes of alpha-linolenic acid (ALA, 18:3n-3) and docosahexaenoic acid (DHA, 22:6n-3), both n-3 PUFAs, are often insufficient in pregnant and lactating women in developing countries [27]. Few human studies have investigated the effect of dietary fats on bone health and those that did yielded inconsistent results [28-30]. Several rodent studies also found conflicting results on the effects of maternal fatty acid (FA) intake on the bone development of offspring [31-35].

Even though it has been suggested that ID and n-3 fatty acid deficiency (FAD) interact directly via iron-dependent hepatic desaturase and elongase enzymes [36, 37], data on potential interactions between ID and low n-3 FA status, particularly in relation to functional health outcomes, are scarce. To the best of our knowledge, no studies have been published on the effect of a maternal combined iron and n-3 FA deficiency on the bone development of the offspring in either animals or humans. Therefore, the aim of this study was to investigate the effects of pre- and postnatal iron and n-3 PUFA depletion, alone and in combination, on bone development in rats, and to determine whether effects are sex-specific.

3.2 Materials and methods

3.2.1 Animals and diets

The animal trial was conducted at the vivarium of the Preclinical Drug Development Platform of the North-West University (NWU), Potchefstroom, South Africa, following the 3R's principles for animal research. Ethical approval was obtained from the AnimCare Ethics Committee of the Faculty of Health Sciences of the NWU (NWU-00270-16-A5).

The purified experimental diets were obtained commercially from Dyets Inc. (Bethlehem, USA) and were based on the American Institute of Nutrition (AIN) 93G purified diets for laboratory rodents for growth, pregnancy and lactation [38]. All diets were isocaloric and contained 10% fat. The basal AIN-93G formulation (control diet) contained 40 mg iron/kg, soybean oil at 70 g/kg diet and hydrogenated coconut oil at 30 g/kg [39-41]. ID diets were modified to contain 15 – 18 mg iron/kg diet [42], while FAD diets contained 81 g/kg diet hydrogenated coconut oil and 19 g/kg diet safflower oil [43-45]. Ingredients of the diets are presented in Table 1 and the analysed fatty acid and iron composition of the diets is presented in supplemental table 1.

Table 3-1: Ingredients of experimental diets based on the AIN-93G diet

	Control	ID	n-3 FAD	ID+n-3 FAD
Cornstarch (g/kg)	397.5	397.5	397.5	397.5
Casein (g/kg)	200	200	200	200
Dyetrose – Dextrinised cornstarch (g/kg)	132	132	132	132
Sucrose (g/kg)	100	100	100	100
Soybean oil (g/kg)	70	70	-	-
Hydrogenated coconut oil (g/kg)	30	30	81	81
Safflower oil (g/kg)	-	-	19	19
Vitamin mix (g/kg)	10	10	10	10
Mineral mix (g/kg)	35	35 ¹	35	35 ¹

1: Mineral mix was modified in the ID diets to contain 15 – 18 mg iron/kg diet

3.2.2 Experimental design

Fifty-six female Wistar rats (21 ± 3 days of age) were pair-housed in standard solid-floor cages with Alpha-Dri bedding provided by LBS Serving Biotechnology Ltd (Horley, UK) (< 2.00 ppm iron). All rats had *ad libitum* access to laboratory diets and demineralised water (18 M Ω) and received a control diet for two weeks of preconditioning. At five weeks of age the rats were randomly allocated to one of four diet groups: 1) Control; 2) ID (iron deficiency); 3) n-3 FAD (n-3 fatty acid deficiency); or 4) ID+n-3 FAD (iron and n-3 fatty acid deficiency). (Figure 1 provides a schematic diagram of the experimental design.) Rats that were allocated to one of the n-3 FAD diet groups (n-3 FAD and ID+n-3 FAD), were placed on an n-3 FAD diet for seven weeks before mating. At nine weeks of age the rats that had been allocated to one of the ID diet groups (ID or ID+n-3 FAD) were placed on either an ID or an ID+n-3 FAD diet for three weeks before mating. At 12 weeks of age, female rats were mated with 12-week-old males and they maintained their pre-pregnancy diets throughout pregnancy and lactation.

Litters were culled to eight pups to maintain nutritional adequacy at three to five days after birth, with ideally four males and four females per litter. The remaining pups were weaned from the dams at postnatal day (PND) 21 and randomly allocated to receive either the control diet (not for the purposes of this study) or to be maintained on their respective experimental diets for three weeks until PND 42-45, when 24 (12 male and 12 female) offspring from each group (n = 96) were euthanised by decapitation and tissue samples were collected.

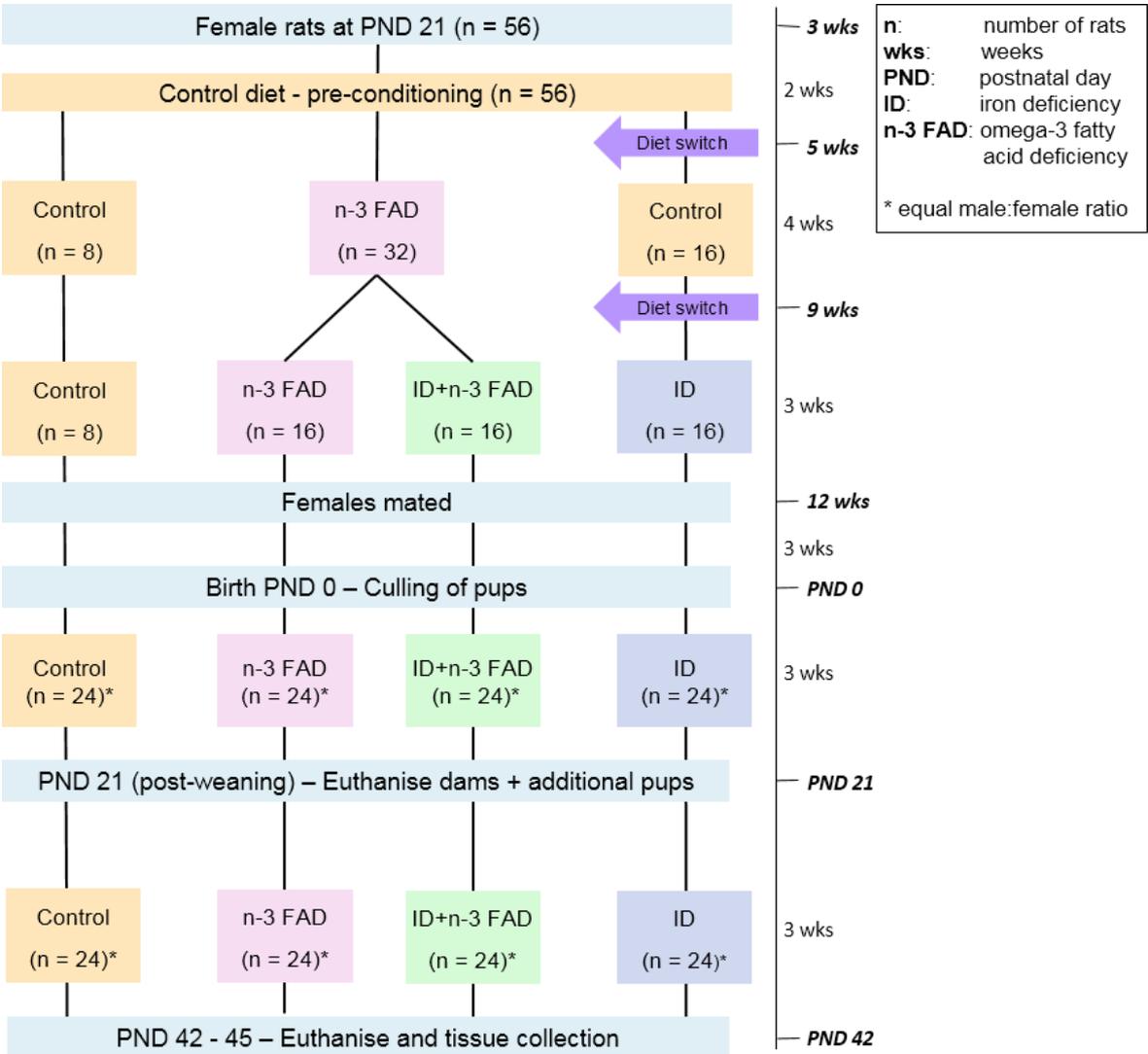


Figure 3-1: Schematic diagram of experimental design

3.2.3 Sample collection, storage and analyses

After decapitation, trunk blood was collected in 4 ml ethylenediaminetetraacetic (EDTA)-coated vacutainer tubes (BD, Plymouth, UK) and centrifuged within one hour at 3000 x g for 10 minutes at 4°C to separate plasma from buffy coat and red blood cells (RBCs). Red blood cells were washed twice with 0.15 mol/L NaCl by centrifugation at 3000 x g for 10 minutes. Plasma and RBC aliquots were stored at -80°C until analysis.

Haemoglobin (Hb) concentrations were measured in whole blood using a portable HemoCue® Hb 201+ photometer (HemoCue AB, Angelholm, Sweden).

Total phospholipid FA analyses in RBC were performed at the CEN of the NWU as described previously [39]. Lipids were extracted from 300 µL RBC with chloroform:methanol (2:1, v:v; containing 0.01% BHT) by a modification of the method of Folch *et al* (1957) [46]. The lipid extracts were concentrated and the neutral lipids separated from the phospholipids by thin layer chromatography (TLC) (Silica gel 60 plates, 10 x 20 cm, Merck) and eluted with diethyl ether:petroleum ether:acetic acid (30:90:1, v:v:v). The lipid band containing phospholipids was removed from the TLC plate and transmethylated with methanol:sulphuric acid (95:5, v:v) at 70°C for two hours to yield fatty acid methyl esters (FAME). The resulting FAME was extracted with water and hexane. The organic layer was evaporated, redissolved in hexane, and analysed by quadrupole (QP) gas chromatography electron ionisation mass spectrometry (GC-EI-MS) on an Agilent Technologies 7890A GC system equipped with an Agilent Technologies 5975C VL mass selective detector. The GC separation of FAME was carried out on an HB-5MS capillary column (30 m x 0.25 mm i.d.; Agilent J&W)

using helium as the carrier gas at a flow rate of 0.9 mL/min. The GC injector was held at a temperature of 250°C and the MS source and QP were maintained at temperatures of 230°C and 150°C, respectively. The injection volume of the sample solution was 1 mL, using a split ratio of 1:25 for the samples. The oven temperature was started at 140°C and programmed at +3°C/min from 140 to 220°C, held at 220°C for two minutes, then programmed at +3°C/min to 230°C and held at 230°C for 10 minutes. The total analysis time was 45 minutes. MS with 70 eV EI was carried out in full scan acquisition mode and all mass spectra were acquired over the m/z range of 50–750. Quantification of FAME was done using the selected ion extraction method. Data analysis was performed using Agilent Quantitative Analyses software (Version B.05.02/Build 5.2.365.0). Relative percentages of FA were calculated by taking the concentration of a given FA derivative as a percentage of the total concentration of all FA identified in the sample.

The lumbar spines and right femurs were collected with surrounding tissue, placed into 50 ml and 15 ml Falcon tubes, respectively, with phosphate-buffered saline (PBS) and stored at -20°C until analysis. Analysis of bone mineral density was done *ex vivo* using areal dual X-ray absorptiometry (DXA) (Hologic Discovery W DXA scanner with APEX system software version 2.3.1) by an experienced radiographer at the CEN of NWU.

The left femurs were collected and cleaned of any surrounding tissue, placed into 15 ml Falcon tubes with PBS and stored at -20°C until analysis. Three-point bending tests were performed at the mid-diaphysis of the left femur using a servohydraulic machine at the School of Mechanical and Nuclear Engineering at NWU. Bones were consistently oriented for testing in the anterior-posterior direction. The bottom supports (3 mm wide with rounded points) were placed 8 mm apart. Mechanical properties, such as ultimate

load, ultimate displacement (amount of displacement at the point of ultimate load), ultimate stress (ultimate load over cross-sectional area), and the load and stress at the point of transition from the elastic to the plastic region, were determined from force deformation curves.

3.2.4 Statistical analyses

Data were analysed and expressed using IBM Statistical Program for Social Sciences (SPSS) Statistics software (version 25) and Excel 2016. Data were examined for normality of distribution (using q-q plots, histograms, and the Shapiro-Wilk test) and the presence of outliers (using box plots). Homogeneity of variance was examined by the Levene's test. Variables that deviated significantly from normality and/or variance homogeneity were transformed prior to inferential statistical analysis using analysis of covariance (ANCOVA).

The effects of pre- and postnatal ID and n-3 FAD, as well as their interactions, on BMD and indices of bone strength at PND 42-45 were determined using 2 x 2 x 2 ANCOVA (iron [sufficient vs. deficient] x n-3 FA [sufficient vs. deficient] x sex [male vs female. When no significant interaction with sex was obtained, sex was included in the models as a covariate. As body weight can influence bone development [47], and ID and/or n-3 FAD may influence body weight, it was also included as a covariate. In the presence of a significant main effect or interaction, between-group differences were determined using one-way ANCOVA followed by Bonferroni's post hoc tests. Data are expressed as

means \pm SEM (parametric data) or geometric means \pm SEM (nonparametric data), and the significance of all tests was set at $P < 0.05$.

3.3 Results

3.3.1 Growth, iron and n-3 fatty acid status of the offspring

Pre- and post-natal ID and n-3 FAD resulted in significantly lower body weight at PND 42-45 and weight gain from PND 21, with no significant ID \times n-3 FAD interactions (Table 2). Neither ID nor n-3 FAD on their own, however, significantly reduced body weight at PND 42-45 or weight gain from PND 21 compared to controls, but resulted in an additive reduction in the ID and n-3 FAD combination diet.

Table 3-2: Offspring body weight, iron and n-3 fatty acid status and left femur size indicators at post-natal day 42-45

	Control	ID	n-3 FAD	ID + n-3 FAD	P-value		
					n-3 ID	n-3 FAD	ID \times n-3 FAD
¹ Body weight at PND 45, g	190 \pm 1.04 ^a	172 \pm 1.04 ^{ab}	188 \pm 1.04 ^a	157 \pm 1.03 ^b	<0.001	0.047	0.110
¹ Weight gain from PND 21, g	133 \pm 1.05 ^a	114 \pm 1.04 ^{ab}	125 \pm 1.05 ^a	105 \pm 1.03 ^b	<0.001	0.009	0.724
¹ Haemoglobin, g/dL	13.5 \pm 0.34 ^a	9.5 \pm 0.33 ^b	13.0 \pm 0.47 ^a	8.6 \pm 0.27 ^b	<0.001	0.053	0.614
² RBC n-3 FA, % total FA	6.7 \pm 0.10 ^a	6.6 \pm 0.10 ^a	0.6 \pm 0.11 ^b	0.7 \pm 0.11 ^b	0.406	<0.001	0.272
² RBC n-6 FA, % total FA	42.5 \pm 0.10 ^c	42.1 \pm 0.10 ^c	48.8 \pm 0.10 ^a	47.0 \pm 0.10 ^b	0.004	<0.001	0.085
² RBC n-6/n-3 FA, % total FA	6.3 \pm 1.03 ^b	6.4 \pm 1.03 ^b	78.8 \pm 1.09 ^a	64.7 \pm 1.13 ^a	0.260	<0.001	0.202
³ Left femur weight, g	0.61 \pm 0.02 ^{abc}	0.57 \pm 0.02 ^{cd}	0.61 \pm 0.01 ^{ab}	0.52 \pm 0.02 ^d	<0.001	0.232	0.053
³ Left femur length, mm	17.9 \pm 0.12 ^a	17.8 \pm 0.27 ^a	17.7 \pm 0.16 ^a	17.0 \pm 0.15 ^b	0.022	0.007	0.140
³ Left femur area, mm ²	4.3 \pm 0.13	4.1 \pm 0.13	4.0 \pm 0.12	3.8 \pm 0.13	0.056	0.058	0.897

Two-way ANCOVA was used to test the effects of ID, n-3 FAD and ID \times n-3 FAD interactions, adjusted for sex. Body weight, weight gain, RBC n-3 FA, n-6 FA and n-6/n-3 FA were log transformed to perform ANCOVA. Between-group differences were determined using one-way ANCOVA followed by Bonferroni's post hoc test (adjusted for sex). Values are means \pm SEM or geometric means \pm SEM for log transformed variables. Means in a row with different superscripts without a common letter differ ($P < 0.05$).

ID: iron deficiency, n-3 FAD: omega-3 fatty acid deficiency, FA: fatty acids; n-6 FA: omega 6 fatty acids; PND: post-natal day; RBC: red blood cell.

1: n=24 for all groups, 2: n=24 (Control, n-3 FAD and ID x n-3 FAD); n=22 (ID), 3: n=15 (Control); n=12 (ID); n=19 (n-3 FAD); n=13 (ID x n-3 FAD).

Pre- and post-natal ID resulted in significantly lower Hb concentrations at PND 42-45 (Table 2). Offspring in the ID groups (ID and ID + n-3 FAD) had significantly lower Hb concentrations compared to offspring in the iron-sufficient groups (control and n-3 FAD).

Pre- and post-natal n-3 FAD resulted in a significantly lower percentage of n-3 FA, a higher percentage of n-6 FA and a higher n-6/n-3 FA ratio in total phospholipids of RBC (Table 2). Offspring in the n-3 FAD groups (n-3 FAD and ID + n-3 FAD) had significantly lower percentages of n-3 FA, compared to offspring in the n-3 sufficient groups (Control and ID). The n-3 FAD also resulted in significantly higher percentages of n-6 FA and higher n-6 FA to n-3 FA ratios.

Pre- and post-natal ID also resulted in significantly lower femur weight and shorter length and showed a possible trend towards a smaller femur mid-diaphysis cross-sectional area (P=0.056). Pre- and post-natal n-3 FAD resulted in significantly shorter femur length and a possible trend towards smaller cross-sectional area (P=0.058). A combined ID and n-3 FAD diet significantly lowered femur weight and length compared to the control diet (Table 2). No diet x sex interactions were found in any of these parameters.

3.3.2 Bone mineral density and bone strength

Pre- and post-natal ID resulted in significantly lower BMD in the spine and right femur (Table 3), and pre- and post-natal n-3 FAD resulted in significantly lower BMD in the right femur, adjusted for sex and body weight at PND 45. The ID diet alone did not significantly lower BMD in the spine ($P=0.528$), but in combination with n-3 FAD, BMD in the spine showed a trend towards a lower BMD ($P=0.065$, adjusted for sex and body weight) compared to the control group, indicating a possible additive effect of ID and n-3 FAD. In the right femur, the ID diet alone did not significantly lower BMD ($P=1.00$); however, the combination with n-3 FAD resulted in lower femur BMD compared to the control diet ($P=0.027$, adjusted for sex and body weight), also indicating an additive effect of ID and n-3 FAD. There were no differences in the BMD of the right femur between the n-3 FAD and control group ($P=1.00$). No diet x sex interactions were found on BMD in the spine or femur.

Pre- and postnatal ID also resulted in significantly lower stiffness, ultimate load, transition load, ultimate stress and transition stress measured in the left femur, even after adjusting for sex and body weight. Pre- and post-natal n-3 FAD did result in significantly higher ultimate displacement (showing greater elasticity), but a post hoc test showed no significant differences between groups when adjusted for sex and body weight. The ID diet alone did not significantly lower stiffness ($P=0.192$); however, in combination with n-3 FAD, it resulted in a possible trend towards lower stiffness ($P=0.071$, adjusted for sex and bodyweight). Effects observed in femur stiffness remained significant after adjusting for the cross-sectional area as well. Ultimate load and ultimate stress were significantly lower in both ID groups (ID and ID + n-3 FAD) compared to offspring in the iron-sufficient groups (control and n-3 FAD). After adjusting for cross-sectional area as well, there was no longer a statistical difference in ultimate

load between the control group and the ID group ($P=0.111$) and a possible trend towards a difference between the control group and the ID and n-3 FAD combination group ($P=0.081$). No adjustment for cross-sectional area was made when ultimate or transition stress was considered, as the area was used to calculate the stress. Transition load and transition stress showed no significant differences between the control group and the ID group. A pre- and post-natal n-3 FAD did not have any effect on bone strength parameters in offspring at PND 42-45. No diet x sex interactions were found on any of the bone strength parameters.

3.4 Discussion

This is the first study, to our knowledge, to investigate the effects of pre- and post-natal ID and n-3 FAD, alone and in combination, on bone development in rats. This study showed that pre- and post-natal ID resulted in lower BMD in the spine and femur, as well as bone strength parameters (stiffness, ultimate load, transition load, ultimate stress and transition stress) in offspring at PND 42-45, even after adjusting for sex and body weight. In this study, pre- and post-natal n-3 FAD alone did not have any effect on BMD or bone strength parameters; however, it had an additive effect on BMD (spine and femur) and femur stiffness when combined with ID. Furthermore, observed effects of ID and n-3 FAD were not sex-specific.

Similar studies have shown that ID can lower BMD and bone strength parameters when rats are fed an iron-deficient diet. In these studies, an ID diet was introduced post-weaning (PND 21) and continued for four to five weeks. The control diets contained the

same amount of iron as our study (standard AIN-93G mineral mix) but the iron content of the experimental diets was 5 – 8 mg iron/kg diet [16, 17] or a more severe ID (amount not specified) [19]. To our knowledge, this is the first study to investigate the effects of a pre- and post-natal ID on bone development, and results show that a less severe ID (15 – 18 mg iron/kg diet) in early development can lead to detrimental effects on bone. Little is known about the role of iron in bone development, but it has been suggested to play a role in bone development by acting as a cofactor of enzymes involved in collagen bone matrix synthesis and the conversion of vitamin D to its active form [12].

Conflicting results have been found on the effects of maternal FA status on bone development in the offspring, with some studies showing positive effects of n-3 FA status [32-35] and others showing no effects [31]. Inconsistent results may be attributed to different n-3 FA concentrations, different time periods exposed to diets, different strains of animals used and different outcomes and time points of outcomes measured. Most of the previous studies focused on n-3 FA supplementation, whereas our study followed a depletion model. Korotkova *et al* (2004) [31] compared different n-6:n-3 FA ratios provided perinatally (for the last 10 days of gestation and throughout lactation), after which offspring were weaned onto ordinary chow and followed up until 30 weeks of age (\pm PND 210). They found no differences in trabecular BMD, but found that different n-6:n-3 ratios perinatally resulted in changes in cortical BMD, bone growth and bone strength parameters later in life. Results from our study found no sex-specific effects, unlike other studies, such as Yin *et al* (2014) [34], which showed higher BMD in female rats, but lower BMD in male rats when receiving a DHA enriched diet during pregnancy and lactation, or Weiler *et al* (2012) [33], which showed effects in female guinea pig offspring (PND 21) only, when receiving a long chain PUFA enriched diet during

lactation. Perinatal n-3 FAD may influence bone development by decreasing calcium absorption or by influencing the differentiation of mesenchymal stem cells into osteoblasts [23-26].

This is the first study, to our knowledge, to investigate the effects of pre-and post-natal ID and n-3 FAD in combination on bone development. Results show that ID and n-3 FAD do not interact in BMD or bone strength, but have additive effects.

Similarities in the pathophysiologic responses between the human and rat skeleton make the rat a valuable model for research on bone health [48]. Studies in humans and computer simulations have shown that achieving a higher PBM early in life will decrease the risk of osteoporosis later in life [2, 4, 49]. The tracking of bone strength indicators from childhood to adolescence further supports the notion that prevention of osteoporosis should begin in the early stages of bone development [50-52]. Increasing bone density or bone strength early in life may also have additional current benefits for children or adolescents [2].

The strengths of this study include the 2 x 2 factorial design investigating the combined effects of ID and n-3 FAD on bone development. Most nutrient deficiencies seldom occur in isolation and it is likely that women of child-bearing age may suffer from ID and n-3FAD [53, 54]. Another strength of our study is the amount of iron and n-3 FA provided in the diets to represent an ID and/or n-3 FAD similar to what can be expected in a human context, and not severe nutrient deficiencies. Using a rat model, bone strength could be determined using three-point bending tests, which is not possible in a

human context. Also, it would not be ethical to initiate nutrient deficiencies purposely in women of reproductive age.

Limitations in this study include the inability to follow offspring up until late adulthood, considering that this study formed part of a larger study. If offspring could have been followed up until late adulthood, it could have been determined whether effects remain unchanged and fracture risk later in life is affected. In the statistical analyses sample sizes for all groups were not equal, owing to some of the bones that broke during harvesting of samples, and some technical difficulties with the three-point bending tests.

In conclusion, this study found in rats that a pre- and post-natal ID, alone and in combination with n-3 FAD, has detrimental effects on the bone development of offspring in early adolescence. An n-3 FAD might have an additive effect by further decreasing BMD and femur stiffness. Outcomes were not sex-specific. Additional research is needed to confirm whether these effects are long-lasting, and whether effects can be reversed when offspring are fed an iron-sufficient diet, or if dams receive iron supplementation during pregnancy and lactation.

Conflict of interest: None

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Table 3-3: Bone mineral density and bone strength in offspring at post-natal day 42-45

	Control	ID	n-3 FAD	ID + n-3 FAD	P-value		
					ID	n-3 FAD	ID x n-3 FAD
¹ Spine BMD, g/cm ²	0.138 ± 0.002	0.128 ± 0.002	0.136 ± 0.003	0.120 ± 0.002	0.017	0.194	0.794
² Right femur BMD, g/cm ²	0.154 ± 0.002 ^a	0.149 ± 0.001 ^{ab}	0.151 ± 0.001 ^{ab}	0.143 ± 0.002 ^b	0.022	0.046	0.479
³ Stiffness, N/mm	114.7 ± 7.6 ^{ab}	80.2 ± 7.4 ^{ab}	110.5 ± 6.4 ^a	72.2 ± 6.0 ^b	0.003	0.554	0.716
³ Ultimate load, N	43.2 ± 1.7 ^a	32.4 ± 1.6 ^b	42.4 ± 1.2 ^a	31.2 ± 1.2 ^b	<0.001	0.884	0.659
³ Transition load, N	31.4 ± 1.6	22.5 ± 1.4	30.9 ± 1.4	21.9 ± 1.5	0.011	0.754	0.720
³ Ultimate displacement, mm	0.76 ± 0.04	0.80 ± 0.03	0.86 ± 0.04	0.86 ± 0.06	0.089	0.011	0.549
³ Transition displacement, mm	0.30 ± 0.03	0.32 ± 0.04	0.30 ± 0.02	0.33 ± 0.03	0.158	0.607	0.966
³ Ultimate stress, Pa	10.1 ± 0.45 ^a	8.0 ± 0.33 ^b	10.7 ± 0.44 ^a	8.2 ± 0.33 ^b	<0.001	0.516	0.714
³ Transition stress, Pa	7.3 ± 0.35 ^{ab}	5.6 ± 0.42 ^b	7.8 ± 0.47 ^a	5.8 ± 0.39 ^b	0.004	0.492	0.574

Two-way ANCOVA was used to test the effects of ID, n-3 FAD and ID x n-3 FAD interactions, adjusted for sex and body weight. Between-group differences were determined using one-way ANCOVA followed by Bonferroni's post hoc test (adjusted for sex and body weight). Values are means ± SEM and means in a row with different superscripts without a common letter differ ($P < 0.05$).

ID: iron deficiency, n-3 FAD: omega-3 fatty acid deficiency, BMD: bone mineral density.

1: n=24 (Control and ID x n-3 FAD); n=22 (ID); n=23 (n-3 FAD), 2: n=24 (Control, ID and n-3 FAD); n=23 (ID x n-3 FAD), 3: n=15 (Control); n=12 (ID); n=19 (n-3 FAD); n= 13 (ID x n-3 FAD).

Supplemental table 3.1: Iron and fatty acid analyses of diet groups (Covance laboratories)

	Control	ID	n-3 FAD	ID+n-3 FAD
Iron (ppm)	41.3	15.2	43.4	15.6
Total Fatty Acids (g/100g)	9.78	9.78	9.76	9.76
Saturated Fatty Acids (g/100g)	3.82	3.82	7.53	7.53
Monounsaturated Fatty Acids (g/100g)	1.45	1.45	0.33	0.33
Polyunsaturated Fatty Acids (g/100g)	3.95	3.95	1.32	1.32
Trans Fatty Acids (g/100g)	0.083	0.083	0.039	0.039
Omega 3 Fatty Acids (g/100g)	0.495	0.495	0.009	0.009
Omega 6 Fatty Acids (g/100g)	3.64	3.64	1.37	1.37
Omega 9 Fatty Acids (g/100g)	1.41	1.41	0.327	0.327
20:3 Eicosatrienoic (g/100g)	<0.007	<0.007	<0.007	<0.007
20:4 Arachidonic (g/100g)	<0.007	<0.007	<0.007	<0.007
20:5 Eicosapentaenoic (g/100g)	<0.007	<0.007	<0.007	<0.007
22:5 Docosapentaenoic (g/100g)	<0.007	<0.007	<0.007	<0.007
22:6 Docosahexaenoic (g/100g)	<0.007	<0.007	<0.007	<0.007

CHAPTER 4: SUMMARY AND CONCLUSION

4.1 Summary and conclusion

To the researchers' knowledge, this is the first study to investigate the effects of ID and n-3 FAD during early development on bone development in offspring. The first hypothesis was proven partially, as results showed that a pre- and post-natal ID has negative effects on the BMD and bone strength of offspring in early adolescence. A pre- and post-natal n-3 FAD might have an additive effect with iron by further decreasing BMD and bone strength. The second hypothesis was also proven, as outcomes were not sex-specific.

4.2 Strengths and limitations

The 2 x 2 factorial design is a great strength of this study, because most nutrient deficiencies seldom occur in isolation and it is likely that women of child-bearing age may suffer from ID and n-3 FAD (Briend *et al.*, 2011, Stoltzfus, 2011). The researchers were therefore able to investigate the effects of ID and n-3 FAD alone and in combination, and determine whether there are any interactions or additive effects.

Another strength of this study is the amount of iron (half the recommended amount for growing rats) and the FA composition in the diets, which potentially reflect an ID and/or n-3 FAD diet as can be found in a human context. This allows the results to be more translatable to humans.

Using a rat model, more invasive techniques such as three-point bending tests to determine bone strength could be used, which is not possible in a human context. Also, it would not be ethical to purposely initiate nutrient deficiencies in women of reproductive age. The 3R principles were followed to ensure the humane use of animals.

The three-point bending test however, has some limitations as well. Even though it is most commonly used to determine the bone strength of rodent bones, owing to the comparable way the load is applied in long bones *in vivo* (Jepsen *et al.*, 2015), it might not be the most practical test. During the three-point bending testing, it was observed that many of the bones did not break in a similar way, probably because bones do not have a uniform circumference or a flat side that can be supported on the bottom rods. This resulted in many test results being invalid, and therefore the different groups were not equal in the statistical analyses. Some bones also had to be used for the development and optimisation of the three-point bending test, especially for the apparatus designed for this study. Fortunately, this apparatus was designed with moveable rods, as to be able to adjust the length for the testing of rat bones of different sizes.

Other limitations in this study include the inability to follow offspring up until late adulthood, considering that this study formed part of a larger study. If offspring could have been followed up until late adulthood, it could have been determined whether effects remain and fracture risk later in life is affected.

Even though the DXA scans have been analysed using animal-specific software, rat femurs and spines at PND 21 (which were collected as part of the larger study) were still too small to provide accurate measurements. If rat bones at PND 21 could have been analysed, perhaps more clarity about the reversibility of effects could have been provided. Tracking of BMD from childhood to early adolescence could also have been explored.

4.3 Recommendations on future research

The next step would be to compare the effects of ID and n-3 FAD on bone development in offspring at PND 42-45 that maintained their respective diets after weaning (this study), with offspring that switched to a control diet after weaning (PND 21). Samples of these offspring have already been collected as part of the larger study, and will be analysed in the near future. This will determine whether effects (as seen in a pre-and post-natal ID) on bone development at PND 42-45 are reversible in offspring switched to a control diet post-weaning.

Bone samples from the second experiment of the larger study, which follows a repletion model, can be analysed to determine whether the effects of a pre- and post-natal ID on bone development in the offspring can be reversed if dams receive iron supplementation during pregnancy and lactation. Possible effects of a pre-and post-natal ID and n-3 FAD, alone and in combination, on the BMD and bone strength of the dams can also be investigated.

It is recommended for future research to investigate the optimal methods to determine bone strength by comparing various biomechanical tests, and the testing environments. The practicality of a torsion test can also be investigated, because the way the load is applied is also comparable with *in vivo* long bone models (Jepsen *et al.*, 2015).

Ideally, the experiment should be repeated, and offspring followed up until late adulthood, to investigate whether the effects remain long term. The tracking of BMD and bone strength from early adolescence to late adulthood can then be determined. These findings would provide crucial insight into the possibility of optimal nutrition in early life as a strategy to prevent disease later in life.

The main goal of this research is to eventually translate it to the human context. Once a better understanding of the role of iron and n-3 FA in bone development is obtained, human trials can

investigate the effects of iron and n-3 supplementation in pre-natal women on the bone development of the child.

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ANNEXURES

5.1 Annexure A: Animal housing and additional methodology

5.1.1 Animal housing

The animal trial was conducted at the vivarium of the Preclinical Drug Development Platform (PCDDP) of the North-West University (NWU), Potchefstroom, South Africa on Wistar rats bred in-house. Rats were kept under a reversed 12/12h light/dark cycle (lights on at 18:00) at $22 \pm 1^\circ\text{C}$ and $55 \pm 10\%$ relative humidity. During the experiment, rats were pair-housed in a standard solid floor cage on Alpha-Dri bedding provided by LBS Serving Biotechnology Ltd. The iron content of the bedding was tested by NP Analytical Laboratories and confirmed as less than 2.00 ppm (Annexure E). Cages were enriched with plastic tubes to provide a solid resting and nesting area, nesting material (Kimtek trace element free paper) and chewing devices (to avoid tooth overgrowth due to the use of powdered diets). All rats had *ad libitum* access to purified laboratory diets (AIN-93 with modifications as specified in section 5.3.4 and demineralized water (18 M Ω) and were monitored daily using study-specific monitoring sheets (Annexure F).

5.1.2 Experimental design (detailed)

Fifty-six female Wistar rats at 21 ± 3 days of age (postnatal day [PND] 21) were housed in pairs and randomly allocated (in pairs) to one of four diet groups, as shown in Figure 1-1. The diet groups were: 1) Control (n = 8); 2) ID (n = 16); 3) n-3 FAD (n = 16); or 4) ID+n-3 FAD (n = 16)). All the rats were placed on the control diet at PND 21 for a two week period of preconditioning. At the end of the preconditioning phase (at five weeks of age) the rats that had been randomly allocated to one of the n-3 FAD diet groups (n-3 FAD and ID+n-3 FAD) were placed on a n-3 FAD diet for seven weeks before mating, in order to imitate a chronic n-3 FAD diet (Moriguchi *et al.*, 2000, Ikemoto *et al.*, 2001, Reinwald *et al.*, 2004, McNamara *et al.*, 2009, Mathieu *et al.*, 2011). Three weeks before mating, at nine weeks of age, the female rats that had been allocated to one of the ID diet groups (ID and ID+n-3 FAD), were placed on an ID or ID+n-3 FAD diet (Mihaila *et al.*, 2011, Greminger and Mayer-Proschel, 2015).

At 12 weeks of age the female rats were placed in individual cages and mated with 12-week-old male breeders of the same strain (maintained on standard laboratory chow) using monogamous breeding (one male bred with one female). The researchers accustomed males to purified AIN93G diets a few days prior to transferring them to the females' cages. Mating (conception) was confirmed by the presence of a vaginal plug (gestational day [GD] 0). After conception, the females were maintained on their pre-pregnancy diet throughout pregnancy. Maternal body-

weights and food intake were recorded to the nearest 0.1 g every second day during the gestational period. During the mating period, however, the rats were minimally handled and only during the light phase, as mating took place during the dark phase. Furthermore, any interruption of the reversed light-dark cycle (dark phase during the day) was minimised by switching off the corridor light in front of the animal room to avoid light entering when the door opened.

Dams were allowed to deliver spontaneously at approximately GD 22 (PND 0). Dam weights after parturition, litter size and litter weights at birth were recorded. Within three to five days after birth the litters were culled to eight pups (to maintain nutritional adequacy), with ideally four males and four females per litter [eight pups/litter; minimum of three litters/group]. Thus, the remaining two to five pups from each litter (from approximately 10 litters per group) were euthanized at birth and samples were collected for analysis (not for the purpose of this MSc study).

The dams stayed with their pups and were maintained on their respective experimental diets throughout lactation. At the start of the post-weaning period (PND 21) the dams were euthanized. Eight pups (four male and four female) from each group (n = 32) were then euthanized and samples were collected. The remaining pups were weaned from the ID, n-3 FAD and ID+n-3 FAD diet groups and were randomly allocated in pairs of same-sex littermates to receive either the control diet (n = 24/group, male:female = 1:1) or maintained on their respective experimental diets (n = 24/group, male:female = 1:1) for three weeks until PND 42-45. During this period, the littermates were housed in pairs by sex. All offspring were weighed three times per week. At PND 42-45, 24 (12 male and 12 female) offspring from each group (n = 168) were euthanized and samples were collected.

5.1.3 Ethical considerations

The 3R (Replacement, Reduction and Refinement) principals were used to ensure that the research had been conducted in a humane way (Singh, 2012). The depletion experiments were done using rodent models, as *in vitro* models would not consider the complex physiological and biochemical nature of the role of pre- and postnatal nutrition in bone development. Furthermore, it would not be ethical to purposely make or leave women of childbearing age deficient in vital nutrients, and therefore it would not be possible to perform this type of study in humans.

Animal numbers were based on the recognized standard of 12 rats per group as based on previous studies of the same kind (for the purpose of the large study) (Ahmad *et al.*, 2002,

Beard *et al.*, 2003, Baumgartner *et al.*, 2012a). All harvested tissue was utilized for biochemical analysis, as this study forms part of a large study.

The rats were housed in same-sex sibling pairs in transparent solid floor cages, allowing for visual interaction. Even though animals were housed in standard solid floor cages with bedding, the cages were enriched with plastic pipes and nesting material (Kimtek trace element free paper). Powdered diets may affect tooth growth, leading to overgrowth. Chewing devices were therefore also provided.

Animals were monitored on a daily basis, by making use of a study-specific NWU vivarium monitoring checklist (Annexure F), to ensure that animals did not experience distress. A different monitoring sheet was used for pups PND 1 to PND 20 (Annexure G). During the iron and n-3 FA depletion period, reduced weight gain was expected. Depleted pups may gain weight more slowly in relation to animals receiving a sufficient diet. In order to ensure that the animals did not suffer from inadequate weight gain, all animals were weighed three times per week. In addition to body weight change, body condition was also monitored, by making use of a body condition score technique (Hickman and Swan, 2010). The decision to euthanize was made based on the monitoring score sheet. Rats that were found to be suffering, with a score between 10 and 15, were to be considered by an animal technologist for humane euthanasia and with a score above 15 would immediately have to undergo humane euthanasia. No rats used in this study had a score of above 10.

The blood iron concentrations (haemoglobin levels) of all dams and offspring were monitored at critical stages during the experiment in a venous blood spot obtained via tail vein nick. A haemoglobin (Hb) concentration below 4 g/dl was considered the humane endpoint at which rats would be euthanized (Koziol *et al.*, 1982). Furthermore, rats were handled regularly to get them used to human touch in order to minimize stress during cognitive and behavioural testing, blood drawings and endpoints.

Both experiments were executed in three stages. The first stage of each experiment served as a pilot study and was therefore completed successfully before the second and third stages of the experiment started.

5.1.4 Experimental diets

The purified experimental diets were obtained commercially from Dyets Inc. (Bethlehem, USA) and were based on the American Institute of Nutrition (AIN) 93G purified diets for laboratory rodents formulation (Reeves *et al.*, 1993), with modifications in Fe content and fat source. All

diets were isocaloric and contained 10% fat. The basal AIN-93G formulation (control diet) contained 35 mg iron/kg, soybean oil at 70 g/kg diet and hydrogenated coconut oil at 30 g/kg (Baumgartner *et al.*, 2012a, Fitsanakis *et al.*, 2009, Grill *et al.*, 2001).

The n-3 FAD diets contained hydrogenated coconut oil at 81 g/kg diet and safflower oil at 19 g/kg diet (Moriguchi and Salem, 2003, Moriguchi *et al.*, 2001, Moriguchi *et al.*, 2013, Harauma *et al.*, 2010). ID diets contained 15-18 mg Fe/kg diet (Greminger and Mayer-Proschel, 2015).

All diets were custom-prepared and stored at -20°C until use. The FA composition of the diets was confirmed in spot samples from each batch of diets by using gas chromatography tandem mass spectrometry (GC-MS-MS). See annexure H for the analysis of the FA composition of the different diets. The iron concentrations in the diets were confirmed in spot samples from each batch of diets by using atomic absorption spectrometry (AAS).

5.2 Annexure B: Harvesting and storage of samples

Material and equipment

- 1 x 50 ml labelled Falcon tube per rat filled $\frac{3}{4}$ with phosphate buffered saline (PBS)
- 1 x 15 ml labelled Falcon tube per rat filled $\frac{3}{4}$ with PBS
- 1 x 15 ml labelled empty Falcon tube per rat
- Gloves and personal protective equipment as specified by the vivarium
- Pliers, scalpels, surgical scissors or other equipment to remove muscles and bones
- Additional PBS in spray bottle
- - 20 °C freezer

Process

1. Decapitation by the animal technician and removal of any other organs harvested.
2. Remove the skin of the rat (if not yet done) and clean with PBS spray. (Figure 5-1).

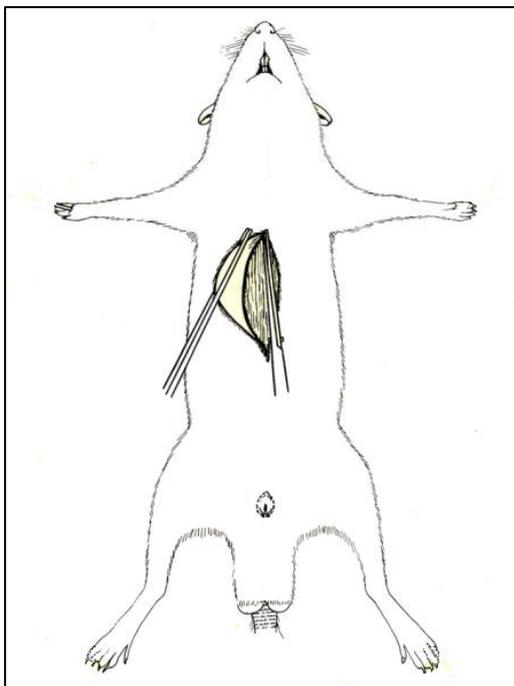


Figure 5-1: Removal of rat skin - image adapted from Jones (2017)

3. Remove left gluteus maximus and left rectus femoris, vastus medialis and vastus lateralis, combined, for purposes other than this study. (Figure 5-2).

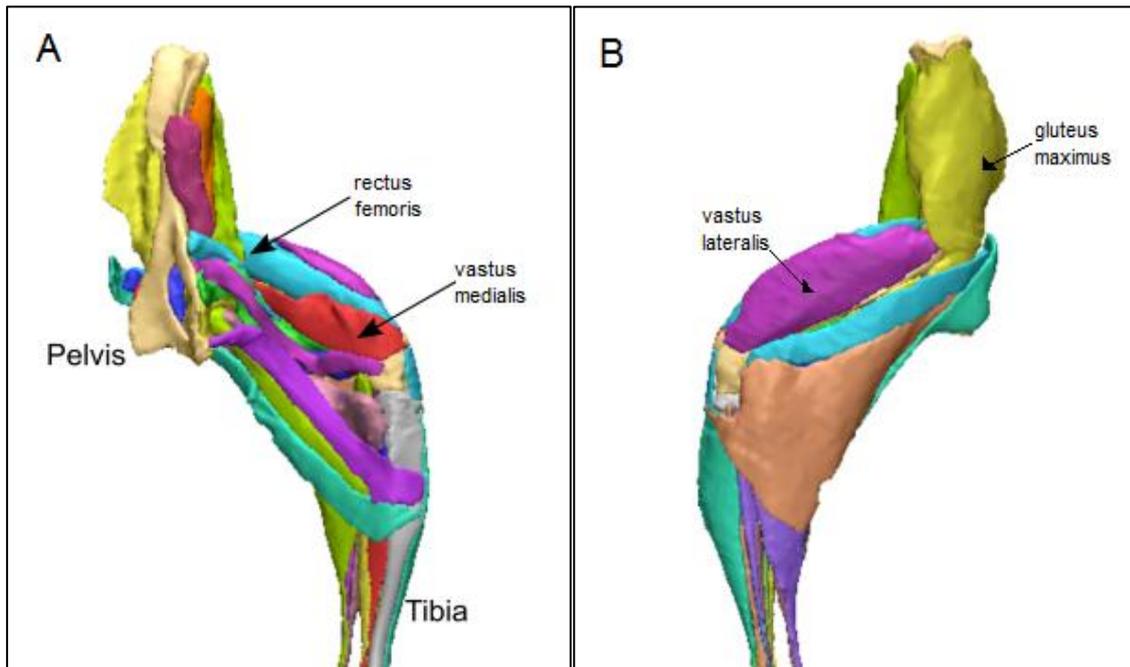


Figure 5-2: Medial (A) and lateral (B) view of rat muscles to be removed for other purposes - images adapted from Charles et al. (2016)

4. Remove the left femur of the rat and clean off any surrounding tissue. (Figure 5-3).

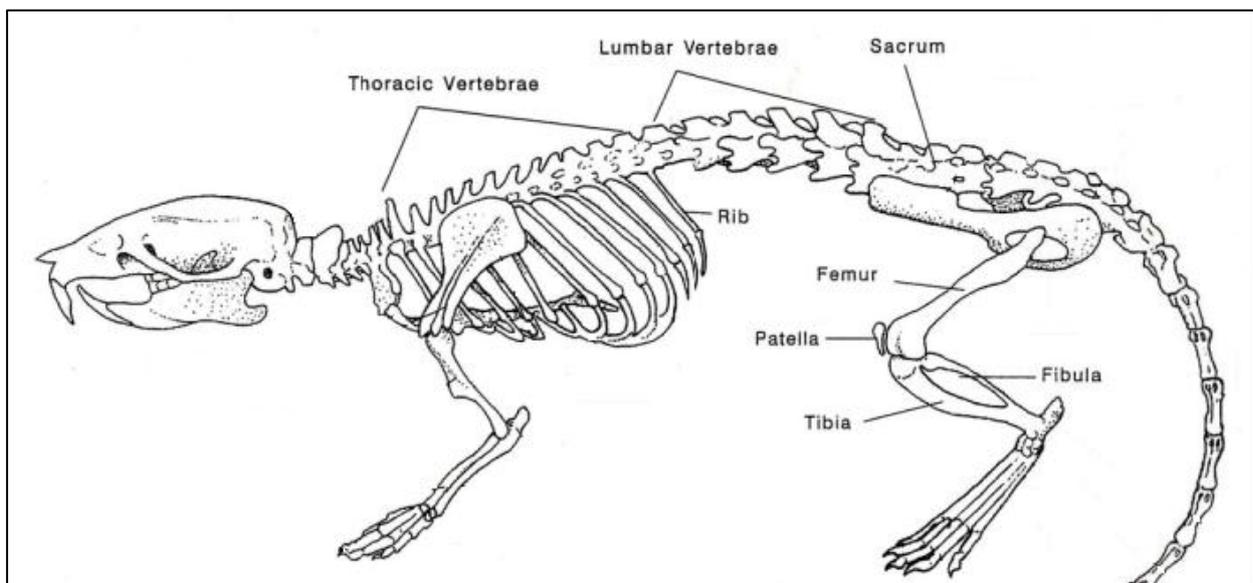


Figure 5-3: Rat skeleton - image adapted from Jones (2017)

5. Place cleaned left femur in 15 ml empty Falcon tube and close. Keep sample on ice until it can be stored in a freezer (-20 °C).

6. Remove the right femur of the rat while keeping the surrounding tissue attached (rectus femoris, vastus medialis and vastus lateralis). Place right femur in the 15 ml Falcon tube filled with PBS with the femur head in the upright position. (Figure 5-4). Ensure that some air remains in the Falcon tube to allow for expansion with freezing. Keep sample on ice until it can be stored in a freezer (-20 °C).

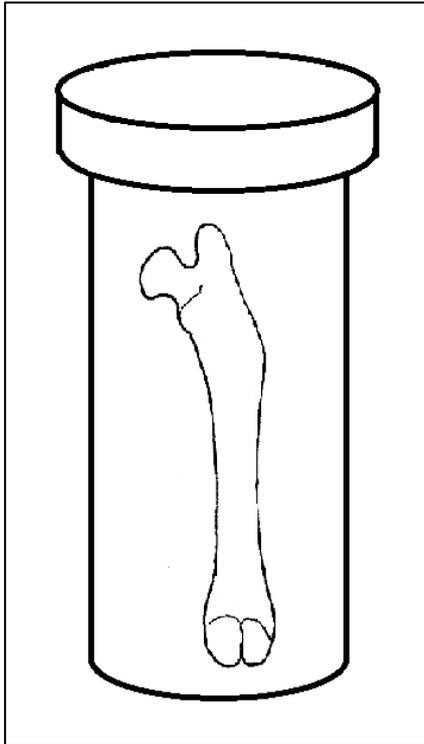


Figure 5-4: Right femur (with surrounding tissue) placed in 15 ml Falcon tube, filled with PBS, with femur head facing upwards

7. Remove the lumbar vertebrae (with surrounding tissue) of the rat while ensuring that parts of the thoracic vertebrae (and ribs) and the sacrum are included to allow for identification of landmarks during DXA scans. (Figure 5-3). Place the spine in the 50 ml Falcon tube filled with PBS with the thoracic vertebrae in the upright position. (Figure 5-5). Ensure that some air remains in the Falcon tube to allow for expansion with freezing. Keep sample on ice until it can be stored in a freezer (-20 °C).

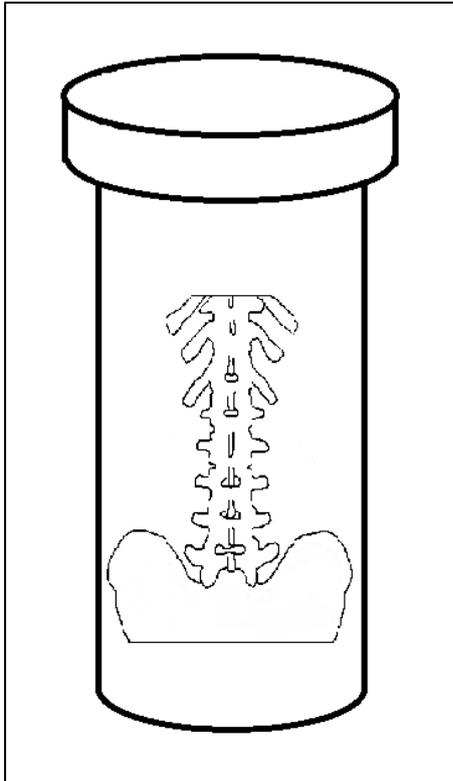


Figure 5-5: Spine (with surrounding tissue) placed in 50 ml Falcon tube, filled with PBS, with thoracic vertebrae facing upwards

5.3 Annexure C: Analysis of bone mineral density in L1-5 vertebrae and right femur *ex vivo* using DXA

Material and equipment

- DXA (Hologic Discovery W DXA scanner [serial number: 84106] with APEX System Software version v.2.3.1 for small animals)
- Right femurs and spines stored with surrounding tissue in 15 and 50 ml Falcon tubes, respectively, filled with phosphate buffered saline (PBS) and frozen at -20 °C.
- Pliers
- Additional PBS
- Gloves
- - 20 °C freezer
- 4 – 6 °C fridge

Process

1. Transfer samples (maximum 50 per day) from the freezer to the fridge (4 – 6 °C) at 16:00 on the day prior to testing.
2. Remove samples from the fridge and keep at room temperature at 07:30 on the day of testing.
3. Ensure that samples are aligned similarly in the Falcon tubes. For the right femurs, the femur head is placed towards the opening of the Falcon tube. The spine is placed so that the ribs are directed at the opening of the Falcon tubes. (Figure 5-6).

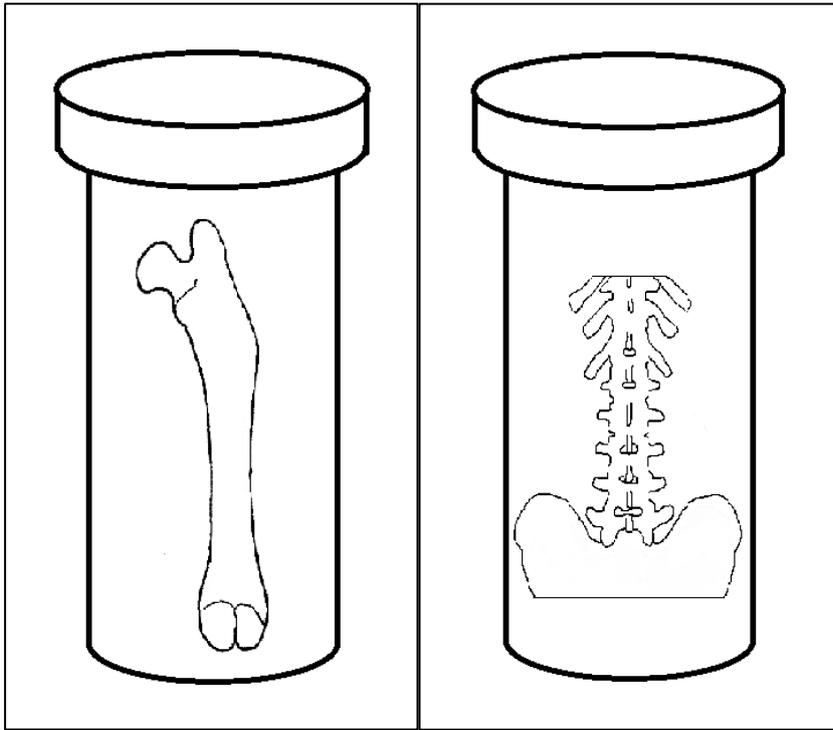


Figure 5-6: Alignment of right femur and spine for DXA scans

4. Fill the Falcon tubes completely with PBS so that no air bubbles will be present during the DXA scans.
5. Measurements can start at 8:30 when samples are at room temperature.
6. The samples are placed in similar positions on the DXA by an experienced radiographer and measurements are taken.
7. After the measurements have been completed, remove some PBS from the Falcon tubes to allow for expansion and return to the freezer.

5.4 Annexure D: Analysis of bone strength of left femur using a biomechanical three-point bending test

Material and equipment

- Servohydraulic machine (Instron model 1026) connected to a computer
- Three-point bending apparatus modified for the size of the rat bones (Figure 5-9)
- Scale (Sartorius BP 1105 [maximum 110 g; d = 0.1 mg])
- Digital ruler (Mitutoyo Absolute digimatic)
- Left femurs stored in 15 ml Falcon tubes stored at – 20 °C
- Gloves
- Permanent marker (fine point)
- -20 °C freezer
- 4 – 6°C fridge

Process

1. Transfer samples (maximum 50 per day) from the freezer to the fridge (4 – 6 °C) at 16:00 on the day prior to testing.
2. Remove samples from the fridge and keep at room temperature at 07:30 on the day of testing.
3. Ensure that samples are at room temperature at 8:30.
4. Weigh all samples on the scale to the nearest 0.1 mg.
5. Measure and record the length of the diaphysis (between the head of the femur and the distal epiphysis – inside of the medial epicondyle) of each left femur at the medial side of the femur with the digital ruler. Mark the midpoint with a fine point marker. (Figure 5-7).

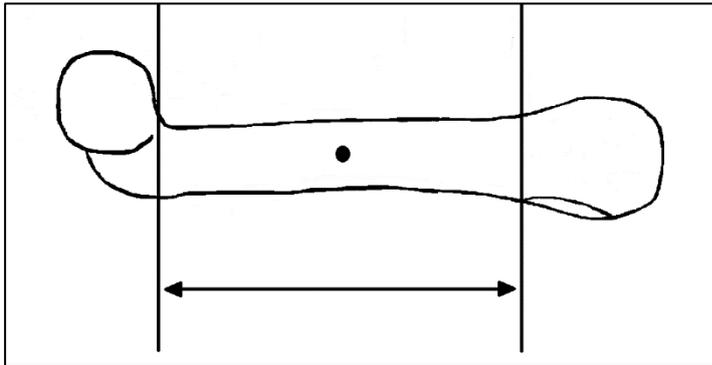


Figure 5-7: Measuring the length of the diaphysis of the left femur

6. Measure and record the mid-diaphysis length on the midpoint marked. Measure in the anterior-posterior direction (the thinnest part). (Figure 5-8)

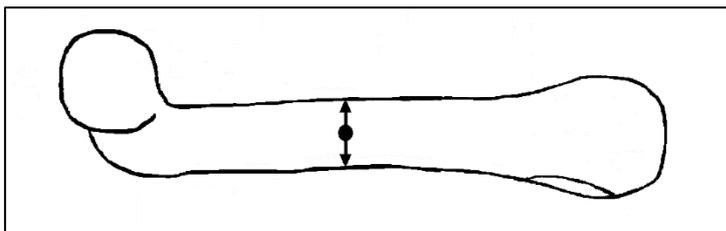


Figure 5-8: Measuring the mid-diaphysis length of the left femur in the anterior-posterior direction

7. Measure and record the mid-diaphysis length of the left femur in the medial-lateral direction on the midpoint marked.
8. Secure the modified apparatus in the correct position on the servohydraulic machine. For the machine available at the Mechanical Engineering department at the NWU, the following steps can be followed:
 - a. Ensure that all power supplies are switched on.
 - b. Open the compressed air valve that is situated behind the MTS Landmark® machine in the laboratory.

- c. Move the top clamp to the desired position and place the “bending rod” in its correct location (Figure 5-9).
- d. Close the clamps by setting the “on-off” switch to “ON”.

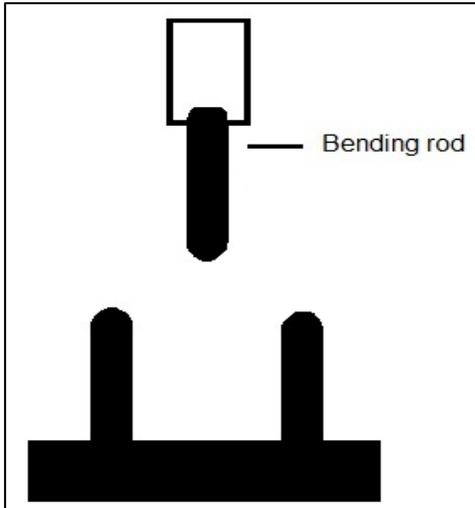


Figure 5-9: Three-point bending test apparatus modified for rat bones, indicating the bending rod

9. Place the left femur in the anterior-posterior direction (anterior side is facing upwards to the bending rod and the medial side is facing the person conducting the test) on the bottom rods, ensuring that the marked midpoint on the medial side is in the centre of the bottom rods. The bending rod must not be touching the femur yet. (Figure 5-10).

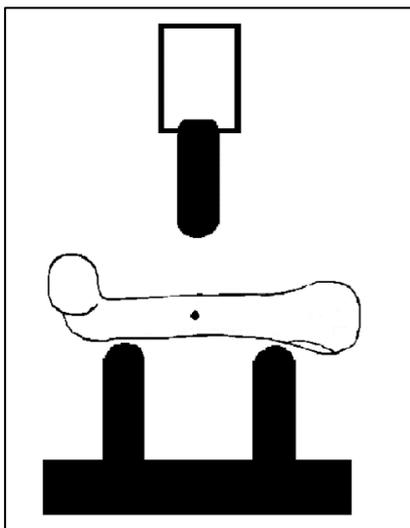


Figure 5-10: Left femur placed on modified three-point bending apparatus in anterior-posterior direction

10. Ensure that the load and displacement readings are zero. For the software on the machine at the NWU reset both "LOAD" and "EXTENSION" values to zero on the electronic board.
11. Prepare the software for running the three-point bending test. The software on the computer at the NWU can be prepared as followed:
 - a. Ensure that the computer is on.
 - b. Open the "Free Serial Port Monitor" shortcut on the computer's desktop.
 - c. Click on "File" and create a "New Session".
 - d. Select "Serial Port Monitor" followed by "Next".
 - e. Select COM3 (this will be the displacement data), followed by "Next".
 - f. Select "Console View", followed by "Next" and "Finish".
 - g. COM3's Console View is active and the user can set it aside.
 - h. Repeat steps b) to g), but this time select "COM4" (this will be the load data).
 - i. Both COM3 and COM4's Console View windows should be open.
12. Lower the bending rod with the servohydraulic machine until a load of 0.5 N is reached. Let the bending rod rest there for 30 seconds.
13. Reset the load and displacement readings so that the test can start at 0 mm displacement.
14. Start to log data and run the test. For the machine at the NWU follow these steps:
 - a. Open the "Shortcut to Tensileauto" shortcut on the computer's desktop: the software will automatically start reading values.
 - b. Minimize the IMP ControlTrak Version 5.01 window.
 - c. COM3 and COM4's Console View windows will be logging data.
 - d. Start the tensile test by moving the "ON-OFF-ON" switch to the bottom.
15. Continue the test until the bone has broken completely. (Stop COM3 and COM4.) Place bone in the Falcon tube and return to the freezer.
16. Transfer or save the logged load and distention data.
 - a. Copy the data from COM3 and COM4 to Notepad and save the Notepad.
 - b. Close the IMP ControlTrak Version 5.01 window and then both COM3 and COM4.
17. Move the top clamp to its original position and repeat steps 8 to 16 for the next specimens.

18. The raw data can now be processed further.

19. After the bone has broken, measure the cross-sectional area of the bone:

- a. Measure the thickness of the bone in the anterior-posterior and the medial-lateral direction at the place where the bone broke and determine the average:

$$\text{Thickness}_{\text{average}} = (\text{Thickness}_{\text{anterior-posterior}} + \text{Thickness}_{\text{medial-lateral}}) / 2$$

- b. Determine the average of the diameter in the anterior-posterior direction and the diameter in the medial-lateral direction:

$$\text{Diameter}_{\text{average}} = (\text{Diameter}_{\text{anterior-posterior}} + \text{Diameter}_{\text{medial-lateral}}) / 2$$

- c. Calculate the cross-sectional area:

$$\text{Cross-sectional area} = \frac{1}{4} \pi (\text{Diameter}_{\text{average}}^2 - (\text{Diameter}_{\text{average}} - 2t)^2)$$

Sample No.: L1619523-1

Received: 07/27/2016

Page 2 of 2

Reported: 08/08/2016

ADC07116 ALPHA-DRI

Test Code	Assay / Analyte	Result	Units
ORGP	Organophosphate pesticides	Thiodan	< 0.0200 ppm
		Trithion	< 0.0200 ppm
RSPB	Organochlorine pest.&PCB's	Heptachlor Epoxide	< 0.0200 ppm
		Heptachlor	< 0.0200 ppm
		DDE	< 0.0200 ppm
		Lindane	< 0.0200 ppm
		Endrin	< 0.0200 ppm
		Mirex	< 0.0200 ppm
		Alpha-BHC	< 0.0200 ppm
		Delta-BHC	< 0.0200 ppm
		Aldrin	< 0.0200 ppm
		Dieldrin	< 0.0200 ppm
		DDT	< 0.0200 ppm
		Chlordane	< 0.0200 ppm
		Methoxychlor	< 0.0200 ppm
		Beta-BHC	< 0.0200 ppm
		PCB	< 0.150 ppm
AFTX	Aflatoxin screen, ELISA	Aflatoxins	< 5.0 ppb

Person responsible for report content: Lynn Loudermilk, Director.

The test code located next to each assay is a method reference code. Results are for samples submitted only. This report shall not be reproduced, except in its entirety, without the written permission of NP Analytical Laboratories.

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The symbol "<" or the words "less than" signifies that no analyte was measured at or above the stated lower limit of quantitation of the procedure under the conditions employed. The use of the symbol ">" or the words "greater than" signifies that the analyte was determined to be present in an amount greater than the stated level. Samples submitted to NP Analytical Laboratories for testing are retained for a minimum of thirty (30) days after the analysis report is issued when sample stability permits. Requests for extended storage must be made to NP Analytical Laboratories prior to or at the time of sample submission.

5.6 Annexure F: Monitoring sheets for dams

Study title: Novel interactions between iron and n-3 fatty acids: The effects of maternal depletion and repletion on offspring development and health							Year:				
Ethics no.: No.NWU-00335-15-A5		Project head: Prof. Marius Smuts			Observer / student: Erna Kemp			Animal ID:			
Parameter	Score	1	2	3	4	5	6	7	8	9	
		Date	Date	Date	Date	Date	Date	Date	Date	Date	
		Time	Time	Time	Time	Time	Time	Time	Time	Time	
Appearance	Normal	0									
	Lack of grooming	1									
	Dull coat, ocular/nasal discharge	2									
	Piloerection, hunched up	3									
Body Condition Score	Rat is well conditioned	0									
	Rat is under conditioned – segmentation of vertebral column prominent/dorsal pelvis pins easily palpable	1									
	Rat is emaciated – vertebral column and dorsal pelvis pins prominent and may be visible	2									
Food & water intake	Normal	0									
	<5% weight loss	1									
	Noted intake, 5-15% weight loss	2									
	No food or water intake	3									
Clinical signs	Normal	0									
	Slight changes	1									
	Respiratory increase ↑ 30%	2									
	Respiratory increase ↑ 50%	3									
Natural behaviour	Normal	0									
	Minor change	1									
	Less mobile alert, isolated	2									
	Vocalisation, restless or still	3									
Provoked behaviour	Normal	0									
	Minor depression	1									
	Moderate change	2									
	Reacts violently/weakly, precomatose	3									
Teeth growth Malocclusion	Normal (no visible malposition or length of teeth)	0									
	Slight overgrowth or uneven growth of incisors	1									
	Visible overgrowth or uneven growth of incisors, without signs of inability to chew food, weight loss or sores developing near the mouth.	2									
	Severe overgrowth or uneven growth of incisors, without signs of inability to chew food, weight loss or sores developing near the mouth.	3									
TOTAL SCORE		0-20									
Other	Observation and/or comment (tick box <input type="checkbox"/> if written on reverse side)		1 <input type="checkbox"/>	2 <input type="checkbox"/>	3 <input type="checkbox"/>	4 <input type="checkbox"/>	5 <input type="checkbox"/>	6 <input type="checkbox"/>	7 <input type="checkbox"/>	8 <input type="checkbox"/>	9 <input type="checkbox"/>
Decision	✓ = normal / ? = monitor carefully / ! = seek advice / ✕ = intervene immediately										
Signature <i>(please sign/initialise with each observation per column)</i>			1	2	3	4	5	6	7	8	9
00 - 04 = Normal		10 - 14 = Suffering, provide relief, observe regularly. Seek opinion from technologist. Consider humane euthanasia									
05 - 09 = monitor carefully, consider intervention		15 - 20 = Sever pain; intervene immediately per humane endpoint, reconsider experimental protocol									

**Observations and/or comments, corresponding to the column on the front page of the monitoring sheet
(see reverse side)**

1.	
2.	
3.	
4.	
5.	
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9.	

5.7 Annexure G: Monitoring sheet for litter

Study title:		Novel interactions between iron and n-3 fatty acids: The effects of maternal depletion and repletion on offspring development and health				Year:		
Ethics no.:		No. NWU-00335-15-A5	Project head:	Prof. Marius Smuts	Observer / student:	Erna Kemp	Litter ID:	
PND	Date	Developmental Milestones			No. Pups	Comments / observations	Decision	Sign
D 1		No fur, slight whiskers, eyelids are sealed. Eyes can only be seen as dark spots beneath the skin. Milk Observable in stomach.						
D 2		Ears flattened against the head, pups are vocal, limbs and digits not fully developed.						
D 4		Ears begin developing and separating from the head, colour should be distinguishable.						
D 6		Ears start to protrude, digits begin to separate and soft toe nails become visible.						
D 8		Pups covered in fuzz, movement more squirming than walking.						
D 10		Ears are fully separated from head, pups should start crawling.						
D 12		Ear canal opens pup can now hear, pups should be walking.						
D 14		Pups eyes should be open, full covering of fur should be present, nipples may be visible on females.						
D16		Pups more active, begin to show rearing behaviour, can sample rat chow, pups also start grooming						
D 18		Pups display normal rat behaviour if slightly hyperactive, pups should be readily eating chow.						
D 20		Pups are fully developed and show normal rodent behaviour.						
Decision		✓ = normal ? = monitor carefully ! = seek advice × = intervene immediately		Any Decision Other than "Normal": Observation and/or comment on reverse side				

Additional Observations and/or comments, corresponding to the row on the front page of the monitoring sheet (see reverse side)

D 1	
D 2	
D 4	
D 6	
D 8	
D 10	
D 12	
D 14	
D 16	
D 18	
D 20	

5.8 Annexure H: Analysis of diets

	Control	ID	n-3 FAD	ID+n-3 FAD
Iron (ppm)	41.3	15.2	43.4	15.6
Saturated Fatty Acids (g/100g)	3.82	3.82	7.53	7.53
Total Cis Unsaturated Fatty Acids (g/100g)	5.4	5.4	1.65	1.65
Monounsaturated Fatty Acids (g/100g)	1.45	1.45	0.33	0.33
Polyunsaturated Fatty Acids (g/100g)	3.95	3.95	1.32	1.32
Trans Fatty Acids (g/100g)	0.083	0.083	0.039	0.039
Omega 3 Fatty Acids (g/100g)	0.495	0.495	0.009	0.009
Omega 6 Fatty Acids (g/100g)	3.64	3.64	1.37	1.37
Omega 9 Fatty Acids (g/100g)	1.41	1.41	0.327	0.327
Total Fatty Acids (g/100g)	9.78	9.78	9.76	9.76
4:0 Butyric (g/100g)	<0.007	<0.007	<0.007	<0.007
6:0 Caproic (g/100g)	0.025	0.025	0.063	0.063
8:0 Caprylic (g/100g)	0.251	0.251	0.681	0.681
10:0 Capric (g/100g)	0.188	0.188	0.503	0.503
12:0 Lauric (g/100g)	1.39	1.39	3.73	3.73
14:0 Myristic (g/100g)	0.535	0.535	1.4	1.4
14:1 Myristoleic (g/100g)	<0.007	<0.007	<0.007	<0.007
15:0 Pentadecanoic (g/100g)	<0.007	<0.007	<0.007	<0.007
15:1 Pentadecenoic (g/100g)	<0.007	<0.007	<0.007	<0.007
16:0 Palmitic (g/100g)	1.06	1.06	0.855	0.855
16:1 Palmitoleic (g/100g)	0.008	0.008	<0.007	<0.007
17:0 Heptadecanoic (g/100g)	0.008	0.008	<0.007	<0.007
17:1 Heptadecenoic (g/100g)	<0.007	<0.007	<0.007	<0.007
18:0 Stearic (g/100g)	0.526	0.526	0.742	0.742
9c 18:1 Oleic (g/100g)	1.39	1.39	0.327	0.327
18:2 Linoleic (g/100g)	3.64	3.64	1.37	1.37
20:0 Arachidic (g/100g)	0.024	0.024	0.016	0.016
18:3 Gamma Linolenic (g/100g)	<0.007	<0.007	<0.007	<0.007
20:1 Eicosenoic (g/100g)	0.015	0.015	<0.007	<0.007
18:3 Linolenic (g/100g)	0.495	0.495	0.009	0.009
18:4 Octadecatetraenoic (g/100g)	<0.007	<0.007	<0.007	<0.007
20:2 Eicosadienoic (g/100g)	<0.007	<0.007	<0.007	<0.007
22:0 Behenic (g/100g)	0.025	0.025	0.008	0.008
22:1 Erucic (g/100g)	<0.007	<0.007	<0.007	<0.007
20:3 Eicosatrienoic (g/100g)	<0.007	<0.007	<0.007	<0.007
20:4 Arachidonic (g/100g)	<0.007	<0.007	<0.007	<0.007
20:5 Eicosapentaenoic (g/100g)	<0.007	<0.007	<0.007	<0.007

24:0 Lignoceric (g/100g)	0.009	0.009	<0.007	<0.007
22:5 Docosapentaenoic (g/100g)	<0.007	<0.007	<0.007	<0.007
22:6 Docosahexaenoic (g/100g)	<0.007	<0.007	<0.007	<0.007
Total 18:1 trans (g/100g)	0.014	0.014	0.018	0.018
Total 18:1 cis (g/100g)	1.49	1.49	0.345	0.345
Total 18:2 trans (g/100g)	0.028	0.028	0.023	0.023
Total 18:3 trans (g/100g)	0.045	0.045	<0.007	<0.007

5.9 Annexure I: Ethical approval of larger study and sub-study



Private Bag X6001, Potchefstroom
South Africa 2520

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

The Applicant / Primary Investigator

Animcare Animal Research Ethics Committee
Tel: 018 299 2234
Fax: 018 299 2225
Email: Tiaan.Brink@nwu.ac.za

13 November 2015

Dear Prof Smuts

AnimCare approval of your application

Ethics Number: NWU-00335-15-S5

Kindly use the ethics reference number provided above in all correspondence or documents submitted to the AnimCare secretariat.

Project Title: Novel interactions between iron and n-3 fatty acids: The effects of maternal depletion and repletion on offspring development and health
Project Head: Prof Marius Smuts
Student: not applicable - umbrella project
Application type: New Application - Large Project

Details of Ethics Approval

You are kindly informed that, following review and discussion of your application at the AnimCare meeting held on *22 Sep 2015 at 09:00 in Room 237, building G16*, and also following your satisfactory response to any corrections or clarifications that may have been requested, the aforementioned application was approved.

Project Category (<i>impact on animal wellbeing</i>)	Not applicable	0	1	2	3	4	5
						X	

Approval Period	Starting date: 2015-11-13	Expiry date: 2016-11-13
------------------------	---------------------------	-------------------------

Conditions of Approval (*if applicable*)

1. Sub-projects needs to be registered if any such studies are undertaken within this large projects, for example when new postgraduate students enrol for studies under this large project.

After ethical review:

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

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

report template is obtainable from Hildah Melamu at Hildah.Melamu@nwu.ac.za. Annually a number of projects may be selected randomly for an external audit.

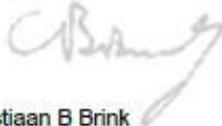
Please note that the AnimCare has the prerogative and authority to ask further questions, seek additional information, require further modification or monitor the conduct of your research.

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

AnimCare complies with the South African National Health Act 61 (2003), The South African National Standards (SANS) document 10386:2008 and the Ethics in Health Research: Principles, Structures and Processes (SANS).

We wish you the best as you conduct your research. If you have any questions or need further assistance, please contact the Ethics Office at Hildah.Melamu@nwu.ac.za or tel. 018 299 2089.

Yours sincerely



Prof Christiaan B Brink
Chair: AnimCare Animal Research Ethics Committee



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Web: <http://www.nwu.ac.za>

Institutional Research Ethics Regulatory Committee

Tel: +27 18 299 4849

Email: Ethics@nwu.ac.za

ETHICS APPROVAL CERTIFICATE OF STUDY

Based on approval by AnimCare Animal Research Ethics Committee (AREC-130913-015) after being reviewed at the meeting held on 24/08/2016, the North-West University Institutional Research Ethics Regulatory Committee (NWU-IRERC) hereby approves your study as indicated below. This implies that the NWU-IRERC grants its permission that provided the special 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: Effects of pre- and postnatal iron and n-3 fatty acid depletion, alone and in combination, on bone development in rats

Study Leader/Supervisor: Dr Jeannine Baumgartner

Student: Ms Estelle Strydom

Ethics number:

N W U - 0 0 2 7 0 - 1 6 - A 5

Institution Study Number Year Status
Status: S = Submission, R = Re-Submission, P = Provisional Authorisation, A = Authorisation

Application Type: New Application - Category 0

Commencement date: 2016-02-17

Category: 0

Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation up to a maximum period of three years.

Special conditions of the approval (if applicable):

- Any research at governmental or private institutions, permission must still be obtained from relevant authorities and provided to the AnimCare. Ethics approval is required BEFORE approval can be obtained from these authorities.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- The study leader (principle investigator) must report in the prescribed format to the NWU-IRERC via AnimCare:
 - annually (or as otherwise requested) on the monitoring of the study, and upon completion of the study
 - 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.
- Annually a number of studies may be randomly selected for an external audit.
- The approval applies strictly to the proposal as stipulated in the application form. Would any changes to the proposal be deemed necessary during the course of the study, the study leader must apply for approval of these amendments at the AnimCare, prior to implementation. Would there be deviation from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.
- The date of approval indicates the first date that the study may be started.
- In the interest of ethical responsibility the NWU-IRERC and AnimCare retains 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 AnimCare or that information has been false or misrepresented,
 - the required amendments, annual (or otherwise stipulated) report and reporting of adverse events or incidents was not done in a timely manner and accurately,
 - new institutional rules, national legislation or international conventions deem it necessary.
- AnimCare can be contacted for further information or any report templates via Ethics-AnimCare@nwu.ac.za or 018 299 2197.

The IRERC would like to remain at your service as scientist and researcher, and wishes you well with your study. Please do not hesitate to contact the IRERC or AnimCare for any further enquiries or requests for assistance.

Yours sincerely

Prof LA

Du Plessis

Digitally signed by

Prof LA Du Plessis

Date: 2017.02.27

12:12:03 +02'00'

Prof Linda du Plessis

Chair NWU Institutional Research Ethics Regulatory Committee (IRERC)

5.10 Annexure J: Author guidelines for Nutrition Research

GUIDE FOR AUTHORS

INTRODUCTION

Nutrition Research publishes research articles, communications, and reviews on all aspects of basic and applied **nutrition**. The mission of *Nutrition Research* is to serve as the journal for global communication of nutrition and life sciences research on **diet** and **health**. The field of **nutritional sciences** includes, but is not limited to, the study of nutrients during growth, reproduction, aging, and disease.

Articles covering basic and applied research on all aspects of nutritional sciences are encouraged, including: nutritional biochemistry and metabolism; metabolomics, nutrient and gene interactions; nutrient requirements in health and disease; digestion and absorption; nutritional anthropology and epidemiology; the influence of socioeconomic and cultural factors on nutrition of the individual and the community; the impact of nutrient intake on disease response, work performance and behavior; the consequences of nutritional deficiency on growth and development, endocrine and nervous systems, and immunity; food intolerance and allergy; nutrient drug interactions; nutrition and aging; nutrition and cancer; obesity; diabetes; and intervention programs.

A principal focus of the journal is to publish research that advances the understanding of **nutrients** and **health protectants** in food for improving the human condition. Of interest are manuscripts on the development of biomarkers for assessing how dietary components influence health status in the human.

The journal also encourages **submission** of manuscripts describing investigations in animal models and cell cultures that utilize methodologic approaches or techniques in biochemistry, immunology, molecular biology, toxicology, and physiology. Epidemiologic studies on nutrient and phytochemical intakes in human populations and novel analytical techniques for these compounds are within the scope of the mission for *Nutrition Research*.

Dr. Bruce A. Watkins, Editor-in-Chief (baw@purdue.edu or bawatkins@ucdavis.edu)
Angela Ranalli-Curtis, Managing Editor (alrcurtis@gmail.com)

Nutrition Research
Department of Nutrition
University of California, Davis
One Shields Avenue
3135 Meyer Hall
Davis, CA 95616-5270, USA

BEFORE YOU BEGIN

Ethics in publishing

Please see our information pages on [Ethics in publishing](#) and [Ethical guidelines for journal publication](#).

Nutrition Research has a policy to follow all aspects of publication ethics and depends on the authors of submitted manuscripts to provide complete information on conflict of interests for the execution of research and data collection. The editorial office and publishers of *Nutrition Research* rely on the authors and their respective institutions to follow the policies to preserve scientific integrity in research and support publication ethics. For information on Ethics in publishing and Ethical guidelines for journal publication see <http://www.elsevier.com/publishingethics> and <http://www.elsevier.com/journal-authors/ethics>.

Declaration of interest

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. If there are no conflicts of interest then please state this: 'Conflicts of interest: none'. [More information](#).

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Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an electronic preprint, see '[Multiple, redundant or concurrent publication](#)' section of our ethics policy for more

information), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service [CrossCheck](#).

Authorship

All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

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Language (usage and editing services)

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the [English Language Editing service](#) available from Elsevier's WebShop.

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Our online submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail.

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Referees

Please submit the names and institutional e-mail addresses of several potential referees. For more details, visit our [Support site](#). Note that the editor retains the sole right to decide whether or not the suggested reviewers are used.

PREPARATION

Research articles and Reviews should generally not exceed 6000 words and Communications should not exceed 2,500.

Each manuscript submitted must provide a title page, list of abbreviations, abstract page, introduction, methods and materials, results, discussion, list of references, and appropriate presentation of data in tables and figures. In some cases, the results and discussion sections can be combined (e.g., communications).

Text must be in 12-point font (Times New Roman or Arial), double-spaced, with 1-inch margins. Consecutive line numbers must be included in the left margin, starting with the title page and ending with the reference section. Page numbers must be included in the bottom right-hand corner of each page. Text must be aligned to the left only and include 2 hard returns at the end of each paragraph, heading, and subheading.

Text should be clear and concise. Tables, figures and references must be cited in sequence in the text. Past tense should be used in reference to the work on which the paper is based, while present tense is normally limited to existing knowledge and prevailing concepts. Previous knowledge and new contributions should be clearly differentiated.

Peer review

This journal operates a single blind review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of two independent expert reviewers to assess the scientific quality of the paper. The Editor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final. [More information on types of peer review](#).

Use of word processing software

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the [Guide to Publishing with Elsevier](#)). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Embedded math

If you are submitting an article prepared with Microsoft Word containing embedded math equations then please read this related support information (http://support.elsevier.com/app/answers/detail/a_id/302/). Displayed formulae should be numbered consecutively throughout the manuscript as (1), (2), etc. against the right-hand margin of the page. In cases where the derivation of formulae has been abbreviated, it is of great help to the referees if the full derivation can be presented on a separate sheet not to be published.

Article structure

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Glossary

Please supply, as a separate list, the definitions of field-specific terms used in your article.

Abbreviations and Symbols

Use only standard abbreviations (Scientific Style and Format, The CBE Style Manual for Authors, Editors, and Publishers, 6th ed. Council of Biology, Chicago IL 1994). Abbreviations should not be used in the title or major headings. The full term for which an abbreviation stands for should precede its first use in the text.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

- **Title.** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
- **Author names and affiliations.** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
- **Corresponding author.** Is responsible for ensuring that all research protocols were approved and for ethical execution of the research. Will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that the e-mail address is given and that contact details are kept up to date. The requirements for the corresponding author are detailed in the Author Submission Checklist which must be completed for manuscript submission.**
- **Present/permanent address.** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract (limited to 250 words) should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Experimental Diets

All studies that include experimental diets must provide a table that lists the ingredients and enough detail for the nutrient content of those diets. Reference to established diets (such as AIN 93G) is appropriate when the major ingredients are listed and the premix levels are provided (actual details of each vitamin and mineral source listed is not necessary in this case). Diets that are developed with different lipid sources should provide a fatty acid compositional analysis of the lipids. In addition, studies that test a botanical or phytochemical ingredient should provide enough chemical compositional analysis as well as the amount of the active compounds

Statistical Methods

Tests of statistical analysis must be fully described. Statements about statistical significance of results must be accompanied by indications of the level of significance. This information must be included where numerical and graphic presentation of data is made in the manuscript in footnotes to tables and in the captions of figures rather than in the text only. Also in the statistical methods section of the manuscript, indicate how the data are presented. For example, means - standard deviation must be shown. Always take special care to present only the significant figures for a measurement and appropriate sample size relevant to a power analysis

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Formatting of funding sources

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